ADIPOSE TISSUE STROMAL VASCULAR FRACTION

Redrawing the lines

J. A. van Dongen

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Colophon

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The development of facial lipofilling from a historical point of view

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GENERAL INTRODUCTION

History of lipofilling

Transplantation of adipose tissue was first described by Gustav Neuber in 1893.[1] Neuber successfully used resected fat grafts to fill depressed scars in the face and noted that only small fat grafts were suitable.[1] In those days, fat grafts were only used to restore loss of volume of soft tissue defects as a result of trauma or congenital defects. In 1910, Lexer was the first to use fat grafts aesthetically to correct wrinkles and fill the infraorbital area.[2] Two years later, Holländer and Veriag von Velt treated two cases of facial lipoatrophy with fat grafts to improve the natural appearance of the face.[3] In 1919, Brunning introduced syringes to transplant fragmented adipose tissue and thus no incision was needed on the recipient site.[4] It took several years before syringes were used to harvest adipose tissue by means of liposuction. In 1926, Miller published about infiltration of fat grafts through cannulas to corrects scars in the head and neck region.[5] Yet, the early ancestor of the modern liposuction technique with a blunt hollow cannula were Arpad and Giorgio Fischer in 1975.[6] Their liposuction technique was, however, rather invasive and traumatic for patients.[6] Two years later, Ilyouz modified the liposuction technique introduced by Arpad and Giorgio Fischer and created the foundation of modern liposuction technique.[7] He used tumescent infiltration fluid prior to liposuction to create a less invasive and traumatic procedure. Liposuction was then performed through a 0.5 to 1 cm incision with a blunt rounded tip cannula in 1,326 patients. Several other plastic surgeons followed Ilyouz's example of tumescent liposuction; some with small modifications.[8-11] In contrast, Fournier and Otteni proposed a dry liposuction technique *i.e.* without tumescent infiltration. Adipose tissue obtained by dry liposuction showed histological comparable results with tumescent liposuction but was less time-consuming.[10] On the other hand, tumescent liposuction showed less anaemia, but more seroma in comparison with dry liposuction.[12] Further instrumental improvement was made by Toledo in 1988 by presenting disposable syringes with different sizes for harvesting adipose tissue.[13] A great advantage of disposable syringes for aspiration of adipose tissue is the reduced risk of contamination or biofilms to grow.[14] A year later a new technique of injection of adipose tissue, called lipofilling, was shown by Fournier.[15] However, liposuction and subsequently lipofilling did not develop very fast in the early and mid-twentieth century mainly due to large variability in results. The inconsistent results were caused by a high variety in volume retention due to donor variations and direct use of unprocessed lipoaspirate mixed with infiltration fluid as well as over-injection of target sites limiting graft survival. At that time, fat grafting was also hampered by legislation issues from the food and drug administration (FDA) because it was considered unclear whether lipofilling would *e.g.*

promote risk for breast cancer in mastectomized patients. Coleman understood that consistent results of lipofilling could be achieved by developing better techniques and instruments for harvesting, processing and injecting lipoaspirate.[16-18] He developed very fine cannulas to harvest and inject smaller particles of adipose tissue and showed it was possible to transplant adipose tissue in areas were only small amount of fat are needed, such as in the hands or in the face. Injection of smaller sized graft fragments using smaller holes in harvesting cannulas as well as for injection could increase volume retention.[19] Smaller sized fat grafts most likely engraft better due to faster connection to donor vasculature and no diffusion barrier resulting in more living cells in the core. Yet, the results remained rather unpredictable and the fate of the injected adipocytes was still unknown.

The fate of injected adipocytes

To date, two main theories about the fate of injected adipocytes have been described in literature. The first theory is the 'host cell replacement theory' described by Neuhof and Hirshfeld in 1923.[20] The 'host cell replacement theory' stated that transplanted adipose tissue completely necrotized in a few months after transplantation and is replaced by fibrotic tissue or newly formed metaplastic adipocytes. The transplanted adipose tissue functions as a scaffold for ingrowth of newly formed fibrotic or adipose tissue both from recipient origin.[20] The second theory is the 'cell survival theory' introduced by Peer in 1955.[21] The 'cell survival theory' assumed that (part of) the fat graft survived after transplantation. Survival of transplanted cells in fat grafts depends on capillary ingrowth of recipient vessels and formation of anastomosis between donor and recipient vessels.[21] Formation of anastomosis or angiogenesis is induced by the occurrence of hypoxia due to the delay between harvesting and injecting lipoaspirate.[22,23] However, mature adipocytes are sensitive to hypoxia and trauma induced by harvesting, processing and injection of fat grafts resulting in apoptosis.[24-26] Adipocytes that are located more favourably *i.e.* close to newly formed anastomosis in the periphery and therefore shorter exposed to hypoxia tend to survive longer.[21] On the other hand, adipocytes secrete more vascular endothelial growth factor under hypoxia stimulating angiogenesis.[27]

The survival of more cells in the periphery of transplanted adipose tissue was later confirmed by Carpaneda and Ribeiro showing that five days postoperative after transplantation three zones *i.e.* peripheral zone, intermediate zone and central zone surrounded by a collagen capsule could be distinguished.[28]

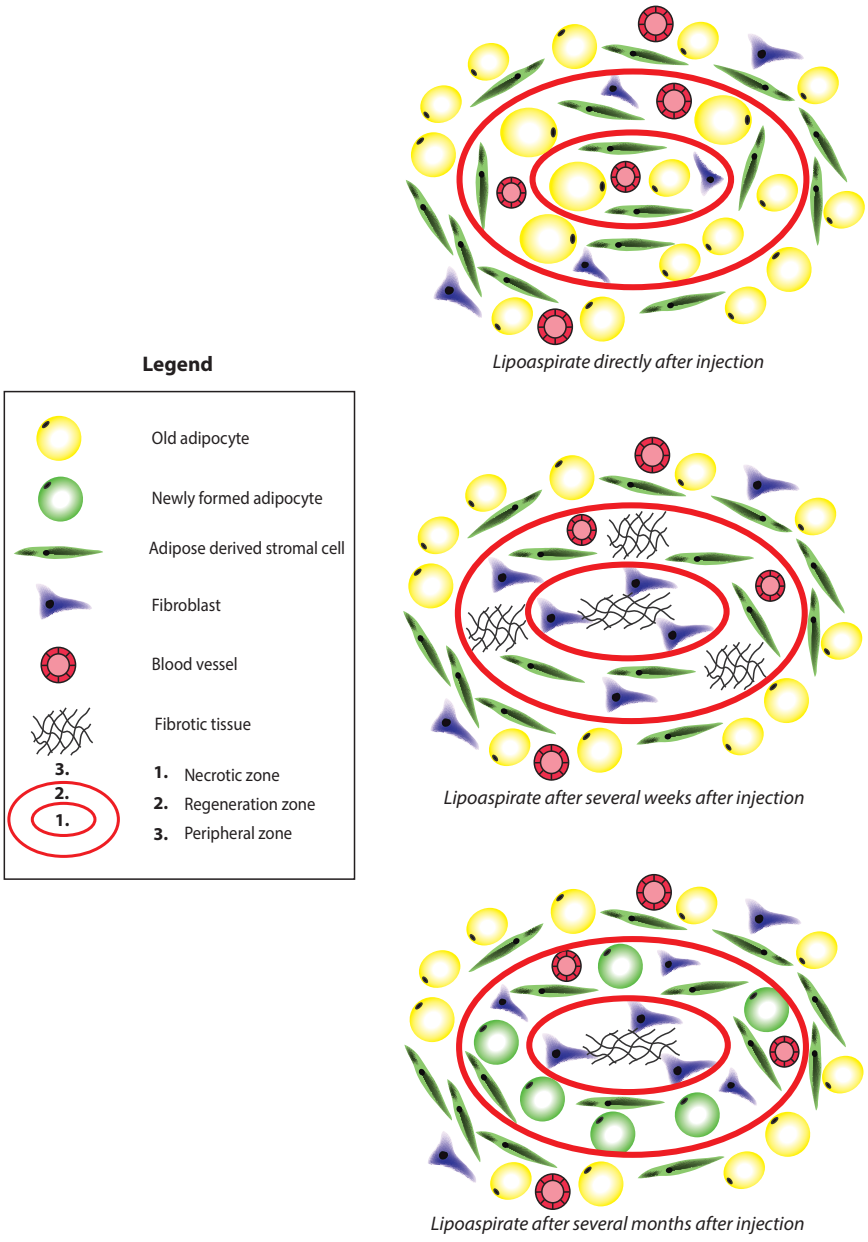

Figure 1. Schematic overview of the fate of lipoaspirate after injection based on a combination of the host cell replacement theory and cell survival theory. Lipoaspirate contains three zones: peripheral zone (almost all adipocytes and adipose derived stromal cells survive), regeneration zone (most adipocytes die but are replaced by newly formed small adipocytes induced by surviving adipose derived stromal cells) and necrotic zone (all adipocytes and adipose derived stromal cells die and are replaced by fibrotic tissue or absorbed).

Twenty-one days postoperative, the three zones were more clearly characterized: peripheral zone showed viable adipocytes with only a few pseudocysts, the intermediate zone was characterized by infiltration of inflammatory cells as well as more pseudocysts, while the central zone was characterized by necrosis and mainly pseudocysts. Sixty days postoperative, the transplanted fat grafts were reduced in volume (60 percent of the viable cells died) with still a collagen capsule presented. The central necrotic zone now was twice as small as the peripheral zone showing an inflammatory process with fatty pseudocysts and formation of collagen.[28] However, viability evaluated by only morphology or presence of nuclei is not sufficient to distinguish between living and dead adipocytes because an adipocyte is too large to evaluate in a single histological section.

Eto et al. used perilipin, a protein that covers lipid droplets, to discriminate between living (positive for perilipin) and dead adipocytes (negative for perilipin) after fat grafting in a mouse model.[24] He showed that the fate of injected adipocytes could be explained by a combination of the 'host cell replacement theory' and the 'cell survival theory'. In line with the 'cell survival theory', Eto et al. showed that transplanted fat grafts contained three zones: peripheral zone, regeneration zone and necrotic zone (Fig. 1). In the peripheral zone, almost all adipocytes and adipose derived stromal cells (ASCs) survive (cell survival). In line with the 'host cell replacement theory' most adipocytes die within one day postoperative in the regeneration zone but are replaced by newly formed small adipocytes induced by surviving ASCs (cell replacement). In the necrotic zone, all adipocytes and ASCs die without regeneration and dead space will be filled with scar formation or absorbed (Fig. 1).[24] Moreover, mature adipocytes are larger and more fragile than ASCs and have therefore less resistance to hypoxia and trauma induced by harvesting, processing and injection of fat grafts.[29-31] Nowadays, many animal and human studies have been performed to study the fate of injected fat grafts with a variety of explanations and therefore no clear conclusion.[32] Although, many studies have shown the important role of processes like fibrosis, inflammation and neoangiogenesis.[32]

Adipose derived stromal cells

In 2001, Zuk et al. discovered that adipose tissue is a rich source of mesenchymal stem cells (MSCs) or ASCs, which are easily enzymatically isolated and expanded in culture.[33] Adipose tissue consists of two large components: adipocytes (parenchyme) and stroma.[34,35] The stroma consists of a cellular part called stromal vascular fraction (SVF) held together by extracellular matrix (ECM). The SVF comprises all non-adipocyte cell types *e.g.* ASCs, endothelial cells, smooth muscle cells, immune cells and fibroblasts.[36,37] ASCs are located around vessels in SVF as precursor cell types *e.g.* pericytes and in

bigger vessels as supra-adventitial cells, however, controversy remains to their exact nature and location.[38-41] The discovery of ASCs was a breakthrough in understanding the regenerative capacity conveyed by grafted adipose tissue. *In vitro*, ASCs showed to be capable of multilineage differentiation potential in adipogenic, osteogenic, chondrogenic and myogenic cell lineages.[33,42] Moreover, ASCs could be cultured upon multiple passages without inducing senescence. The multilineage differentiation capacity and extensive culture period suggested that ASCs are a stem cell-like or progenitor cell type. Latter, several other studies showed the capability of ASCs to differentiate into neuron-like cells[43], cardiomyocytes[44], hepatocytes[45], pancreatic cells[46] and epithelial cells.[47] However, these in vitro proof of concepts might not reflect the ASCs' physiological behaviour. The multilineage differentiation ability of ASCs has led research groups to believe that the therapeutic effect of ASCs is through differentiation into target cells, while other studies have shown that the plethora of secreted paracrine factors, exosomes and cytokines results in stimulation of important processes regarding facial lipofilling *e.g.* angiogenesis and skin tissue regeneration.[48-52]

Clinical use of ASCs as cell-based therapy is expensive and time-consuming due to enzymatic isolation and culture.[53] Also, cultured ASCs changes significantly in terms of phenotype and function after adherence to tissue culture plastic, while the real phenotype of uncultured ASCs remains unknown. After multiple days of culture, the *in vivo* phenotype of ASCs emerges to an *in vitro* phenotype by losing CD34 expression and gaining CD105 expression.[38,54] Functionally, the ASCs secretome changes upon culture. During culture, many aspects affect the secretion of cytokines and growth factor such as hypoxia (*e.g.* increased vascular endothelial growth factor secretion) or 3D versus 2D culture (*e.g.* upregulation of genes related to cell adhesion and wound healing in 3D culture).[55,56] In this way, relating *in vitro* experimental data to clinical evidence and vice versa is difficult.

Till recently, the Gold standard to isolate SVF containing ASCs from adipose tissue is by means of enzymes, requires a laboratory and is thus time-consuming and expensive.[33] Enzymatic digestion of adipose tissue yields a heterogeneous cell suspension containing only cells *e.g.* ASCs, endothelial cells, smooth muscle cells, pericytes, fibroblasts and immune cells. Due to expensive and time-consuming enzymatic isolation procedures, intra-operative non-enzymatic or mechanical isolation procedures increased popularity. Mechanical isolation procedures to isolate SVF only use centrifugation and shear stress to disrupt the larger and weaker cells *i.e.* adipocytes.

Lipofilling and skin rejuvenation

In 2006, Coleman was the first to describe that lipofilling was more than a permanent filler.[57] Coleman described that the overlying skin of transplanted adipose tissue showed signs of improvement *e.g.* less scarring caused by acne and decreased pores size.[57,58] He surmised that the observed skin rejuvenation was facilitated by the presence of ASCs in fat grafts.[57]. In 2013, Tonnard et al. showed three clinical cases with reduced dark coloration of the lower eyelid and reduced number of wrinkles of the décolleté and perioral after treatment of Nanofat.[59] This was the first case report showing skin rejuvenation after the use of emulsified lipoaspirate. Since the publication of the Nanofat procedure, an increase interest in facial skin rejuvenation using regenerative components of adipose tissue *e.g.* SVF has been.[59-69] In most studies, SVF has been used as additive to lipofilling to increase the ratio of regenerative cells/per ml of lipoaspirate injected in facial planes. The rationale behind SVF enriched lipofilling is to enhance skin rejuvenation. However, most of these studies lack a proper study design with objective outcomes. Thus far, no solid conclusion could be made whether SVF as additive to facial lipofilling or as monotherapy is effective for skin rejuvenation, although a large commercial market already exists.

AIM AND OUTLINE OF THE THESIS

The aim of studies described in this thesis is to design and develop a new intra-operative mechanical isolation procedure to isolate SVF; to evaluate the skin rejuvenative effect of SVF on a decreased skin quality caused by aging and scarring using our newly developed mechanical isolation procedure to isolate SVF. Using *in vitro* experiments, this thesis aims to identify the regenerative components of SVF responsible for stimulating important processes related to skin rejuvenation.

To date, the Gold standard for SVF isolation is enzymatic isolation. However, an increasing number of countries forbids by legislation the use of enzymes to manipulate tissue. To bypass legislation, a growing number of alternative mechanical isolation procedures are being developed. **Chapter 2** comprises a systematic review of the available intra-operative enzymatic and mechanical isolation procedures. In this systematic review, procedure characteristics and composition of isolated SVF are compared. **Chapter 3** describes the development and validation of our own developed mechanical isolation procedure to isolate SVF, the so-called Fractionation of Adipose Tissue (FAT) procedure. After several years of gaining experience using the FAT procedure, minor practical issues were discovered which led to development of a new version of the FAT procedure. **Chapter 4** describes the validation of the latest

FAT procedure using additional validation techniques, while **chapter 5** shows the immunomodulative effect of SVF on chondrocytes. In **chapter 6**, an extensive overview is given of protocols to use and validate the FAT procedure.

A decrease in skin quality can be caused by a physiological process *i.e.* ageing or pathophysiological processes *e.g.* scarring or fibroproliferative diseases. Although the mechanism of (patho)-physiological processes affecting skin quality differs highly, many studies suggest that lipofilling or any component of adipose tissue *e.g.* SVF provides skin rejuvenation regardless the nature of decreased skin quality. In **chapter 7** an extensive overview is given of the role of lipofilling as anti-scarring treatment. In this review, both the clinical evidence as well as the mechanism of action based on *in vitro* and animal experiments is described. In **chapter 8**, a multicenter prospective randomized placebo-controlled trial is performed to study the effect of SVF on scarring in a mamma-reduction model. Of each patient, one breast serves as control, while the wound of the other breast receives SVF. We hypothesized that early intervention to improve wound healing would result in a less visible scar. In **chapter 9**, a randomized placebo-controlled trial is performed investigating the effect of lipofilling on facial skin rejuvenation due to ageing. Our hypothesis is that the addition of platelet-rich plasma, a known stimulator of ASCs, results in an improved skin elasticity in comparison with lipofilling alone. To further increase skin elasticity, we hypothesized that the addition of SVF to facial lipofilling in combination with platelet-rich plasma would benefit. **Chapter 10** shows the results of a randomized placebo-controlled trial comparing SVF in combination with lipofilling and platelet-rich plasma to lipofilling and platelet-rich plasma alone. SVF is obtained by means of the FAT procedure. **Chapter 11** systematically describes the clinical evidence of using adipose tissue as a treatment for facial skin rejuvenation due to ageing.

SVF is believed to be pro-regenerative by secreting a plethora of released factors by ASC. Additionally, SVF contains extracellular matrix which is able to bind and release secreted factors by ASCs and thus functions as a natural scaffold to guide tissue regeneration. In **chapter 12**, an off the shelve product is created based on gelated extracellular matrix incubated with released factors from ASCs. We hypothesized that this hydrogel would be able to stimulate important processes regarding wound healing and skin rejuvenation *i.e.* angiogenesis as well as fibroblast migration and proliferation. In **chapter 13**, the toxicity of the hydrogel as a 3D *in vitro* culture model was studied.

Finally, in **chapter 14**, the findings of this thesis and implications are being summarised and discussed as well as directions for future perspectives are underlined.

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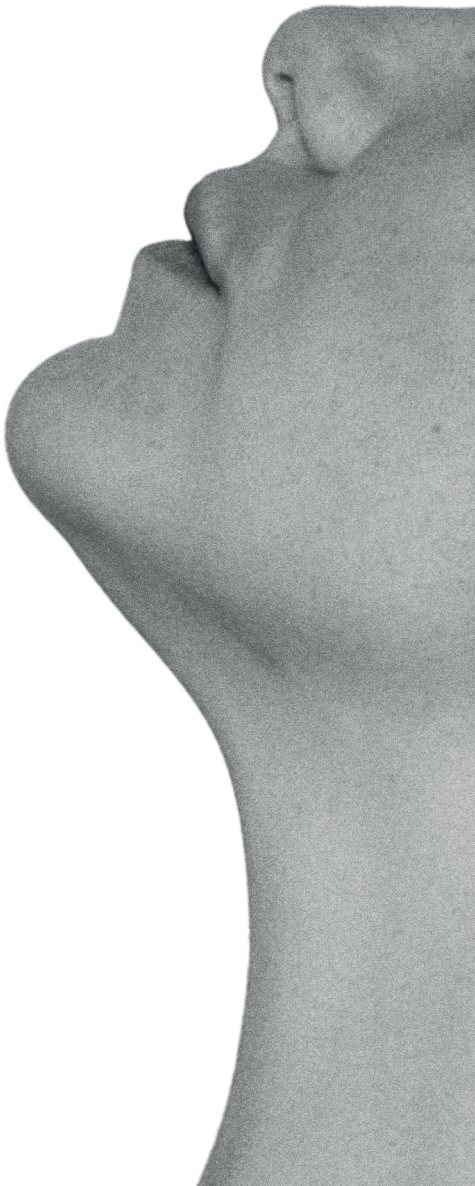
PART I

Isolation procedures of stromal vascular fraction



02

Comparison of intraoperative procedures for isolation of clinical grade stromal vascular fraction for regenerative purposes: a systematic review

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ABSTRACT

Introduction

Intraoperative application of stromal vascular fraction (SVF) of adipose tissue, requires a fast and efficient isolation procedure of adipose tissue. This review was performed to systematically assess and compare procedures currently used for the intraoperative isolation of cellular SVF (cSVF) and tissue SVF (tSVF) which still contains the extracellular matrix.

Methods

Pubmed, EMBASE and The Cochrane Central Register of controlled trials databases were searched for studies that compare procedures for intraoperative isolation of SVF (searched 28th of September, 2016). Outcomes of interest were cell yield, viability of cells, composition of SVF, duration, cost and procedure characteristics. Procedures were subdivided in procedures resulting in a cSVF or tSVF.

Results

Thirteen out of 3038 studies were included, evaluating eighteen intraoperative isolation procedures, were considered eligible. In general, cSVF and tSVF intraoperative isolation procedures had comparable cell yield, cell viability and SVF composition compared to a non-intraoperative (*i.e. culture lab-based collagenase protocol)* control group within the same studies. The majority of intraoperative isolation procedures are less time consuming than non-intraoperative control groups, however.

Conclusion

Intraoperative isolation procedures are less time-consuming than non-intraoperative control group with similar cell yield, viability of cells and composition of SVF and therefore more suitable for use in the clinic. Nevertheless, none of the intraoperative isolation procedures could be designated as preferred procedure to isolate SVF.

INTRODUCTION

Adipose tissue seems to be an outstanding source for regenerative therapies, since it is an easy accessible source for adipose-derived stem or stromal cells (ASCs). Adipose tissue can easily be harvested with liposuction, a low risk procedure that can be performed under local anesthesia. Several clinical trials have been published using ASCs for soft tissue reconstruction [1], cardiac repair [2], pulmonary repair [3] and cartilage repair [4]. All these trials show promising results for future use of ASCs in tissue repair and regeneration.

To harvest ASCs, adipose tissue or lipoaspirate is subjected to enzymatic dissociation followed by several centrifugation steps [5], which is a relative long-lasting procedure that cannot be performed during surgery. The cell population obtained by this enzymatic digestion and centrifugation is the stromal vascular fraction (SVF), containing ASCs, endothelial cells, supra-adventitial cells, lymphocytes and pericytes [5,6]. ASCs *in vivo* are characterized as CD31min/CD45min/CD34pos/CD90pos/CD105low cells [7]. After isolation, the SVF can either be used directly in clinical procedures or can be cultured to increase the number of cells before using them in the clinic [8,9]. In case of cell culturing, only ASCs and their precursor cells (supra-adventitial cells and pericytes) are able to adhere and survive [10,11]. Upon passaging *in vitro*, the phenotype of ASCs starts to deviate from their *in vivo* phenotype (Spiekman et al., 2016): in this process CD34 surface expression is lost, while CD105 expression is up-regulated to mention a few [7,12]. Alternatively, administration of the enzymatically prepared vascular stromal fraction of adipose tissue might have a therapeutic capacity that is similar to cultured ASCs. Although, no formal scientific evidence exists, the consensus is, that the therapeutic benefit of SVF predominantly relies on the abundantly present ASCs.

The current protocol to isolate and culture ASCs from adipose tissue involves enzymatic digestion with collagenase. This is a laborious and time consuming protocol and requires a specialized culture lab (Good Manufacturing Practice facilities (cGMP)), which is not available in most peripheral hospitals [13]. Therefore, intraoperative procedures for SVF isolation are warranted, in particular systems that do not employ enzymatic treatment, such as mechanical dissociation.

At present, several (commercial) procedures are available for intraoperative isolation of SVF [14,15]. These intraoperative isolation procedures differ in various aspects: isolation of a single cell SVF (cellular SVF (cSVF)) resulting in a pellet with hardly any volume or isolation of SVF cells containing intact cell-cell communications (tissue SVF (tSVF). Most of the enzymatic intraoperative isolation procedures result in a cSVF, because

of the loss of cell-cell communications and extracellular matrix. In most of the non-enzymatic intraoperative isolation procedures the cell-cell communications remain intact, resulting in an end product with more volume (tSVF). Different studies assessed the cell yield and phenotype of the isolated cSVF or tSVF of the various intraoperative isolation procedures compared to other intraoperative (commercial) procedures or to the gold standard for SVF isolation (non-intraoperative culture lab-based collagenase protocols which require cGMP facilities for clinical use, referred to as 'non-intraoperative isolation protocol'). Recently, new intraoperative isolation procedures are introduced and tested. It is not clear yet if intraoperative isolation procedures generate a similar quality and quantity of SVF as non-intraoperative isolation protocols. Next to this, the distinction between end products of intraoperative isolation procedures, *e.g.* cSVF and tSVF have never been studied. Therefore, a systematic review was performed to assess the efficacy of intraoperative isolation procedures of human SVF based on number of cells, cell viability and composition of SVF. In addition, duration and costs of the intraoperative isolation procedures were compared.

MATERIAL & METHODS

Protocol and registration

This study was performed using the PRISMA protocol [16]. The search strategy for this systematic review was based on a Population, Intervention, Comparison, and Outcome (PICO) framework [17]. The study was not registered.

Eligibility criteria

Studies were included when at least two different types of intraoperative isolation procedures or one intraoperative isolation procedure with a non-intraoperative isolation protocol were assessed using human adipose tissue to isolate SVF. Studies need to use the adipose fraction of lipoaspirate. Studies only evaluating centrifugation forces, sonication or red blood cell (RBC) lysis buffer were excluded. Studies focusing on processing methods of adipose tissue for the use in fat grafting were excluded as well as case reports, case series and reviews. Searches were not limited to date, language or publication status (Table 1).

Information sources and search

Pubmed, EMBASE (OvidSP) and The Cochrane Central Register of controlled trials databases were searched (searched 28th September, 2016). The search was restricted to human studies. The search terms (Table 2) were based on three components: (P)

adipose stromal cell, adipose stem cell, stromal vascular fraction, autologous progenitor cell, or regenerative cell in combination with (I) cell separation, isolation, dissociation, digestion, emulsification, isolation system, cell concentrator and finally connected with (C) enzymatic, non-enzymatic, or mechanical.

Table 1. Inclusion and exclusion criteria.

Inclusion criteria	Exclusion criteria
Clinical trials	Case reports
Comparative studies	Case series
Full text available	Reviews
All languages	Letters to editor
Human studies	Non-comparative studies
	No full text available
≥2 different types of SVF isolation procedures	Processing methods for fat grafting Protocols using centrifugation or RBC lysis buffer only
1 SVF isolation procedure compared with control group Intraoperative procedures	Mesenchymal cells derived from other source than adipose tissue Blood saline fraction used instead of adipose fraction of the lipoaspirate Laboratory based enzyme protocols as experimental group No outcome of interest: SVF composition (CD markers), cell yield, viability of SVF

Study selection and data collection process

Two authors (JAD, AJT) selected studies independently based on the eligibility criteria. Inconsistencies were discussed during a consensus meeting. In case of disagreement, the senior author (MCH) gave a binding verdict.

Data items

Search term was partly based on a Population, Intervention, Comparison, Outcome (PICO) framework. Outcomes of interest were not included in the search term. For this review the outcomes of interest were cell yield, viability of the nucleated cells, composition of the SVF and duration, cost and characteristics of the intraoperative isolation procedures. Effect sizes were calculated on cell yield and viability in studies with a comparison of intraoperative isolation procedures versus regular non-intraoperative isolation protocols. Differences in harvesting procedure were not taken into account.

Table 2. Specific search terms of databases.

Search term Pubmed:
((((Adipose Tissue [Mesh] OR Adipocytes [Mesh] OR Fat [tiab] OR Lipoaspirate* [tiab])) AND (Cell separation [Mesh] OR Isolat* [tiab] OR Dissociat* [tiab] OR Emulsification [tiab] OR Concentrat* [tiab] OR Digest* [tiab] OR Obtained [tiab])) AND (Stem cells [Mesh] OR Stromal cells [Mesh] OR Autologous progenitor cell* [tiab] OR Stromal vascular* [tiab] OR Regenerative cell* [tiab] OR Vascular stroma [tiab])) Restriction: Only human
Search term Embase:
('adipose tissue':ab,ti OR 'adipocytes':ab,ti OR 'fat':ab,ti OR lipoaspirate*:ab,ti AND ('cell separation' OR isolat*:ab,ti OR dissociat*:ab,ti OR 'emulsification':ab,ti OR concentrat*:ab,ti OR digest*:ab,ti OR 'obtained':ab,ti) AND ('stem cells':ab,ti OR 'stromal cells':ab,ti OR 'autologous progenitor cell':ab,ti OR 'autologous progenitor cells':ab,ti OR 'stromal vascular':ab,ti OR 'stromal vascular fraction':ab,ti OR 'regenerative cell':ab,ti OR 'regenerative cells':ab,ti OR 'vascular stroma':ab,ti)) AND [embase]/lim NOT [medline]/lim AND 'article'/it *Restriction: Only EMBASE*
Search term Cochrane Library:
(adipose tissue OR adipocytes OR fat OR lipoaspirate*) AND (cell separation OR Isolat* OR Dissociat* OR Emulsification OR Concentrat* OR Digest* OR Obtained) AND (stem cells OR stromal cells OR autologous progenitor cell* OR stromal vascular* OR regenerative cell* OR vascular stroma)

Risk of bias in individual studies

It is known that the quality of ASCs depends on age and harvest location of the donor [18-21]. The inclusion of young healthy patients may positively affect the results. Therefore, detailed information about demographics are described in this review.

Summary measurements

Effect sizes were calculated of the outcome variables cell yield and percentage of viable nucleated cells from cSVF between enzymatic intraoperative isolation procedures and non-intraoperative isolation protocols (gold standard). The following effect size formula was used: effect size = (difference in mean outcomes between enzymatic intraoperative isolation procedures and gold standard) / (standard deviation of the gold standard). Studies which presented results in mean and standard deviation were analyzed. Intraoperative isolation procedures focusing on tSVF instead of cSVF were not taken into account in the effect size of cell yield, because of different start volumes of lipoaspirate and end volumes of tSVF.

Synthesis of results

In some studies, derivate numbers of graphs are used when the actual number of outcomes was not given. Cell types within the SVF can be distinguished based on CD marker expression or immuno-staining. To compare SVF compositions between different studies and to compare intraoperative procedures with their control (*i.e.* non-intraoperative protocols or other intraoperative procedures) in the same study, only CD marker expression was used. Studies evaluating a single CD marker expression to analyze different cell types were seen as insufficient distinctive and were excluded. Cells were divided into two major groups: CD45min (adipose tissue-derived) and CD45pos (blood derived) cells to analyze the expression of stromal cells, pericytes, vascular endothelial cells/endothelial progenitor cells, endothelial cells, lymphocytes, leukocytes and hematopoietic stem cells. All other cells are placed in the category: other cell types. The CD34pos/CD146pos population is excluded from analysis because of the inability to discriminate between progenitor pericytes and progenitor endothelial cells [22].

Risk of bias across studies

Included studies could present different outcome variables related to SVF analysis. There is a risk that studies did not present a full SVF characterization and thereby bias their results. In order to provide an overview of the used outcome variables per study, a Modified IFATS/ISCT Index Score was used. The risk of publication bias of positive results might be expected in those articles were the authors have benefits in the investigated products. Disclosure agreements were reviewed for each study.

Modified IFATS/ISCT Index Score for the measurement of adipose tissue-derived stromal vascular fraction

Studies were assessed based on the reported outcome variables. The assessment of quality was evaluated based on the position statement of the International Federation of Adipose Therapeutics and Science (IFATS) and the International Society of Cellular Therapy (ISCT) [5]. The IFATS and ISCTS proposed guidelines to develop reproducible standardized endpoints and methods to characterize ASCs and SVF cells. For each of the following characterization methods a grade was given by the authors (JAD, AJT) to an article if the characterization was carried out: viability of nucleated cells, flow cytometry of SVF cells, flow cytometry of ASCs (CD13, CD29, CD31, CD34, CD44, CD45, CD73, CD90, CD105, CD235a), proliferation and frequency (CFU-F) and functional assays (adipogenic, osteogenic and chondrogenic differentiation assays) of ASCs. The maximum score in case of a full characterization was 5.

RESULTS

Included studies

A total of 3038 studies were identified after database searching. 2955 articles were excluded after abstract screening. 59 full text studies were assessed on eligibility criteria. Fourteen studies were excluded based on the use of a non-intraoperative protocol for isolation as experimental method [7,23-35]. Seven studies described isolation protocols in general but gave no results [36-42]. Seven studies were excluded based on the lack of a control group (*i.e.* non-intraoperative isolation protocols or other intraoperative isolation procedures) [10,18,43-47]. Four studies were excluded based on their study design [48-51]. Three studies were excluded based on the use of culture methods to isolate ASCs, because culture methods are incompatible with intraoperative applications [52-54]. Four studies used only centrifugation, centrifugation or RBC lysis buffer as isolation protocol and were thereby excluded [55-58]. Three studies used the blood saline fraction of lipoaspirate and were thereby excluded [59-61]. Four studies did not describe an outcome of interest [62-65]. Four additional studies were identified through other sources (Fig. 1). Thus, thirteen studies with eighteen intraoperative isolation procedures remained for analysis.

Study characteristics

In total, 93 subjects were enrolled in the thirteen studies. Nine studies reported gender of which 95% was female (n=58). Nine studies reported the mean age or age variance of the subjects and ten other studies described the use of infiltration (Table 3). No meta-analysis could be performed because the metrics and outcomes were too diverse.

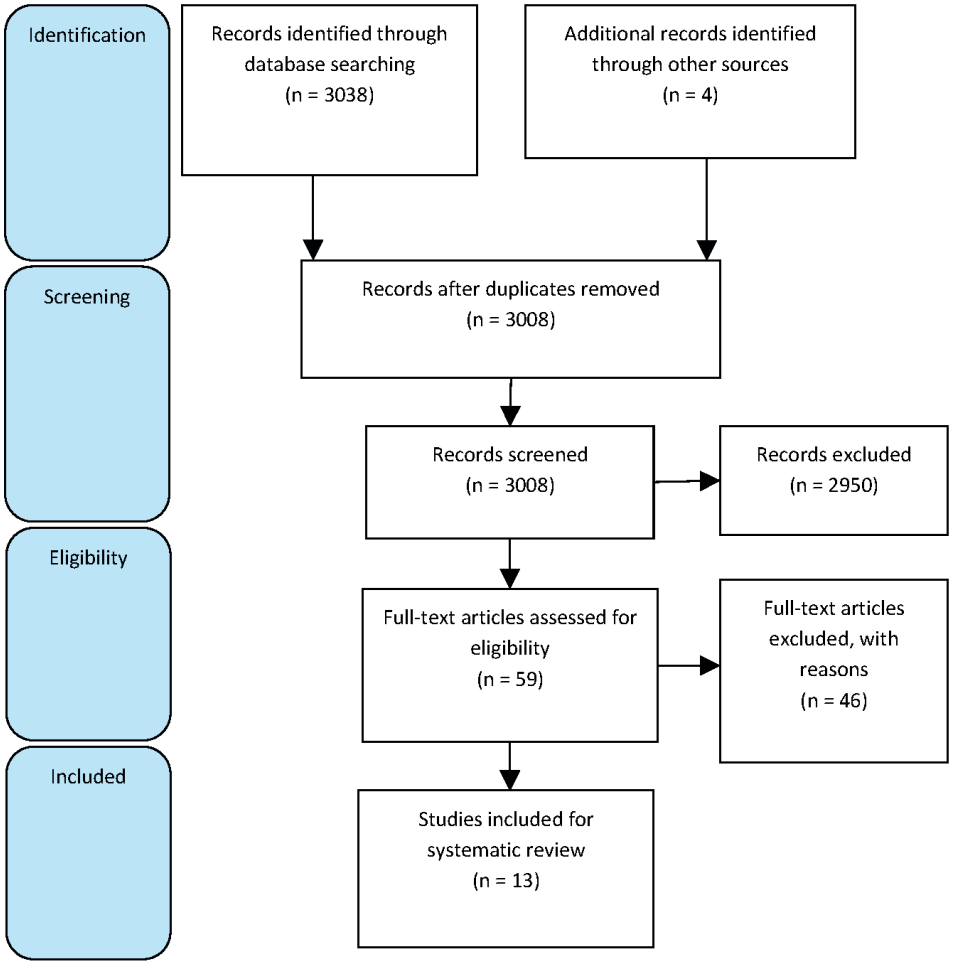

Figure 1. Flow diagram of study selection.

Table 3. Study characteristics.

Name	Author	Female (F)/ Male (M)	Age mean +sd (y)	Age variance (y)	Liposuction
CHA	Aronowitz et al. 2013	5F	-	-	Tumescent liposuction
CYT					
LIPOK					
PNC					
CYT	Aronowitz et al. 2016	5F	32.4 +/-5.9	25-37	Tumescent liposuction
GID SVF2					
LIPOK					
PNC					
LIPOG	Bianchi et al. 2013	4	-	-	Liposuction
TGCIS	Doi et al. 2012	6F	-	-	Liposuction
CYT	Domenis et al. 2015	9	46	41-70	Liposuction
FAST		6	52	19-74	
LIPOK		5	52	41-74	
FAT	van Dongen et al. 2016	11F	-	-	Liposuction
SEPAX	Güven et al. 2012	11F	-	20-65	Tumescent liposuction
CYT	Lin et al. 2008	6F	38.7+/-16.3	18-60	Plastic Surgery
FEF REF SF	Mashiko et al. 2016	10F	41.0+/-8.2	-	Liposuction
STCELL	Millan et al.	3	-	-	Liposuction
SHUF5	Osinga et al. 2015	3F/3M	M57, F61	-	Liposuction
SHUF30					
AIS	SundarRaj et al. 2015	11	30.86	17-47	-
NANO	Tonnard et al. 2013	1F	40	n/a	Abdominoplasty liposuction

AIS Automated Isolation System; CHA-station (CHA-Biotech); CYT Celution System Enzymatic (Cytori); FAST Fastem Corios (Corios); FAT Fractionation of Adipose Tissue procedure; FEF Filtrated fluid of emulsified fat; GID SVF2 (GID Europe); LIPOG Lipogems (Lipogems); LIPOK Lipokit System (Medi-khan); NANO Nanofat procedure; REF Residual tissue of emulsified fat; PNC Multi station (PNC); SEPAX Sepax (Biosafe); SF Squeezed fat; SHUF5 Shuffling 5 times; SHUF30 Shuffling 30 times; STCELL StromaCell; TGCIS Tissue Genesis Cell Isolation System (Tissue Genesis); 1 = used infiltration prior to liposuction, 0 = not mention the use of infiltration or did not use infiltration prior to liposuction

Donor site	Infiltration (1/0)	Cannula (mm)	Pressure
Abdominal	1	2.5 blunt	25-28 mmHg Vacuum
Abdominal, flank, back, arms, buttocks, inner thighs	1	-	-
Abdominal	1	19 cm blunt, 3 mm OD, 5 oval holes (1x2 mm)	manually or clamping
-	0	-	-
Hips, Trochanteric, abdominal	1	3 blunt	0.4 bar
Hips, Trochanteric, abdominal			
Hips, abdominal			
-	1	Sorenson cannula	-
Abdominal	1	-	-
-	0	-	-
Thigh	1	3-mm multiport cannula, holes of 2 mm	-
-	1	-	-
Abdominal	1	4 mm blunt cannula, oval opening 2 x 4mm	manual
Abdominal, thigh, hip	0	-	-
Abdominal	1	3-mm sharp nultiport cannula, holes of 1mm	high-negative

Characteristics of the intraoperative isolation procedures

All intraoperative isolation procedures are divided into two categories: enzymatic and non-enzymatic procedures resulting in cSVF and tSVF respectively (Table 4A and table 4B). Eight of the eighteen intraoperative isolation procedures were based on enzymatic digestion and ten isolation procedures were based on non-enzymatic procedures. Two non-enzymatic procedures, the Residual tissue of emulsified fat procedure and the Fractionation of adipose tissue procedure, are named differently, but are almost identical. One intraoperative isolation procedure, the Filtrated fluid of emulsified fat, is a combined procedure of two other intraoperative isolation procedures *i.e.* the Fractionation of adipose tissue procedure and the Nanofat procedure [66-68].

Start volume versus end product

The Automated isolation system, GID SVF2, Lipokit system and Multi station are enzymatic intraoperative isolation procedure that resulted in large average amounts of SVF (7.2 ml – 20 ml), suggesting inefficient enzymatic digestions [69,70]. The non-enzymatic intraoperative isolation procedures resulted in larger end volumes than only a pellet. Prior the Lipogems procedure, 130 ml of adipose tissue can be obtained to mechanical dissociate to 100 ml of lipoaspirate. Hence, this a reduction of the volume of 1.3 times, suggesting an inefficient mechanical dissociation to our opinion [22]. In contrast, the Fractionation of adipose tissue procedure resulted in a 10.4-fold volume reduction [67]. For all other intraoperative isolation procedures, no data is mentioned about the end volume of the lipoaspirate (Table 4A and table 4B).

Duration and costs

Duration of the intraoperative isolation procedures varied from 5 seconds to 133 minutes (n=12). Isolation with the Automated isolation system was the longest intraoperative isolation procedure [69]. Shuffling lipoaspirate 5 or 30 times through a luer-to-luer lock syringe will take 5 or 30 seconds respectively and were therefore the fastest procedures [71]. In general, the tested non-enzymatic procedures take less time than the enzymatic procedures (Table 4A and table 4B).

The costs of only enzymatic procedures Celution system (2013: $1950 and 2016: $2400), CHA-station ($710), Multi station (2013: $460 and 2016: $250), Lipokit system (2013: $530 and 2016: $450) and GID SVF2 ($1000) are mentioned, the enzymatic Celution system being the most expensive [70,72]. No data of non-enzymatic intraoperative procedures were available (Table 4A and table 4B).

Table 4A. Duration, costs and procedure characteristics of intraoperative isolation procedures focusing on cSVF.

Name	Author	Enzymatic/ Non-enzymatic (E/N)	Automatic/ Manual/ Semi (A/M/S)	Open/ closed (O/C)	Isolation details		Time (min)	Disposable (D)/ reusable (R) cost (Dollar)	Volume processed (ml)	Capacity (ml)	End volume (ml)	Maximum volume processed /maximum end volume
AIS	SundarRaj et al. 2015	E	A	C	Tissue digestion, heating and agitation, three-stage filter system (100 micron, 35 micron, 5 micron porosity)	-	133	-	-	500	10.8 [4-20]	-
CHA	Aronowitz et al. 2013	E	S	C	Fat bag, adapter, centrifugation, shaking incubator and tissue digestion, cell strainer, cell counter	Collagenase	88+/23	D710	80-180	180	-	-
CYT	Aronowitz et al. 2013	E	A	C	Washing (lactated Ringer), tissue digestion and agitation, centrifugation	Celase/ Reagent A	90 +/16	D1950	100-180	360	-	-
	Aronowitz et al. 2016						89.4 [85-93]	D2400 per 120-360 ml	126 [90-150]	360	5 [5]	30
	Domenis et al. 2015						60	D	250	-	Pellet	-
	Lin et al. 2008						90	-	-	-	Pellet	-
GID-SVF2	Aronowitz et al. 2016	E	M	C	Disposable canister for harvesting, filtration, separation and concentration	GIDzyme-50	71.4 [68-75]	D1000 per 20-120 ml	53.2 [32-88]	120	7.2 [6-9]	13.3
LIPOK	Domenis et al. 2015	E	S	C	1200 xg centrifugation (with a weight-mesh filter piston), celltibator	Liberase (collagenase mixture)	-	-	-	-	-	-
	Aronowitz et al. 2013					Collagenase	111+/-18	D530	60-100	100	-	-
	Aronowitz et al. 2016					Time Machine accelerator	120.8 [99-149]	D450 per 100 ml	71.4 [40–97]	400	20 [15-25]	3.9
PNC	Aronowitz et al. 2013	E	M	O	Centrifugation, shaking incubator, clean bench, HEPA filter, UV-lamp	Collagenase	115+/-13	D460	100-150	400	-	-
	Aronowitz et al. 2016						65.4 [59-74]	D250 per 100 ml	105.6 [68-150]	800	12.2 [10.5-15]	10

Table 4A. Continued.

Name	Author	Enzymatic/ Non-enzymatic (E/N)	Automatic/ Manual/ Semi (A/M/S)	Open/ closed (O/C)	Isolation details		Time (min)	Disposable (D)/ reusable (R) cost (Dollar)	Volume processed (ml)	Capacity (ml)	End volume (ml)	Maximum volume processed /maximum end volume
SEPAX	Güven et al. 2012	E	A	C	Tissue digestion, priming and straining, centrifugation, washing	0.15% NB6 GMP Grade Collagenase	90-120	-	40-400	-	Pellet	-
TGCIS	Doi et al. 2012	E	A	C	Tissue digestion, centrifugation, washing, 700 xg centrifugation	0.075% collagenase	65	D	20-60	-	Pellet	-

AIS Automated Isolation System; CHA-station (CHA-Biotech); CYT Celution System Enzymatic (Cytori); GID SVF2 (GID Europe); LIPOK Lipokit System (Medi-khan); PNC Multi station (PNC); SEPAX Sepax (Biosafe); TGCIS Tissue Genesis Cell Isolation System (Tissue Genesis)

Table 4B. Duration, costs and procedure characteristics of intraoperative concentration procedures focusing on tSVF.

Name	Author	Enzymatic/ Non-enzymatic (E/N)	Automatic/ Manual/ Semi (A/M/S)	Open/ closed (O/C)	Isolation details		Time (min)	Disposable (D)/ reusable (R) cost (Dollar)	Volume processed (ml)	Capacity (ml)	End volume (ml)	Maximum volume processed /maximum end volume
FAT	Van Dongen et al. 2016	N	M	O	3000 rpm (radius 9.5 cm) centrifugation, shuffling through a 1.4 mm hole connector, 3000 rpm (radius 9.5 cm) centrifugation	n/a	8-10	R	10	10	0.96 [0.75 - 1.75]	10.4
FAST	Domenis et al. 2015	N	M	-	Filterbag (120 micron filter), 400 xg centrifugation	n/a	-	-	-	-	10	-
FEF	Mashiko et al. 2016	N	M	O	1200 xg centrifugation, shuffling through a connector with three small holes 30 times, 1200 xg centrifugation, fluid of decanting filtration (500-mm pore size) used	n/a	-	-	-	-	-	9.9+/-2.0
LIPOG	Bianchi et al. 2013	N	M	C	Filtering, decantation, stainless steel marbles to mix layers (oil, adipose tissue, blood, saline), washing, decantation, reversing devices, filtering	n/a	20	D	40-130	130	60-100	1.3
NANO	Tonnard et al. 2013	N	M	O	Shufling adipose tissue through a female-to-female luerlok 30 times, filtering	n/a	-	-	-	-	-	-
REF	Mashiko et al. 2016	N	M	O	1200 xg centrifugation, shuffling through a connector with three small holes 30 times, 1200 xg centrifugation, residual tissue of decanting filtration (500-mm pore size) used	n/a	-	-	-	-	-	2.5+/-0.2
SF	Mashiko et al. 2016	N	M	O	1200 xg centrifugation, squeeze using automated slicer, 1200 xg centrifugation	n/a	-	-	-	-	-	2.1+/-0.2
SHUF5	Osinga et al. 2015	N	M	O	Shuffling lipoaspirate through female-to-female luerlok 30 times	n/a	5 sec.	-	10	-	-	-
SHUF30	Osinga et al. 2015	N	M	O	Shuffling lipoaspirate through female-to-female luerlok 30 times	n/a	30 sec.	-	10	-	-	-
STCELL	Milan et al.	N	M	C	1000 xg centrifugation	n/a	-	-	400	500	Pellet	-

FAT Fractionation of Adipose Tissue procedure; FAST Fastem Corios (Corios); FEF Filtrated fluid of emulsified fat; LIPOG Lipogems (Lipogems); NANO Nanofat procedure; REF Residual tissue of emulsified fat; SF Squeezed fat; SHUF5 Shuffling 5 times; SHUF30 Shuffling 30 times

Table 4B. Duration, costs and procedure characteristics of intraoperative concentration procedures focusing on tSVF.

Name	Author	Enzymatic/ Non-enzymatic (E/N)	Automatic/ Manual/ Semi (A/M/S)	Open/ closed (O/C)	Isolation details		Time (min)	Disposable (D)/ reusable (R) cost (Dollar)	Volume processed (ml)	Capacity (ml)	End volume (ml)	Maximum volume processed /maximum end volume
FAT	Van Dongen et al. 2016	N	M	O	3000 rpm (radius 9.5 cm) centrifugation, shuffling through a 1.4 mm hole connector, 3000 rpm (radius 9.5 cm) centrifugation	n/a	8-10	R	10	10	0.96 [0.75 - 1.75]	10.4
FAST	Domenis et al. 2015	N	M	-	Filterbag (120 micron filter), 400 xg centrifugation	n/a	-	-	-	-	10	-
FEF	Mashiko et al. 2016	N	M	O	1200 xg centrifugation, shuffling through a connector with three small holes 30 times, 1200 xg centrifugation, fluid of decanting filtration (500-mm pore size) used	n/a	-	-	-	-	-	9.9+/-2.0
LIPOG	Bianchi et al. 2013	N	M	C	Filtering, decantation, stainless steel marbles to mix layers (oil, adipose tissue, blood, saline), washing, decantation, reversing devices, filtering	n/a	20	D	40-130	130	60-100	1.3
NANO	Tonnard et al. 2013	N	M	O	Shufling adipose tissue through a female-to-female luerlok 30 times, filtering	n/a	-	-	-	-	-	-
REF	Mashiko et al. 2016	N	M	O	1200 xg centrifugation, shuffling through a connector with three small holes 30 times, 1200 xg centrifugation, residual tissue of decanting filtration (500-mm pore size) used	n/a	-	-	-	-	-	2.5+/-0.2
SF	Mashiko et al. 2016	N	M	O	1200 xg centrifugation, squeeze using automated slicer, 1200 xg centrifugation	n/a	-	-	-	-	-	2.1+/-0.2
SHUF5	Osinga et al. 2015	N	M	O	Shuffling lipoaspirate through female-to-female luerlok 30 times	n/a	5 sec.	-	10	-	-	-
SHUF30	Osinga et al. 2015	N	M	O	Shuffling lipoaspirate through female-to-female luerlok 30 times	n/a	30 sec.	-	10	-	-	-
STCELL	Milan et al.	N	M	C	1000 xg centrifugation	n/a	-	-	400	500	Pellet	-

FAT Fractionation of Adipose Tissue procedure; FAST Fastem Corios (Corios); FEF Filtrated fluid of emulsified fat; LIPOG Lipogems (Lipogems); NANO Nanofat procedure; REF Residual tissue of emulsified fat; SF Squeezed fat; SHUF5 Shuffling 5 times; SHUF30 Shuffling 30 times

Cell yield

Thirteen studies evaluated the cell yield of eighteen different intraoperative isolation procedures [22,66-77] (Table 5A and table 5B). The reported cell yield after those different procedures varied between 0.19 – 11.7 x 10^5 cells per ml in enzymatic intraoperative isolation procedures and between 1.8 – 22.6 x 10^5 cells per ml in non-enzymatic intraoperative isolation procedures. Non-enzymatic intraoperative procedures yielded higher number of cells since the cell yield was based on 1ml of end volume, whereas the enzymatic intraoperative isolation cell yield was based on the obtained pellet per 1 ml start volume of lipoaspirate. Of the enzymatic intraoperative isolation procedures, the Celution system, Multi station and Lipokit system were evaluated by more than one group of authors [70,72-74]. Interestingly, obvious different yields were seen using the same procedure in different studies [70,72-74]. Reproducibility is thereby questioned in our opinion. The cell yield using the enzymatic Celution system was significantly higher as compared to the Lipokit system ($p=0.004$), the Multi station ($p=0.049$) and CHA-station ($p<0.001$) [72]. In contrast, Domenis et al. did not find a statistical difference between the enzymatic Celution system and Lipokit system. Moreover, Aronowitz et al. again compared the enzymatic Celution system with the Lipokit system and Multi station. This time, Multi station and the Lipokit system resulted in significant more cells as compared to the Celution system ($p<0.05$) [70].

In the non-enzymatic intraoperative isolation procedures, the Squeezed fat, Residual fluid of emulsified fat and Fractionation of fat procedures resulted in the relative highest cell yields per ml harvested lipoaspirate [66,67]. Non-enzymatic intraoperative isolation procedures such as shuffling (5 times and 30 times), the Nanofat procedure and Fastem did not mention the begin and end volumes, so the relative yield by isolation cannot be calculated [68,71,74]. Osinga et al, reported that most of the adipocytes remain intact after shuffling 5 or even 30 times [71]. Consequently, to our opinion, the effect of shuffling only cannot be stated as an isolation procedure. We deem it possible that the lipoaspirate after both two procedures did not differ from the initial lipoaspirate obtained at the start of the procedure. However, the benefit might be at a different level, because shuffling does improve the injectability of lipoaspirates as shown by Tonnard et al. [68].

Table 5A. Cell yield and viability per milliliter start volume of lipoaspirate of all intraoperative isolation procedures per study.

Enzymatic isolation procedure	Cell yield x10^5 cells/ml	SD	Viability nucleated cells (%)	SD
AIS (SundarRaj, 2015)	1,2	0,5	98%	21
CHA (Aronowitz, 2013)	0,6	0,15	87%	12
CYT (Aronowitz, 2013)	2,4*	0,32	93%	2
CYT (Aronowitz, 2016) [a]	1	0,16	84%*	1
CYT (Domenis, 2015)	11,7	0,5		
CYT (Lin, 2008)	3,7	0,86	89%	1
GID SVF2 (Aronowitz, 2016) [a]	2,9	0,65	69%	6
LIPOK (Domenis, 2015)	5	3		
LIPOK (Aronowitz, 2013)	0,3	0,15	72%	15
LIPOK (Aronowitz, 2016) [a]	6,2*	0,25	50%	10
PNC (Aronowitz, 2013)	1,1	0,49	57%	21
PNC (Aronowitz, 2016) [a]	5,4*	1,64	82%*	5
SEPAX (Güven, 2012)	2,6*	1,2		
TGCIS (Doi, 2012)	7	1,89	81%	

**Significantly best procedure tested in their study (p>0.05); [a] No exact data mentioned in text, data extracted from figures by authors JAD and AJT. AIS Automated Isolation System; CHA-station (CHA-Biotech); CYT Celution System Enzymatic (Cytori); GID SVF2 (GID Europe); LIPOK Lipokit System (Medi-khan); PNC Multi station (PNC); SEPAX Sepax (Biosafe); TGCIS Tissue Genesis Cell Isolation System (Tissue Genesis)*

More interesting than comparing intraoperative isolation procedures evaluated in different studies might be the comparison between an intraoperative isolation procedure and a non-intraoperative isolation protocol (gold standard) starting from the same lipoaspirate. Six studies reported the results of such comparisons (Table 6A) [69,73-77]. The Automated isolation system and Tissue genesis cell isolation system resulted in the same cell yield as the non-intraoperative isolation protocol control (effect size, respectively, 0.07 and 0.00) [69,76]. Sepax isolated a higher cell yield compared to a non-intraoperative isolation protocol (effect size 1.11) (Table 6A) [75]. Lower cell yield was seen after using the Lipokit system compared to the non-intraoperative isolation protocol control (effect size -0.52) [74]. Interestingly, the highest positive as well as the most negative effect sizes were seen with the enzymatic Celution system related to regular isolation with a non-intraoperative isolation protocol [73,74].

Table 5B. Cell yield per milliliter of end volume, viability and concentration of concentration procedures.

Non-enzymatic isolation procedure	Cell yield x10^5 cells/ml	SD	Viability nucleated cells (%)	SD	Start volume	End volume	End as % of start volume	Y:C ratio*
FAST (Domenis, 2015)	4,6	2,9	-	-	-	-	-	-
FAT (van Dongen, 2016)	22,6	4,5	-	-	10	1	10%	2.3
FEF (Mashiko, 2016)	1,8[a]	0,5[a]	39%	9	-	-	10%	0.2
NANO (Tonnard, 2013)	-[b]	-	-	-	100	-	-	-
REF (Mashiko, 2016)	6,5[a]	0,8[a]	91%	3	-	-	39%	2.5
SF (Mashiko, 2016)	8,0[a]	0,4[a]	90%	5	-	-	48%	3.8
SHUF5 (Osinga, 2015)	13		65%	-	10	-	-	-
SHUF30 (Osinga, 2015)	11		63%	-	10	-	-	-
STCELL (Millan, 2015)[a]	18,8	4,7	87%	-	100	15	15%	2.8

** Y:C ratio: Yield: Concentration ratio; relative cell yield per processed lipoaspirate volume. [a] No exact data mentioned in text, data extracted from figures by authors JAD and AJT. [b] Cell yield 2*10^6 cells per 100 ml of processed fat by the Nanofat procedure. No cell yield per ml end product can be calculated. FAST Fastem Corios (Corios); FAT Fat procedure; FEF Filtrated fluid of emulsified fat; NANO Nanofat procedure; REF Residual tissue of emulsified fat; SF Squeezed fat; SHUF5 Shuffling 5 times; SHUF30 Shuffling 30 times; STCELL StromaCell*

Table 6A. Effect sizes of studies evaluating enzymatic intraoperative isolation procedures regarding cell yield.

	Enzymatic isolation procedure			Non-intraoperative isolation protocol			
Study	N	Cell yield x10^5 cells	SD	N	Cell yield x10^5 cells	SD	Effect size
AIS, SundarRaj, 2015	11	1.17	0.5	11	1.15	0.30	0,07
CYT, Domenis, 2015	9	11.7	5.0	16	6.7	3.30	1,52
CYT, Lin, 2008	6	3.7	0.9	3	4.96	0.72	-1,75
LIPOK, Domenis, 2015	9	5.0	3.0	16	6.7	3.30	-0,52
SEPAX, Güven, 2012	6	2.6	1.2	6	1.6	0.90	1,11
TGCIS, Doi, 2012	6	7.0	1.9	6	7.0	2.43	0,00

AIS Automated Isolation System; CYT Celution System Enzymatic (Cytori); LIPOK Lipokit System (Medikhan); SEPAX Sepax (Biosafe); TGCIS Tissue Genesis Cell Isolation System (Tissue Genesis)

Table 6B. Effect sizes of studies evaluating viable nucleated cells.

	Procedure			Non-intraoperative isolation protocol			Effect
Study	**N**	**% viable cells**	**SD**	**N**	**% viable cells**	**SD**	**size**
Enzymatic							
AIS, SundarRaj, 2015	11	97.5	2.8	11	97.3	1.5	0.13
CYT, Lin, 2008	3	89.2	1.1	3	90.8	1.3	-1.23
TGCIS, Doi, 2012	6	80.7	7.1	6	82.4	7.7	-0.22
Non-enzymatic							
FEF, Mashiko, 2016	10	39.3	9.1	10	93.8	1.2	-45.4
REF, Mashiko, 2016	10	90.6	2.8	10	93.8	1.2	-2.67
SF, Mashiko, 2016	10	89.9	4.6	10	93.8	1.2	-3.25
STCELL, Millan, 2014 [a]	3	87.7	8.9	3	74.5	20.1	0.66

[a] *No exact data described in text, data extracted from figures by authors JAD and AJT. AIS Automated Isolation System; CYT Celution System Enzymatic (Cytori); FEF Filtrated fluid of emulsified fat; REF Residual tissue of emulsified fat; SF Squeezed fat; STCELL StromaCell; TGCIS Tissue Genesis Cell Isolation System (Tissue Genesis)*

VIABILITY OF NUCLEATED CELLS

Eight studies described viabilities from 39% to 98% of nucleated cells in the SVF. No big differences in viability were seen between enzymatic and non-enzymatic intraoperative isolation procedures. The Filtrated fluid of emulsified fat procedure showed the lowest viability [66], while the Automated isolation system showed the highest viability of nucleated cells of 98% after isolation (Table 5A and table 5B) [69]. Three enzymatic and three non-enzymatic intraoperative isolation procedures were compared to a non-intraoperative isolation protocol regarding the viability of nucleated cells (Table 6B) [69,73,76]. The viability of five intraoperative isolation procedures was comparable to their non-intraoperative isolation protocol controls; the effect sizes were close to zero in many studies (Table 6B). Only the Filtrated fluid of emulsified fat procedure showed an effect size of -45.4 [66]. In general, viability did not differ between non-intraoperative isolation protocols and the individual intraoperative isolation procedures tested.

Composition of stromal vascular fractions

The SVF compositions is reported in nine studies evaluating six enzymatic procedures and three non-enzymatic procedures. The stromal cell population is larger in the SVF isolated by the enzymatic Celution system, Sepax and Tissue genesis cell isolation

system and the non-enzymatic Residual of emulsified fat and Squeezed fat procedures compared to other intraoperative isolation procedures [66,72,75,76] (Table 7). The percentage of stromal cell population of the SVF isolated by the enzymatic Celution system only differs with 25.2% between two studies [72,74] and 32.8% between two other studies, both evaluated by Aronowitz et al. [70,72]. In general, non-enzymatic procedures yielded same amounts of CD31min/CD34pos stromal cells.

The stromal cell population, including pericytes, ASCs and supra-adventitial cells, are the most important cell types in regenerative therapies because of their paracrine effect and multi-lineage differentiation capacity [10,78].

Pericytes defined using other CD markers than to define the stromal cell population are placed separately in the table. The enzymatic Celution system evaluated by Lin et al. resulted in the lowest percentage of pericytes in the SVF (0.8%), but used more than three CD markers to detect pericytes [73]. SundarRaj et al. resulted in a higher percentage (2.0%) of pericytes in SVF obtained by the Automated isolation system, but used only two CD markers to determine the pericyte population and other cell types [69]. The use of multiple CD markers results in a more specific population than the use of less CD markers and so a lower percentage of that specific cell type *e.g.* pericytes [22]. Bianchi et al. used CD34min/CD146pos/CD90pos to detect the pericyte-like population in the SVF and isolated the highest percentage of pericytes using the non-enzymatic Lipogems procedure as compared to other intraoperative isolation procedures [22]. However, Bianchi et al. mostly used other combinations of CD markers in comparison to other studies [22]. This renders their SVF composition incomparable with SVF compositions obtained by other intraoperative isolation procedures.

Table 7. Stromal vascular fraction composition (CD marker) of intraoperative isolation procedures in all studies.

	Enzymatic isolation procedures					
	AIS (SundarRaj, 2015)	**[a]CHA (Aronowitz, 2013)**	**[a]CYT (Aronowitz, 2013)**	**CYT (Aronowitz, 2016)**	**[a]CYT (Domenis, 2015)**	**CYT (Lin, 2008)**
Adipose derived cells (CD45-)						
Vascular endothelial/Endothelial progenitor cells (CD31+/CD34+)	24%	1.1%	4.2%	-	-	8%
Endothelial cells (CD31+/CD34-)	5%	-	-	-	-	-
Pericytes (CD31-/CD146+) (CD31-/CD34-/+) (CD34-/CD146+/CD90+)	2%	-	-	-	-	0.8%
Stromal cell population (CD31-/CD34+)	25%	28%	43.5%	10.7%	18.3%	-
Blood derived cells (CD45+)						
Lymphocytes (CD31+/CD34-/CD90-/CD105-/CD146-)	-	-	-	-	-	19%
Leukocytes (CD31-/CD34-/CD90-/CD105-/CD146-)	-	-	-	-	-	7.6%
Hematopoetic stem cell (CD31dim/CD34+/CD90-/CD105-/CD146-)	-	-	-	-	-	4.6%
Other cell types (CD markers)		-	-	-	-	-
Unknown	44%	70.9%	52.3%	89.3%	81.7%	60%

									Non-enzymatic isolation procedures			
	GID SVF2 (Aronowitz, 2016)	[a]LIPOK (Aronowitz, 2013)	LIPOK (Aronowitz, 2016)	[a]LIPOK (Domenis, 2015)	[a]PNC (Aronowitz, 2013)	PNC (Aronowitz, 2016)	[a]SEPAX (Güven, 2012)	TGCIS (Doi, 2012)	FAST (Domenis, 2015)	[a]LIPOG (Bianchi, 2013)	[a]REF (Mashiko 2016)	[a]SF (Mashiko 2016)
	-	0.7%	-	-	0.9%	-	25%	24.5%	-	-	-	-
	-	-	-	-	-	-	-	-	-	-	26%	28%
	-	-	-	-	-	-	-	2.2%	-	23.2%	-	-
	8.9%	26.5%	7.2%	20%	22%	9%	40%	43.2%	26.7%	-	43%	39%
	-	-	-	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-	49.4%	-	-
	91.1%	72.8%	92.6%	80%	77.1%	91%	35%	30.1%	73.3%	27.4%	31%	33%

Stromal cell population (CD31min/CD34pos) consists of supra-adventitial cells, ASCs and pericytes, only pericytes defined as CD31min/CD146pos, CD31min/CD34min/pos or CD34min/CD146pos/CD90pos are placed separately in the table. [a] No exact data described in text, data extracted from figures by authors JAD and AJT. AIS Automated Isolation System; (CHA-Biotech); CYT Celution System Enzymatic (Cytori); FAST Fastem Corios (Corios); GID SVF2 (GID Europe); LIPOG Lipogems (Lipogems); LIPOK Lipokit System (Medi-khan); PNC Multi station (PNC); REF Residual tissue of emulsified fat; SEPAX Sepax (Biosafe); SF Squeezed fat;TGCIS Tissue Genesis Cell Isolation System (Tissue Genesis)

The enzymatic procedures: Automated isolation system, Tissue genesis cell isolation system and Sepax isolated more endothelial progenitor cells in comparison to other intraoperative isolation procedures [69,75,76]. Nonetheless, more endothelial progenitor cells were not corresponding to less stromal cells or pericytes. In all differently obtained SVF, the origin of large numbers of cells remains unidentified. This is partly because not every study identified both adipose tissue-derived and blood-derived cell types, but probably not every subpopulation of all cell types is already known as well.

When donor variability is neutralized by the use of the same lipoaspirate, intraoperative isolation procedures resulted in different SVF compositions. Lipogems isolated significantly more pericytes and stromal cells than the non-intraoperative isolation protocol control ($p<0.05$, fig. 2) [22]. The enzymatic Celution system resulted in significantly more endothelial progenitor cells in comparison with the CHA-system, Lipokit system and Multi station, which is not necessarily preferred ($p=0.003$) [72]. All other intraoperative isolation procedures compared with non-intraoperative isolation protocols showed no significant differences.

Modified IFATS/ISCT Index Score for the measurement of adipose tissue-derived stromal vascular fraction

Modified IFATS/ISCT index scores ranged from 1 to 4.6 out of 5. Güven et al. scored 4.6 and presented the most complete characterization of the SVF and ASCs [75] (Table 8). Tonnard et al. scored 2 points, but had only used CD34 as a marker to identify a subpopulation in the SVF [68]. Two studies used other methods than flow cytometry to determine the composition of SVF [67,71]. No studies were excluded based on a low number of outcomes of interest measured by the modified IFATS/ISCT Index Score, because five out of thirteen studies scored less than half of the possible points given. This high number of low scores given to studies underlines the need for standardization.

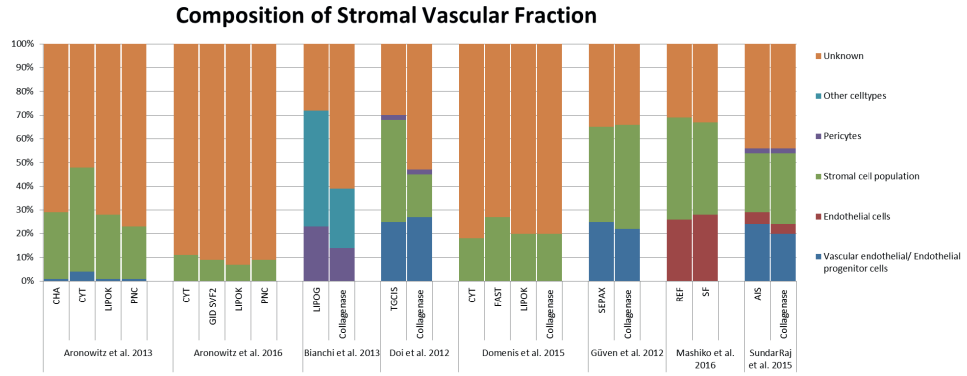

Figure 2. SVF composition (CD marker) of procedures comparing an intraoperative isolation procedure with a non-intraoperative isolation protocol or with other intraoperative isolation procedures within one study. Stromal cell population (CD31min/CD34pos) consists of supra-adventitial cells, ASCs and pericytes, only pericytes defined as CD31min/CD146pos, CD31min/CD34min/pos or CD34min/CD146pos/CD90pos are placed separately in the table. Endothelial cells and vascular/progenitor endothelial cells are described as respectively, CD31pos/CD34min and CD31pos/CD34pos. No exact data described in text by Aronowitz et al., Bianchi et al., Domenis et al., Güven et al. and Mashiko et al., data is extracted from figures by authors JAD and AJT. AIS Automated Isolation System; CHA-station (CHA-Biotech); CYT Celution System Enzymatic (Cytori); FAST Fastem Corios (Corios); GID SVF2 (GID Europe); LIPOK Lipokit System (Medi-khan); PNC Multi station (PNC); REF Residual tissue of emulsified fat; SEPAX Sepax (Biosafe); SF Squeezed fat;Tissue Genesis Cell Isolation System (Tissue Genesis)

Disclosure agreements of included articles

A disclosure agreement of support by the manufacturer was provided in five of the thirteen studies [22,72,73,75,76] (Table 9). The company, which was mostly involved in the studies, was Cytori, the manufacturer of the enzymatic Celution system.

Table 8. Modified IFATS index score for the measurement of adipose tissue-derived stromal vascular fraction.

			Flow cytometry of cultured ASCs										Functional assays			
Studies	**Viability**	**Flow cytometry of SVF**	**CD13**	**CD29**	**CD31**	**CD44**	**CD45**	**CD73**	**CD90**	**CD105**	**CD235a**	**CFU-F**	**Adipogenic**	**Osteogenic**	**Chondrogenic**	**Total Score**
Aronowitz et al. 2013	1	1										1				3.00
Aronowitz et al. 2016	1	1										1				3.00
Bianchi et al. 2013	1	1										0	1/3	1/3	1/3	3.00
Doi et al. 2012	1	1										0				2.00
Domenis et al. 2015	0	1	1/9	1/9		1/9	1/9	1/9	1/9	1/9		1				2.78
Van Dongen et al. 2016	1	0		1/9	1/9	1/9	1/9		1/9	1/9		1	1/3	1/3		3.33
Güven et al. 2012	1	1		1/9		1/9		1/9	1/9	1/9		1	1/3	1/3	1/3	4.56
Lin et al. 2008	1	1										1	1/3	1/3		3.67
Mashiko et al. 2016	1	1										0				2.00
Millan et al.	1	0				1/9		1/9	1/9	1/9		0				1.44
Osinga et al. 2015	1	0										1	1/3	1/3	1/3	3.00
SundarRaj et al. 2015	1	1										1				3.00
Tonnard et al. 2013	0	1										0	1/3			1.33

Table 9. Disclosures of included studies.

Articles	Disclosures
Aronowitz et al. 2013	Loan devices CHA and Cytori
Aronowitz et al. 2016	No disclosures
Bianchi et al. 2013	Lipogems
Doi et al. 2012	Kaneca, Inc
Domenis et al. 2015	No disclosures
van Dongen et al. 2016	No disclosures
Güven et al. 2012	Biosafe SA and loan Sepax
Lin et al. 2008	Cytori Therapeutic Inc
Mashiko et al. 2016	No disclosures
Millan et al. 2014	No disclosures
Osinga et al. 2015	No disclosures
SundarRaj et al. 2015	No disclosures
Tonnard et al. 2013	No disclosures

DISCUSSION

Grafting of lipoaspirates and of SVF in particular, is a rapidly evolving treatment modality for scars and other skin defects, arthritis, neuropathy, diabetic ulcers to mention a few. Many of these, initially small scale, single center studies, are on the verge of expansion to multicenter placebo-controlled double-blind randomized clinical trials. An important prerequisite is the use of an efficient and standardized intraoperative isolation procedure of SVF. This systematic review shows that none of these procedures supersedes other procedures in terms of cell yield, viability and SVF composition while being time and cost efficient too when analyzed using the same lipoaspirate. However, three intraoperative isolation procedures (shuffling 5 times, shuffling 30 times and Lipogems) showed only a minimal reduction of the volume of lipoaspirate, implicating that most of the adipocytes still are intact. Consequently, these three procedures are methods of processing rather than isolation procedures [22,71]. Moreover, there is a wide variation in cell yield, viability of cells and composition of SVF when all intraoperative isolation procedures are compared together. Study characteristics showed small and varied sample sizes regarding the number, sex and age of the donors. It is known that the cell yield and viability of SVF differ among donors, depending on age, harvest location and co-morbidities, such as obesity, of the donors [18-21,79]. This interdonor variability is a possible explanation for the variations

found between several studies. To avoid variation bias, isolation procedures should be investigated using identical lipoaspirates in the same study. There are, however, differences between non-enzymatic and enzymatic isolated SVFs on a different level. Non-enzymatic isolation procedures resulted in larger volumes (tSVF) than the resulting pellets (cSVF) after enzymatic intraoperative isolation procedures. Because the final products of both types of isolation procedures are different, the clinical purpose of the use of SVF is an important factor which isolation procedure suits best. In some cases, such as the intra-articular injection of SVF in temporomandibular joints requires very small volumes, whereas the end volume of SVF enriched lipofilling is less relevant. Isolation procedures of SVF of adipose tissue are based on reduction of large volume containing tissue or cells, such as ECM and/or adipocytes to concentrate the stromal vascular fraction. Non-enzymatic isolation of SVF results in a smaller volume of adipose tissue containing intact ECM and cell-cell communications between SVF cells (tSVF), because the shear forces are too low to disrupt cell to cell and cell to ECM adhesions [12,80]. Therefore, the tissue structure of lipoaspirate is still intact in the tSVF. Enzymatic procedures, however, likely result in a single cell cSVF, because enzymes likely disrupt all cell-cell interactions and ECM (Fig. 3) [15]. This is may not happen in the Automated isolation system, GID SVF2, Lipokit system and Multi station, possibly due to insufficient enzymatic digestion [69,70].

Clinical use of tSVF has several advantages over the use of cSVF in different clinical applications of regenerative medicine. It is well known that single cells migrate within 24 hours after application [81]. The ECM, containing a microvasculature structure, might function as a natural scaffold for cells like ASCs and most likely also augments rapid vascularization and reperfusion. This will probably increase cell retention rates after injection and enhance clinical effects. In case of early scar formation, wound healing, or organ fibrosis, tSVF might therefore be more an appropriate therapy, which implicates that non-enzymatic procedures are more suitable as compared to enzymatic isolation procedures. In case of excessive pre-existing scar formation, the ECM in the SVF might not be appropriate and therefore the application of a cSVF or ASCs might be more eligible. ASCs could remodel excessive scar formation by immunomodulation or instruction of resident cells.

Characterization of subpopulations in the SVF depends upon selection of appropriate markers. Selection of an insufficient number of markers will give a disfigured image of the actual SVF composition (Fig. 3). SVF of adipose tissue can be divided into two major subpopulations based on the expression of CD45, which is a hematopoietic cell marker: adipose derived (CD45min) and blood derived (CD45pos) [7]. Adipose derived cell populations can be divided into endothelial cells (CD31pos) and stromal

cells (CD31min) [7]. Three important subpopulations of the stromal cell population (CD45min/CD31min) are supra-adventitial cells: CD34pos/CD146min, pericytes: CD34pos/min/CD146pos and ASCs: CD34pos/CD90pos/CD105low [7,11,12,82]. Supra-adventitial cells and pericytes are both identified as precursor cells of ASCs, although there remains some controversy about this item [11,12,80,83]. Ideally, to discriminate between those three cell types within the CD45min/CD31min subpopulation, CD146 and/or CD90 markers should be used additionally. However, in most studies two CD markers or inappropriate combinations of CD markers have been used to determine cell types; only Lin et al. used all the aforementioned combinations [73]. Because Lin et al. focus mainly on blood derived cells and not on the stromal cell population or pericytes, this did not affect their results. Doi et al. ascribed CD31min/CD34min/CD45min to the pericyte population, so therefore the CD34pos pericytes will be missed [76]. SundarRaj et al. and Güven et al. used CD34pos/CD31min to determine the number of ASCs [69,75], while pericytes and supra-adventitial cells also express CD34. Therefore, the number of ASCs contains pericytes and supra-adventitial cells as well [7,11]. To cover pericytes, supra-adventitial cells and ASCs, Domenis et al., Aronowitz et al. and Mashiko et al. used CD34pos/CD31min/CD45min to determine the stromal cell population [66,70,72,74]. CD34pos is frequently used as a marker to describe cells with stem cell characteristics in both hematopoietic and non-hematopoietic stem cells [84]. The differences in use of CD marker expression to determine pericytes and the stromal cell population might be a possible explanation for the large variations found in SVF between different studies. No solid conclusions could be made about which isolation procedure generates the most stromal cells or pericytes.

Unfortunately, a limited number of commercially available intraoperative SVF isolation procedures not yet have reached scientific validation at an acceptable level. The American Society for Aesthetic Plastic Surgery (ASAPS) and the American Society of Plastic Surgeons (ASPS) published a position statement in 2012 on fat grafting and stem cells [85]. All specialized equipment for the use of stem cell extraction should be fully verified regarding efficacy and safety before use in clinical settings. In 2013, the IFATS and ICTS proposed guidelines with standardized endpoints and methods to verify and compare SVF isolation procedures [5]. None of the included studies fully verified their isolation procedure according to these IFATS and ICTS guidelines. Moreover, viability was measured in different ways among studies (*e.g.* directly on obtained SVF or after an extra non-intraoperative isolation protocol) and lipoaspirate was processed differently prior to isolation (*e.g.* centrifugation or decantation). For those reasons, we propose new adjusted IFATS and ICTS guidelines to validate intraoperative isolation procedures (Fig. 3). All intraoperative isolation procedures should be validated using centrifuged adipose tissue to determine the actual volume of lipoaspirate prior to isolation. It is

known that increased centrifugal forces have a harmful effect on the viability of fat grafts [86,87]. However, the use of centrifuged adipose tissue is necessary to determine the actual cell yield after an isolation procedure. Furthermore, cell viability of tSVF should be determined directly on tSVF, instead of using an extra non-intraoperative isolation protocol which possibly results in more cell damage. However, the proposed adjusted standardized endpoints and methods by IFATS and ICTS are time-consuming and expensive since it requires cultured ASCs. In order to quickly verify isolation procedures intraoperatively during clinical trials, the end product of non-enzymatic intraoperative isolation procedures should be centrifuged to separate the oily fraction from the tSVF and pellet fraction based on density. For enzymatic intraoperative isolation procedures, microscopy can be used to visualize single cells. In this way, isolation procedures can be quickly evaluated during clinical trials.

A large number of SVF isolation procedures without applying a full verification according to the IFATS and ICTS guidelines is available [14]. Oberbauer et al. presented a narrative overview of enzymatic and non-enzymatic intraoperative SVF isolation procedures [14]. In twenty-one out of thirty (both enzymatic as well as non-enzymatic) intraoperative isolation procedures reported in their study, there was a lack of verification data. In two studies intraoperative isolation procedures without scientific evidence *e.g.* viability of SVF, flow cytometry of SVF cells and ASCs, were used to treat patients. One study used SVF obtained by ultrasonic cavitation to treat patients with migraine and tension headache [88]. Another study used SVF in combination with platelet rich plasma for meniscus repair [89]. Hence, it cannot be guaranteed that the isolation procedures indeed isolate SVF, which is clinical safe for use. It seems that the use of most SVF isolation procedures with its concomitant clinical application is far ahead of a sound scientific base upon which these procedures should be used.

Moreover, the clinical safety of isolated SVF or ASCs is not clear yet, especially regarding clinical use in patients with any kind of malignancy. It is demonstrated, *in vitro*, that ASCs influence growth, progression and metastasis of cancer cell lines through *e.g.* promoting angiogenesis and differentiation of ASCs into carcinoma-associated fibroblasts [90]. Zimmerlin et al. showed *in vitro* that ASCs influence growth of active malign cell lines, but this is not seen in latent cancer cell lines [91]. Clinical data suggest that the use of isolated SVF or ASCs is safe in patients without an oncological history [92]. *In vitro* studies often use higher concentrations of ASCs as compared to clinical studies and this might be the cause of differences found between *in vitro* and *in vivo* studies [92]. However, to test clinical safety it is important to reach scientific validation of the commercially available procedures at an acceptable level. In this review it become clear

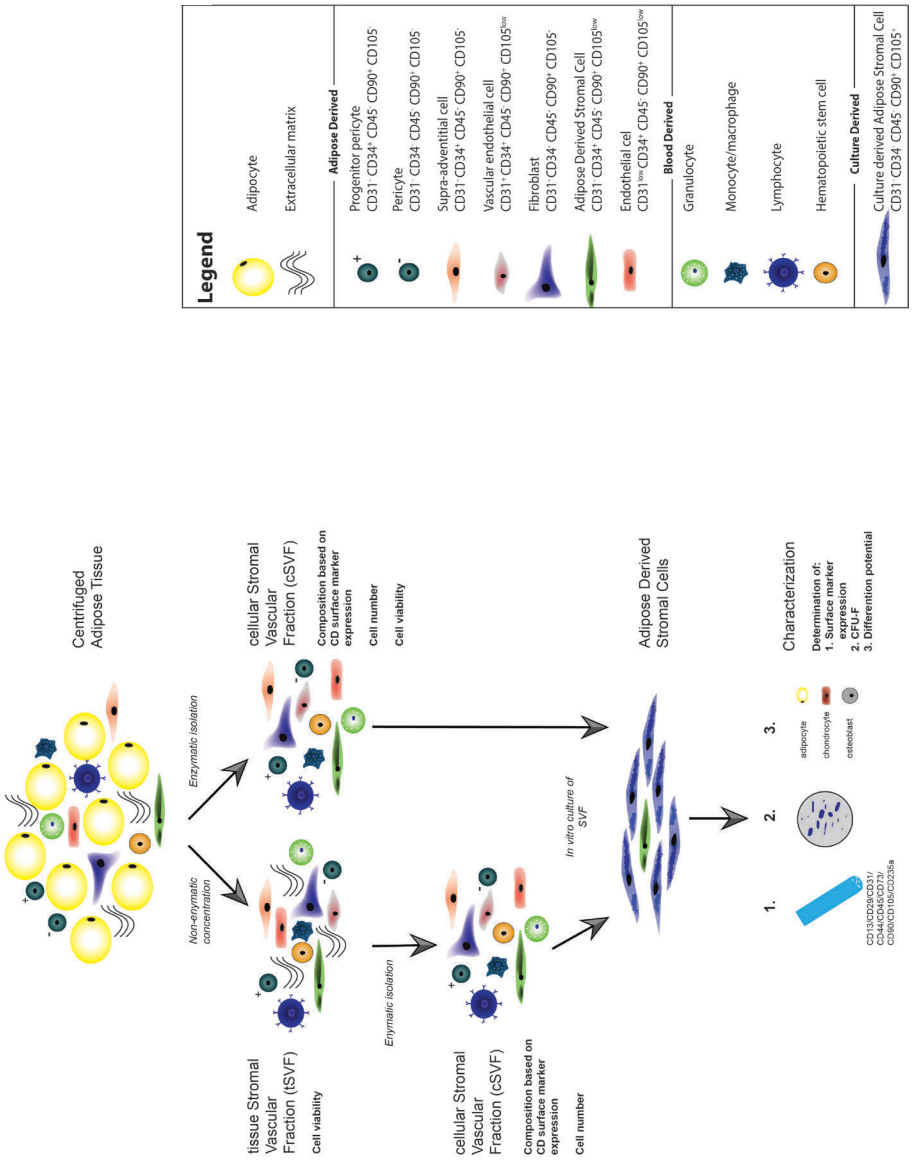

Figure 3. Schematic overview of enzymatic versus non-enzymatic intraoperative isolation and characterization of the obtained cSVF or tSVF.

that the reproducibility of the procedures as well as characterization of the SVF had shortcomings. If this is reached, further scientific research with proper controls with regard to the clinical effect and safety of SVF or ASCs are definitely wanted.

CONCLUSION

There is no evidence thus far that any intraoperative isolation procedure could be designated as preferred procedure for isolating SVF. However, three isolation procedures are rather processing techniques than isolation procedures. Enzymatic and non-enzymatic procedures had comparable results as it comes to cell yield, viability, and SVF composition. Non-enzymatic isolation procedures end products resulted had greater volumes (tSVF) than the pellets (cSVF) of the enzymatic isolation procedures. The results of intraoperative isolation procedures are comparable with those of the gold standard, the collagenase based non-intraoperative isolation protocol. Since intraoperative isolation procedures are less time-consuming, but as efficient as the non-intraoperative isolation protocol, the use of intraoperative isolation procedures seems to be more suitable for clinical purposes. However, only small sample sizes have been used to validate the isolation procedures. To test clinical safety, it is important to reach scientific validation of the commercially available procedures at an acceptable level. Regarding to this review, this level is not yet reached by many procedures.

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03

The Fractionation of Adipose Tissue (FAT) procedure to obtain stromal vascular fractions for regenerative purposes

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ABSTRACT

Autologous adipose tissue transplantation is clinically used to reduce dermal scarring and to restore volume loss. The therapeutic benefit on tissue damage more likely depends on the stromal vascular fraction of adipose tissue than on the adipocyte fraction. This stromal vascular fraction can be obtained by dissociation of adipose tissue, either enzymatically or mechanical. Enzymatic dissociation procedures are time-consuming and expensive. Therefore, we developed a new inexpensive mechanical dissociation procedure to obtain the stromal vascular fraction from adipose tissue in a time sparing way, which is directly available for therapeutic injection. This mechanical dissociation procedure is denoted as the Fractionation of Adipose Tissue (FAT) procedure. The FAT procedure was performed in eleven patients. The composition of the FAT-stromal vascular fraction was characterized by immunohistochemistry. Adipose derived stromal cells isolated from the FAT-stromal vascular fraction were compared with adipose derived stromal cells isolated from non-dissociated adipose tissue (control) for their CD-surface marker expression, differentiation and colony forming unit capacity. Case reports demonstrated the therapeutic effect of the FAT-stromal vascular fraction. The FAT-stromal vascular fraction is an enrichment of extracellular matrix containing a microvasculature and culturable adipose derived stromal cells. Adipose derived stromal cells isolated from FAT-stromal vascular fraction did not differ from adipose derived stromal cells isolated from the control group in CD-surface marker expression, differentiation and colony forming unit capacity. The FAT procedure is a rapid effective mechanical dissociation procedure to generate FAT-stromal vascular fraction ready for injection with all its therapeutic components of adipose tissue: it contains culturable adipose derived stromal cells embedded in their natural supportive extracellular matrix together with the microvasculature.

INTRODUCTION

Nowadays, autologous adipose tissue transplantation is frequently used to reduce dermal scarring[1,2] and to restore volume loss[3] as is the case with soft tissue defects, facial fat atrophy[4,5] and ageing of the face.[6] The therapeutic benefit of autologous adipose tissue transplantation in these aforementioned situations is partly ascribed to the presence of precursors of Adipose Derived Stromal Cells (ASC) in this autologous adipose tissue albeit that this awaits formal proof. ASC are progenitor cells that reside in the Stromal Vascular Fraction (SVF) of adipose tissue attached around the vessels as pericytes and periadventitial cells.[7,8] *In vitro*, these precursors readily adhere to tissue culture plastic after their enzymatic dissociation from other cells in the SVF. During culture, the typical ASC phenotype emerges, *e.g.* the secretion of a plethora of growth factors, cytokines, matrix proteases and extracellular matrix molecules. [9] Furthermore, these cells are able to differentiate into ectodermal[10], endodermal[11] or mesenchymal cell-lineages.[12] Therefore, ASC seem to be suitable for cell-based therapy to repair damaged tissue in organs and aged skin.[13] For example, in cardiology ASC are already used for the clinical experimental treatment of ischemic cardiomyopathy[14] to repair heart muscle cells, in orthopedics ASC are used to treat intervertebrate disc degeneration[15] and in cosmetic facial surgery ASC are reported to be beneficial for skin rejuvenation.[16]

The SVF of adipose tissue comprises all non-adipocyte cells, *e.g.* fibroblasts, leukocytes, endothelial cells, and the connecting extracellular matrix (ECM).[17] As a whole, the SVF potentially harbors more therapeutic capacity than the ASC alone: the ECM can augment tissue remodeling, because it acts as a temporary scaffold that also contains matrix remodeling enzymes and because it serves as a slow-release reservoir of growth factors. Moreover, the fragmented microvasculature may augment reperfusion after injection of SVF.[18-21] Therefore, it would be ideal to harvest this SVF fraction as an intact and injectable 'unit' to be used in a more specific way such as in cell-based therapy. This SVF can be isolated from adipose tissue either by means of enzymatic dissociation or by means of mechanical dissociation. Most enzymatic techniques used thus far for isolating the SVF from adipose tissue use collagenase.[22] Main disadvantage of all these enzymatic techniques is that they are rather time-consuming and expensive, but also that enzymatic treatment disrupts all communicative connections that exist between the cells as well as between the cells and ECM. Moreover, legislation in several countries does not allow to clinically apply cell-based products that are derived with collagenase.

Mechanical dissociation of adipose tissue to isolate the SVF, however, might be more suitable as compared to enzymatic dissociation, because mechanical dissociation of

adipose tissue is a fast time sparing inexpensive method. In this study we describe a new method to mechanically dissociate adipose tissue intra-operatively to get hold of its therapeutic components for injection: this procedure is named the FAT procedure (Fractionation of Adipose Tissue).

MATERIAL & METHODS

Liposuction and FAT procedure

Adipose tissue harvesting was performed with a Sorenson lipo-harvesting cannula (Tulip, Medical Products®, San Diego, Calif. QR link video) during normal liposuction procedures in eleven patients (Fig. 1, Supplemental Content). Adipose tissue was harvested after infiltration with 500ml-modified Klein's solution (per 500ml of saline, 20ml of lidocaine, 2% Epinefrine 1:200.000 and 2ml of bicarbonate was added). For the FAT procedure harvested adipose tissue was centrifuged at 3,000 rpm with a 9.5 cm radius fixed angle rotor for 2.5 min (Medilite™, Thermo Fisher Scientific, NY) at room temperature (RT) after decantation. After one round of centrifugation, the oil and infiltration fluid fractions were discarded, yielding condensed lipoaspirate. One sample of 10ml of condensed lipoaspirate that was centrifuged only once was used as a control, from heron referred as 'control'. One sample of 10ml of condensed lipoaspirate was used for mechanical dissociation. For mechanical dissociation, two 10ml syringes with a total volume of 10ml of condensed lipoaspirate were connected to the Fractionator, a Luer to Luer transfer with three 1.4 mm holes (Tulip) (Fig. 2, Supplemental Content). Mechanical dissociation was performed by pushing the lipoaspirate through the Fractionator forward and backwards thirty times. After mechanical dissociation, the adipose tissue was centrifuged again at 3,000 rpm with a 9.5 cm radius fixed angle rotor for 2.5 min at room temperature (Medilite). This resulted in four different fractions: an oily fraction, a stromal vascular fraction (from heron referred as 'FAT-SVF') and an aqueous fraction containing a small pellet fraction (Fig. 1).

Stromal Vascular Fraction Viability

A live dead staining with Carboxyfluorescein Diacetate Succinimidyl Ester (CFDA-SE) and Propidiumiodide (PI) was performed to measure the FAT-SVF viability compared to the control (n=3). For CFDA-SE and PI staining, pre-warmed 0.001% of CFDA-SE and 0.001% of PI in serum free Dulbecco's Modified Eagle's Medium (DMEM) was used directly on the FAT-SVF and control. After 30 min of incubation under culture conditions cells were washed with Phosphate Buffered Saline (PBS) three times and fixed with 2% paraformaldehyde (PFA). 0.0002% DAPI in PBS was used to stain the

nuclei. As a dead cell control a few samples were fixed with 2% PFA in PBS and then stained with PI. Results were evaluated with a confocal immunofluorescence microscope (Leica TCS SP2 AOBS spectral confocal microscope).

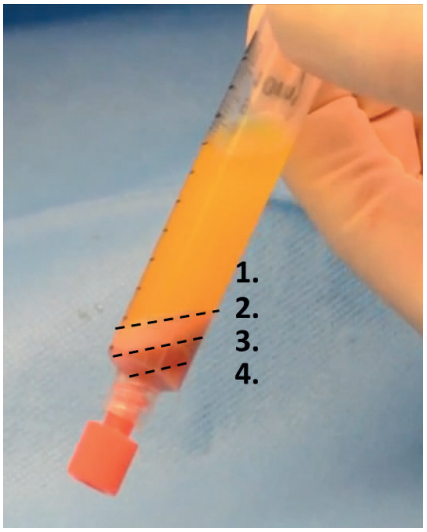

Figure 1. The mechanical dissociation procedure results in four different fractions: **(1)** oily fraction, **(2)** SVF and **(3)** infiltration fluid fraction containing **(4)** a small pellet fraction.

Immunohistochemistry & Masson's Trichrome

Four of the obtained samples of FAT-SVF and control were formalin-fixed and embedded in paraffin. 4 µm slides were deparaffinized and incubated overnight with 0.1 M Tris/HCL buffer (pH 9.0) for α-Smooth Muscle Actin (α-SMA) and Perilipin A, von Willebrand Factor (vWF) staining was pre-incubated with 10 mmol Tris/1 mmol EDTA buffer (pH 9.0). Then, endogenous peroxidase activity was blocked with 30% hydrogen peroxide in PBS for 30 min RT. Samples were washed with PBS three times and incubated with primary antibody and 1% BSA in PBS for 1 hour RT. 1% human serum (HS) for α-SMA and Perilipin A and 1% swine serum for von Willebrandt (vWF) was added to the primary antibody and 1% BSA. Negative controls were incubated without primary antibodies. Subsequently all samples, including negative controls, were washed with PBS three times and incubated with secondary antibody, 1% BSA, 1% HS in PBS for 30 min RT. Only a third antibody in 1% BSA and 1% HS in PBS for 30 min was used in α -SMA staining. Staining was completed with 3,3'-Diaminobenzidine (DAB) incubation (Sigma® Life Science, St. Louis, MO, USA) and hematoxylin

staining of nuclei. Finally, samples were mounted with aqueous mounting agent and visualized under light microscope (Leica Microsystems, DM IL). A Masson's Trichrome staining was performed as well after 4 μm slides (n=4) were deparaffinized. Samples were mounted with toluene solution and visualized under light microscope (Leica Microsystems, DM IL).

Primary antibodies used in this study were directed against alpha smooth muscle actin (αSMA, 1:200, Abcam, Cambridge, UK) to stain smooth muscle cells, vWF (1:200, DAKO, Glostrup, Denmark) and Perilipin A (1:200, Abcam, Cambridge, UK) to stain adipocytes and. Secondary antibodies used in this study were polyclonal Rabbit anti-Mouse for α –SMA (1:100, DAKO, Glostrup, Denmark), polyclonal Swine anti-Rabbit for vWF (1:100, DAKO, Glostrup, Denmark) and polyclonal Goat anti-Rabbit (1:100, DAKO, Glostrup, Denmark) for Perilipin A. Third antibody used was polyclonal Swine anti-Rabbit (1:100, DAKO, Glostrup, Denmark).

Cell Isolation and Culture

Ten of the obtained samples (FAT-SVF & control) were washed with PBS three times. After washing, 0.1% collagenase A in PBS/1% bovine serum albumin (BSA) as dissociation medium was added. The samples were stirred for 1.5 hour in a 37°C water bath. Cells were resuspended in lysisbuffer and placed on ice for 5 min to disrupt erythrocytes. The samples were centrifuged at 8°C, 600xg for 10 min and resuspended in DMEM (BioWhittaker Walkersville, MD): 10% Fetal Bovine Serum (FBS), 1% L-Glutamine, 1% Penicillin/Streptomycin (P/S). Then seeded in a 6-well plate or 25cm^2 tissue culture flask depending on the amount of cells. Cells were counted upon staining with Trypan-blue in a Bürker Turk counting chamber. Cells were cultured at 37°C at 5% CO_2 in a humidified incubator, medium was refreshed twice a week. Morphology of the cells derived from the FAT-SVF and control were compared with after 8 days of culture and visualized with an inverted light microscope with Nomarski phase contrast (Leica Microsystems, DM IL).

Flow cytometry

Cells (n=6) collected from cultures after passage 2-4 were analyzed for CD surface marker expression using flow cytometry. Cells were stained with two different sets of anti-human monoclonal antibodies.

Subset A: CD31- phycoerythrine/cyanine7 (Pe/Cy7; IQ Products, Groningen, the Netherlands), CD45-fluorescein isothiocyanate (FITC; IQ products, Groningen, the Netherlands) and CD90-allophycocyanin (APC; BD Bioscience, San Jose, CA, USA).

Subset B: CD29-APC (eBiosience, Vienna, Austria), CD44-FITC (BD Bioscience, San Jose, CA, USA) and CD105-Pe/Cy7 (eBiosience, Vienna, Austria). As isotype controls, the following monoclonal antibodies were used: Mouse IgG1 kappa-Pe/Cy7, Mouse IgG1 kappa-APC (both eBioscience, Vienna, Austria) and Mouse IgG1 kappa-FITC (Biolegend, San Diego, CA). Cells were mixed well with each antibody and incubated on ice and in dark for 30 min. Isotype cells were used to set the gates on the basis of the staining. A BD FACScalibur system was used to analyze the samples.

Adipogenic, Osteogenic & Smooth Muscle cell differentiation assay

Cells from passage 2-4 (n=6) were collected and cultured in a 24-well plate and incubated in DMEM (containing 10% FBS, 1% L-Glutamine and 1% P/S). After reaching confluence, adipogenic, osteogenic and smooth muscle cell differentiation medium was added to show lipid accumulation, mineralization of bone-like noduli and expression of filamentous actin (F-actin) respectively. Adipogenic differentiation medium consisted of DMEM, 0,1µM dexamethasone, 1 nM insulin, 0,5 mM isobutymethylxanthine (IBMX). Osteogenic medium consisted of DMEM, 0.1µM dexamethasone, 10 mM β-glycerophosphate and 0.05 mM ascorbic acid. Smooth muscle cell differentiation medium consisted of DMEM and 0.1% TGF-beta1. Cells were cultured in differentiation medium for 14 days. Medium was refreshed twice a week. After 14 days, cells were fixed with 2% PFA and stained with Oil Red O (Sigma-Alderich, St. Louis, MO, USA) for adipogenic differentiation, Alizarin Red (Sigma-Alderich, St. Louis, MO, USA) for osteogenic differentiation and Phalloidin-FITC (1:250, Invitrogen™, Thermo Fisher Scientific, NY) in DAPI for 30 min for smooth muscle cell differentiation. Oil Red O staining and Alizarin Red staining were evaluated with a light microscope (Leica Microsystems, DM IL). Phalloidin-FITC staining was evaluated with an immunofluorescence microscope (Leica Microcystems, DM IL).

Colony formation assay

Hundred and thousand cells of FAT-SVF and control (n=3) were plated in a 6-well plate in triplicate and cultured for fourteen days. Cell culture plates were washed with PBS three times and fixed with 2% PFA in PBS for fifteen min. Cells were again washed with PBS three times and stained with 0.05% Crystal Violet (Sigma-Aldrich). After staining, cells were washed with tap water two times and dried inverted. The cloning efficiency or capacity to form colonies from single seeded cells was assessed by determining the area of colonies as well as their intensity of staining with Crystal Violet, both of which are surrogate determinants for the number of cells. Colony area and intensity were analyzed using a plugin for imageJ.[23] Images were taken using Tissue FAXS microscope (TissueGnostics, Vienna, Austria).

Statistical analysis

Immunohistochemistry images and colony formation assay images were analyzed using ImageJ, version 1.4.3.67 (NIH, USA). Descriptive statistics were used to evaluate the cell numbers, α-SMA and vWF expression, CD surface expression scores and colony area and intensity. Data were expressed as mean ± standard deviation (SD). A t-test was performed using Graphpad Prism, version 5.01 (Graph Pad Software Inc., Los Angeles).

RESULTS

Only living cells in the Stromal Vascular Fraction

In the FAT-SVF as well as in the control only living cells (green) were detected (Fig. 2) in contrast to the dead cell (red) controls that contained only dead cells (Fig 3, Supplemental Content).

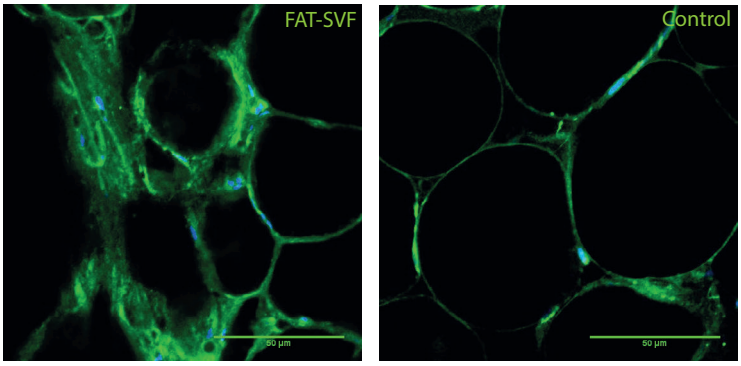

Figure 2. Immunofluorescent images of FAT-SVF and control viability. Living cells turned green, while dead cells turned red after staining with CFDA-SE/PI. Nuclei turned blue after staining with DAPI.

Immunohistochemistry & Masson's Trichrome

The fraction of small vessels visible in FAT-SVF (determined as mean α-SMA positivity of 6.2%, [+/- 5.5] and mean vWF positivity of 7.0%, [+/- 4.2]) was higher than in the controls (mean α-SMA positivity of 0.6%, [+/-0.6] and mean vWF positivity of 0.3%, [+/-0.3]), respectively $p<0.05$ and $p<0.01$ (Fig. 3). Interestingly, in both groups, vessels were present that did not express vWF. Likely, the procedure to generate the FAT-SVF had activated part of the vessels and caused these to release their vWF. Von Willebrand Factor and other 'damage-response' proteins, are stored in the Weibel Palade bodies within endothelial cells, the contents of which are secreted after stress-induced

activation such mechanical shear. Therefore, only quantification of vWF was performed on samples that expressed vWF staining, implying the presence of non-activated EC. Perilipins are lipid-coating proteins that protect against lipase action. Therefore, the presence of Perilipin marks intact adipocytes. After mechanical dissociation, adipocyte 'ghosts' were present in the FAT-SVF occasionally (Fig. 3). Mainly large adipocytes were visible in the control, whereas only smaller ones in the FAT-SVF. Masson's Trichrome staining showed more collagen deposition (blue) between the cells (red) in the FAT-SVF as compared to the control (Fig. 3), implying enrichment of extracellular matrix in the FAT-SVF by disrupting adipocytes compared to controls.

Mechanical dissociation cell yield

Complete mechanical dissociation of adipose tissue was performed intraoperatively within 8 to 10 minutes of extra operating time and resulted in a FAT-SVF with a mean volume of 0.96 ml [range 0.75 to 1.75]. Enzymatic isolation of the FAT-SVF resulted in a mean number of cells of 2.7 $x10^6$ [+/- 1.1 $x10^6$] per 1 ml (Fig. 4). Enzymatic isolation of 10ml of the control resulted in a mean number of cells of 3.5 $x10^5$ [+/-5.1 $x10^6$] per 1 ml ($p<0.001$), a 7.7 times lower number of cells in 1 ml adipose tissue as compared to 1 ml of FAT-SVF (Fig. 4). Three patients were excluded from the study. One patient had an uncountable low number of cells in the FAT-SVF sample. In one FAT-SVF and one control sample of two different patients, we were unable to culture enough cells to reach confluency. Cultured cells had a spindle-shaped fibroblast-like morphology in both samples (Fig. 4, Supplemental Content). There was no visible difference between both groups.

Cells cultured from mechanically dissociated adipose tissue harbor ASC characteristics

For subset A, a mean of 99.8% [+/-0.2] of the FAT-SVF showed expression of CD90 and no expressions of CD31 and CD45 were shown. For the control, a mean of 99.8% [+/-0.1] showed expression of CD90 with also no expressions of CD31 and CD45. There was no significant difference between the two groups ($P>0.05$).

For subset B, a mean of 99.8% [+/0.2], 99.0% [+/0.7] and 95.9% [+/- 4.5] of the FAT-SVF showed expression of CD29, CD44 and CD105. For the control, a mean of 98.2% [+/-3.5], 98.5% [+/1.2] and 96.6% [+/2.6] showed expression of CD29, CD44 and CD105, respectively. No significant differences occurred between the two groups ($P>0.05$). Representative data of CD-surface marker expression is shown in figure 5, Supplemental Content.

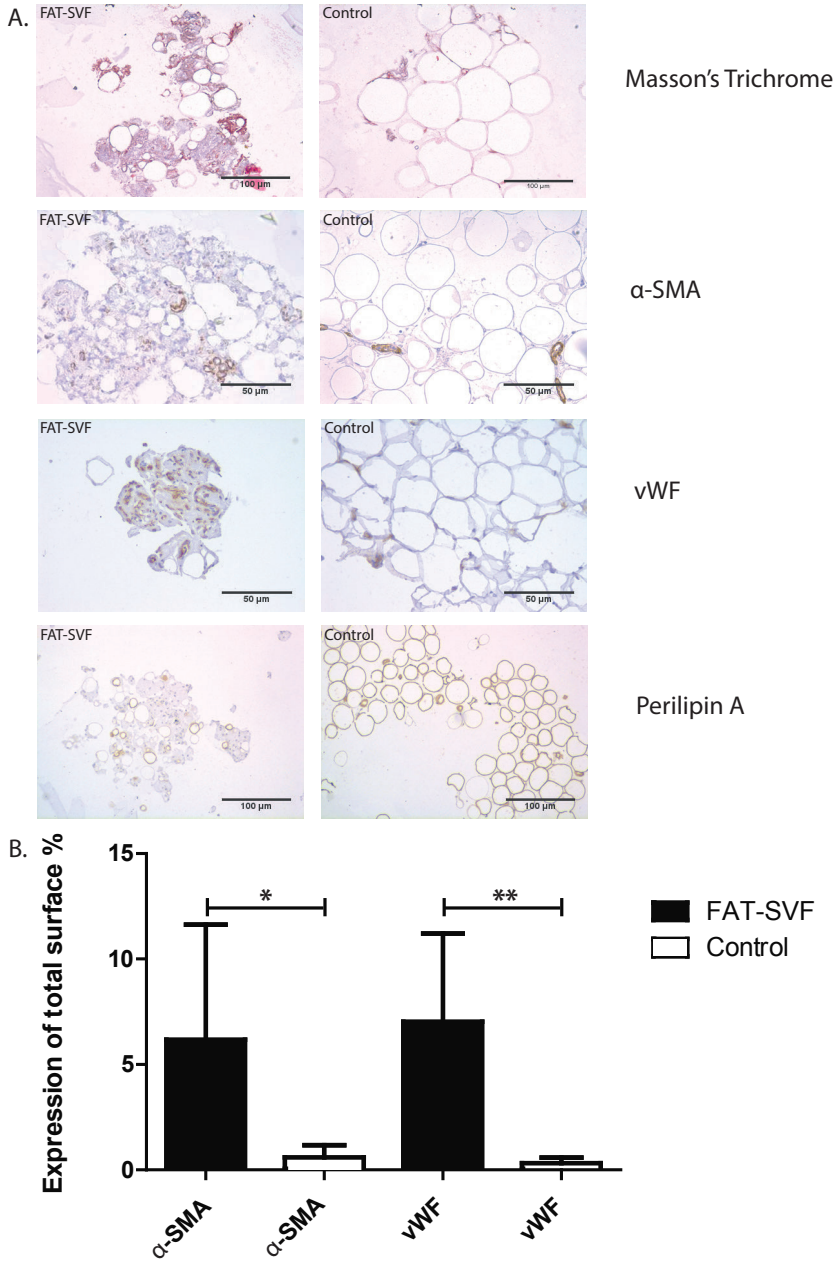

Figure 3. (A) Light microscope images of Masson's Trichrome, α-SMA, vWF and Perilipin A staining of the FAT-SVF and control samples. **(B)** Statistic data of expression of α-SMA and vWF in the FAT-SVF as well as in the control. Results are presented as mean ± standard deviation (SD). *Significant more small vessels were visible in the FAT-SVF as compared to the control ($p<0.05$). **Significant more endothelial cells visible in the FAT-SVF compared to the control ($p<0.01$).

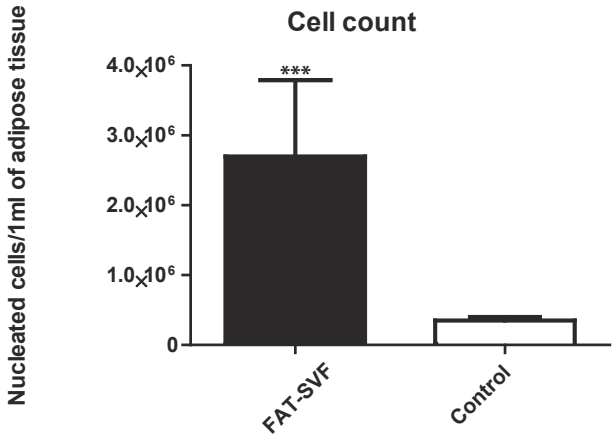

Figure 4. Statistic data of number of nucleated cells per 1 ml of FAT-SVF and control samples. Results are presented as mean ± standard deviation (SD). *** FAT-SVF contains significant more nucleated cells in 1 ml as compared to 1 ml of control ($p<0.001$).

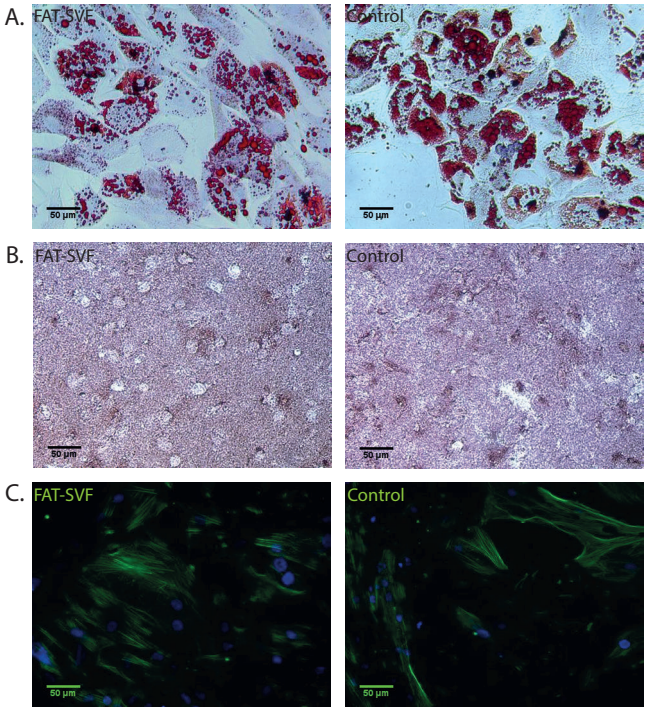

Figure 5. Light microscope and immunofluorescent images of ASC derived from FAT-SVF and control. **(A)** After Oil Red O staining, lipid droplet accumulation turned red. **(B)** After Alizarin Red staining, mineralized bone-like nodules turned red. **(C)** After phalloidin-FITC staining, F-actin turned green. No visual differences between FAT-SVF and control.

Cells cultured from FAT-SVF retain Adipogenic, Osteogenic and Smooth Muscle Cell differentiation capacity

All samples of the FAT-SVF and the control showed significant more lipid droplet formation, mineralized bone-like noduli formation and f-actin expression compared to their negative controls (Fig. 6, Supplemental Content). No significant differences were present between the FAT-SVF and the control (Fig. 5). As ASC are known to express F-actin in all cases, all negative controls were slightly positive as anticipated. One sample of the control group detached, most likely due to contraction of F-actin.

Cells from FAT-SVF retain colony formation capacity

Total colony areas and colony intensities did not differ between ASC derived from the FAT-SVF and controls. This was irrespective of the initial seeding density (P>0.05) (Fig. 6).

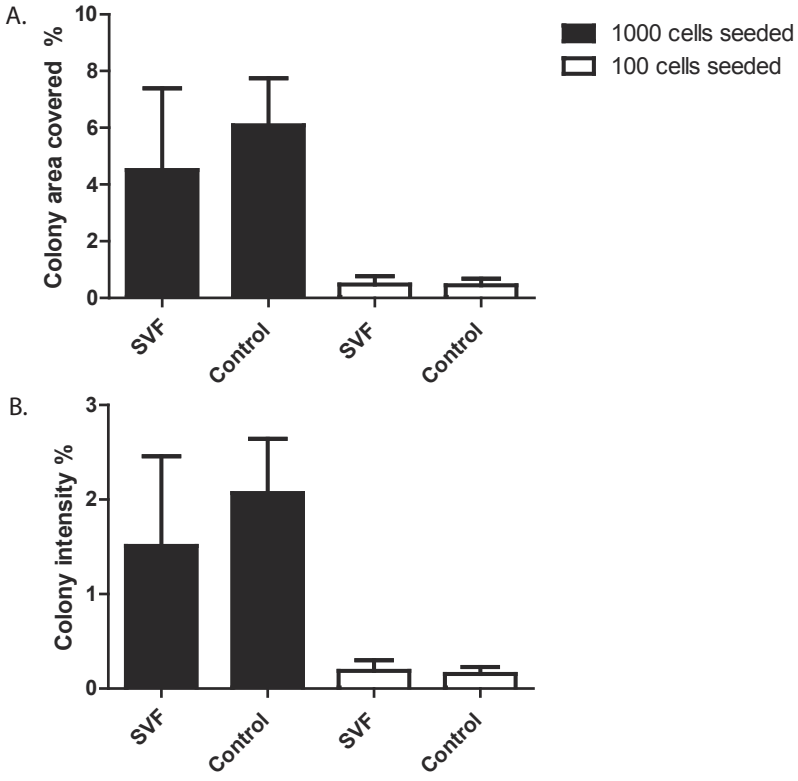

Figure 6. Statistic data of **(A)** total colony area covered and **(B)** colony intensity of ASC derived from FAT-SVF and control. No significant differences in colony area covered and intensity between ASC derived from FAT-SVF and control when hundred or thousand cells were seeded (P>0.05).

DISCUSSION

In this study, we have demonstrated that the FAT procedure is both efficient and reliable intra-operative method to isolate the FAT-SVF from adipose tissue within 8 to 10 min in a simple and reproducible way, suitable for injection: the FAT procedure dissociates lipoaspirate by disrupting adipocytes mechanically and consequently results in a stromal vascular fraction containing culturable ASC as well as small vessels embedded in an extracellular matrix. This FAT-SVF is indicated to increase scar remodeling and might even accelerate wound healing *in vivo.*

ASC derived from the FAT-SVF as well as ASC from the control showed similar expression with regard to the CD-surface markers, had similar like spindle shaped fibroblast-like morphology, the same multipotent differentiation potential and colony formation capacity.

Phenotypically, ASC have two main characteristics. First, ASC possess markers such as mesenchymal markers (CD29+, CD44+, CD90+ and CD105+) and endothelial and hematopoietic markers (CD31- and CD45- respectively).[24] ASC freshly isolated from adipose tissue are CD105-, but become CD105+ after culturing.[17] Therefore, the variation of CD105+ cells can be larger than for other CD surface markers. Furthermore, endothelial cells also express CD105, which is TGF-beta receptor type III, which might influence the FACS results.[17] Second, ASC are able to attach to culture plastic flasks and present as having a spindle shaped fibroblast-like morphology. Functionally, ASC are characterized by their ability to differentiate into adipocytes, osteoblasts and smooth muscle cells.[12,24] Since there are no significant or visual differences in function and phenotype between ASC derived from the SVF and the control, the FAT procedure apparently does not affect the potential of ASC. Furthermore, the FAT procedure results in a FAT-SVF that contains only living cells and significantly more nucleated cells in 1 ml of adipose tissue as compared to non-dissociated lipoaspirate.

The FAT procedure is a reproducible method to generate consistent volumes of injectable FAT-SVF. The composition of the FAT-SVF is histologically heterogeneous: some parts consist of small blood vessels mainly, while other parts consists of ECM only. We surmise that the mechanical disruption of the adipose tissue causes a redistribution of softer (extracellular matrix) and harder (microvasculature) component of the FAT-SVF. The heterogeneity, however, could also be a consequence of inter-patient variation. For this study anonymized samples were obtained. Therefore, future clinical trials might reveal variation among patients. Von Willebrand Factor staining was only present in the non-activated endothelial cells in the FAT-SVF. Activated endothelial cells secrete

their vWF and respond therefore negative to the vWF staining. The ratio between activated and non-activated endothelial cells, however, remains unclear. Quantification of the number of adipocytes as compared to the surface area of FAT-SVF proved methodologically to be impossible. When all adipocytes are mechanically disrupted, the FAT-SVF disintegrates into small unquantifiable parts. When a few adipocytes are still intact, bigger parts of FAT-SVF are visible. In this way only quantification of the bigger parts of FAT-SVF, containing more adipocytes, is possible. So therefore, the number of adipocytes counted is higher as compared to the real number of adipocytes. Interestingly, our method to disrupt adipose tissue yielded an injectable FAT-SVF that contains all the prerequisites for tissue regeneration: ASC, microvasculature and supporting extracellular matrix.

Compared to conventional or automated dissociation methods of adipose tissue, the FAT procedure is a faster and more cost-effective method to produce a FAT-SVF.[25] The dissociation device is small and of simple design *i.e.* even in disposable format can be produced cost effective, in particular in large quantities. The short time necessary for the mechanical dissociation procedure enables the surgeon to use the FAT-SVF during surgery with a minimal delay of operation time. It also likely contributes to the high vitality of cells that are retained in the FAT-SVF. As opposed to enzymatic digestion, where ASC need to survive in an ischemic environment due to the lengthy isolation procedure. Also the use of collagenase or non-autologous material potentially renders enzymatic digestion more sensitive for (bacterial) contamination. Furthermore, enzymatic isolation results in a SVF comprised of a suspension of individual, non-connected cells with a total lack of tissue structure. In general, retention rates of single cell injections (i.e. ASC) are rather low.[26] With mechanical dissociation, the extracellular matrix is retained in the FAT-SVF, which probably functions as a 'microvascularized' scaffold for the ASC thus keeping a matrix for tissue integrity. In this way, it can be expected to increase cell survival after injection, thus reducing the otherwise low reported retention rates.

The interaction between cells and growth factors in FAT-SVF is expected to increase the regenerative potential in cell-based therapy as compared to the single use of ASC. For instance, the combined application of pericytes and endothelial cells has been shown to augment angiogenesis compared to their single use.[27] Additionally, ASC enhance angiogenesis through secreted growth factors such as vascular endothelial growth factor (VEGF), fibroblast growth factor(s) (FGF) and hepatocyte growth factor (HGF)[28,29], in particular under hypoxia.[20] ASC conditioned medium, which is rich in VEGF, FGFs and HGF, both increases the proliferation rate of endothelial cells, while suppressing apoptosis, in particular under hypoxia.[29] Endothelial cell proliferation and

survival are important mechanisms in angiogenesis.[30] Furthermore, the ECM in FAT-SVF influences the migration and morphogenesis of angiogenesis.[31] ECM contains several proteins such as collagen and fibronectin, which are important during the wound healing process.[32] Hence, we surmise that FAT-SVF might also be a suitable cell-based therapy for stimulating wound healing or regeneration of damaged tissue, as we already have experienced in two illustrative cases (rest of series will be published when the prospective clinical trials have been finished). Firstly, a 62-year-old female patient suffered for nine months from prolonged redness and scar formation on her right cheek after a too deep peeling with trichloroacetic acid (TCA) that did not want to subside. Within three weeks after injection with 1 ml of FAT-SVF, the deep red coloured scar became brighter and turned into nearly normal skin (Fig. 7). In the second case, a 33-year-old female patient received an injection of FAT-SVF in the lateral 5 cm of the horizontal incision after a mamma reduction. Two weeks postoperative, full dehiscence of the wounds occurred, after cheese wiring of the barbed suture, except for the area treated by FAT-SVT (Fig. 7).[33] Probably, FAT-SVF injection accelerated wound healing, making the premature scar better resistant to mechanical forces in an earlier phase during wound repair compared to the non-treated wound edges.

Autologous adipose tissue transplantation to replace lost volume or to improve wound healing and revise dermal scars *i.e.* fibrotic lesions is routinely performed nowadays. Ideally, anti-scar treatment would resolve the existing scar which would involve the degradation of excessively deposited ECM, the removal of unwanted cells such as myofibroblasts and finally the restoration of the original tissue architecture of the dermis including its subcutaneous adipose tissue. We argue that initially the filler effect of fat grafts is less relevant than its anti-scarring properties, Thus, enrichment of anti-scarring cells or matrix, which are both present in the FAT-SVF, might favor the process. At later stages, the precursor cells of adipocytes that reside within FAT-SVF, will regenerate the subcutaneous adipose tissue. Treatment of fibrosis in organs such as heart, kidney or liver, or in osteoarthritic joints such a knee or temporomandibular, with bulky lipografts is undesirable, due to tissue damage caused by injection of large volumes. Therefore, the reduction of the volume of lipoaspirate, as can be achieved with our FAT procedure, to generate an injectable ECM-based cellularized therapeutic gel, that act as an instructive scaffold for repair, seems to be a promising alternative.

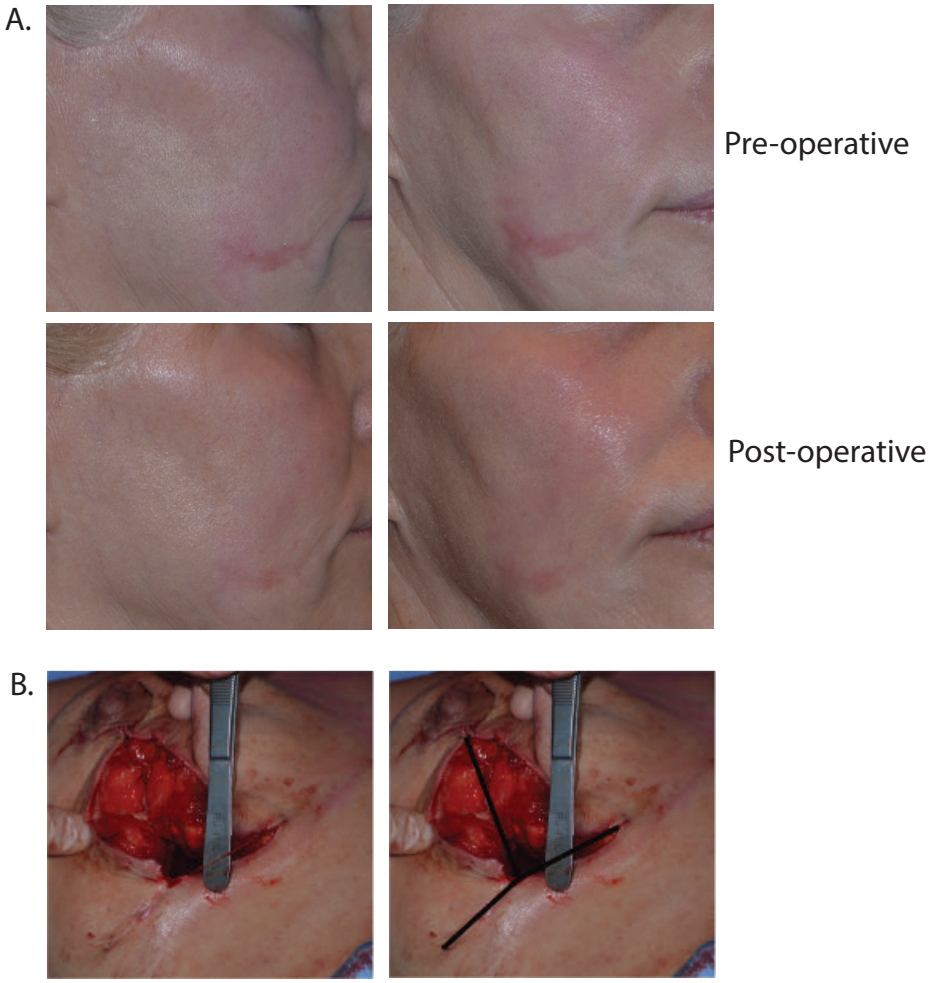

Figure 7. (A) 62-year-old female patient with a nine-month-old scar as a result of a too deep TCA peeling. After injection with FAT-SVF, the dark red colorization of the scar became brighter and resembled to normal skin. **(B)** A 33-year-old female patient with partial dehiscence of the wounds, two weeks postoperative after a mamma reduction. The lateral 5 cm of the horizontal scar was injected with FAT-SVF after the mamma reduction and remained closed. Barbed V-lock suture is still intact and visible in first image. Black lines mark the place of the suture in the second image.

CONCLUSION

The FAT procedure is an effective method to mechanically dissociate lipoaspirate and to create a FAT-SVF with all the therapeutic components of adipose tissue in about 10% of its original volume during a surgical procedure without hardly any time-loss: it contains a 7.7 times higher number of nucleated cells as compared to non-dissociated lipoaspirate and ASC embedded in an extracellular matrix that also contains a microvasculature structure.

Further prospective studies are necessary to assess the therapeutic value of the FAT-SVF as such for e.g. in organ repair, scar revision, osteoarthritis or wound healing. Currently, we are conducting two double blind randomized clinical trials to assess this effect of FAT-SVF on scar formation and skin quality.

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SUPPLEMENTAL CONTENT

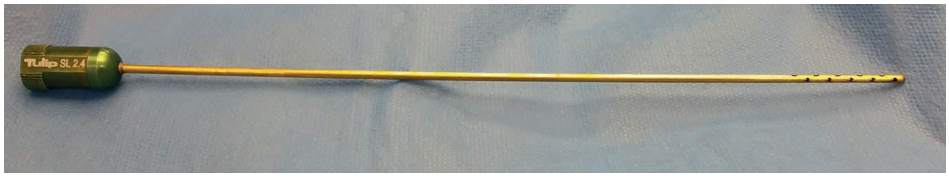

Figure 1. A standard 1-mm diameter Sorenson type lipo-harvesting cannula with smooth outside holes.

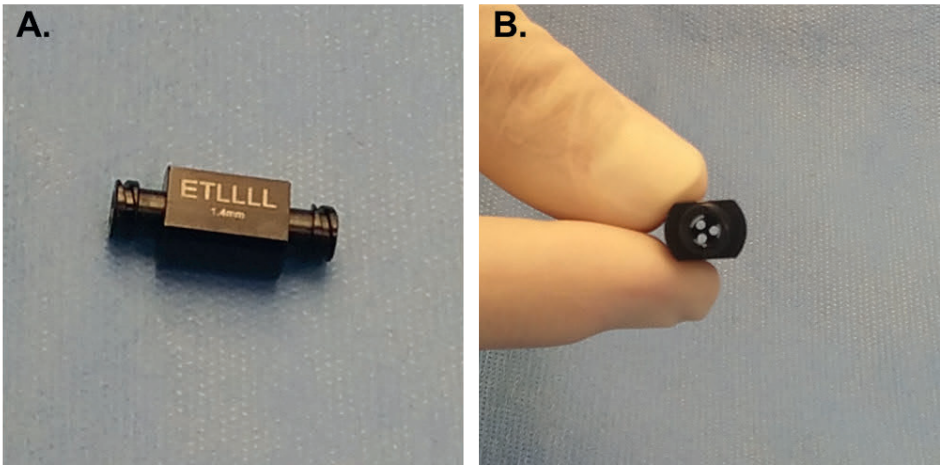

Figure 2. (A) Side view of the Fractionator. **(B)** Three 1.4 mm holes of the fractionator visible.

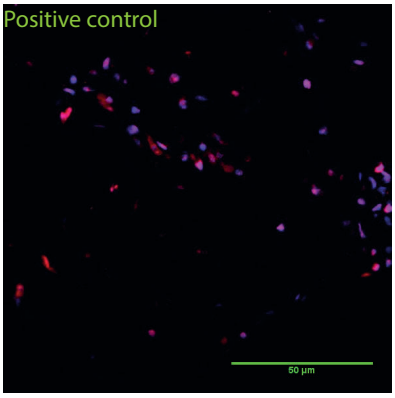

Figure 3. Immunofluorescent images of dead cell control viability. Dead cells turned red after staining with CFDA-SE/PI. Nuclei turned blue after staining with DAPI.

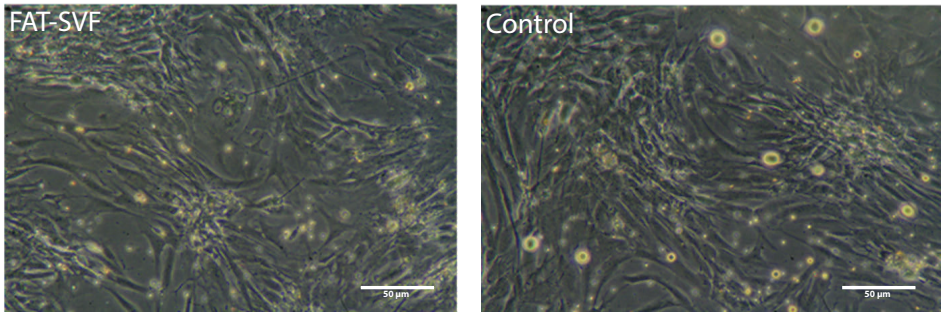

Figure 4. Light microscope images of ASC attached to culture plastic derived from **(A)** FAT-SVF and **(B)** control with a 20x original magnification after 8 days. Spindle-shaped fibroblast-like morphology is visible in both groups, no visual differences between both groups.

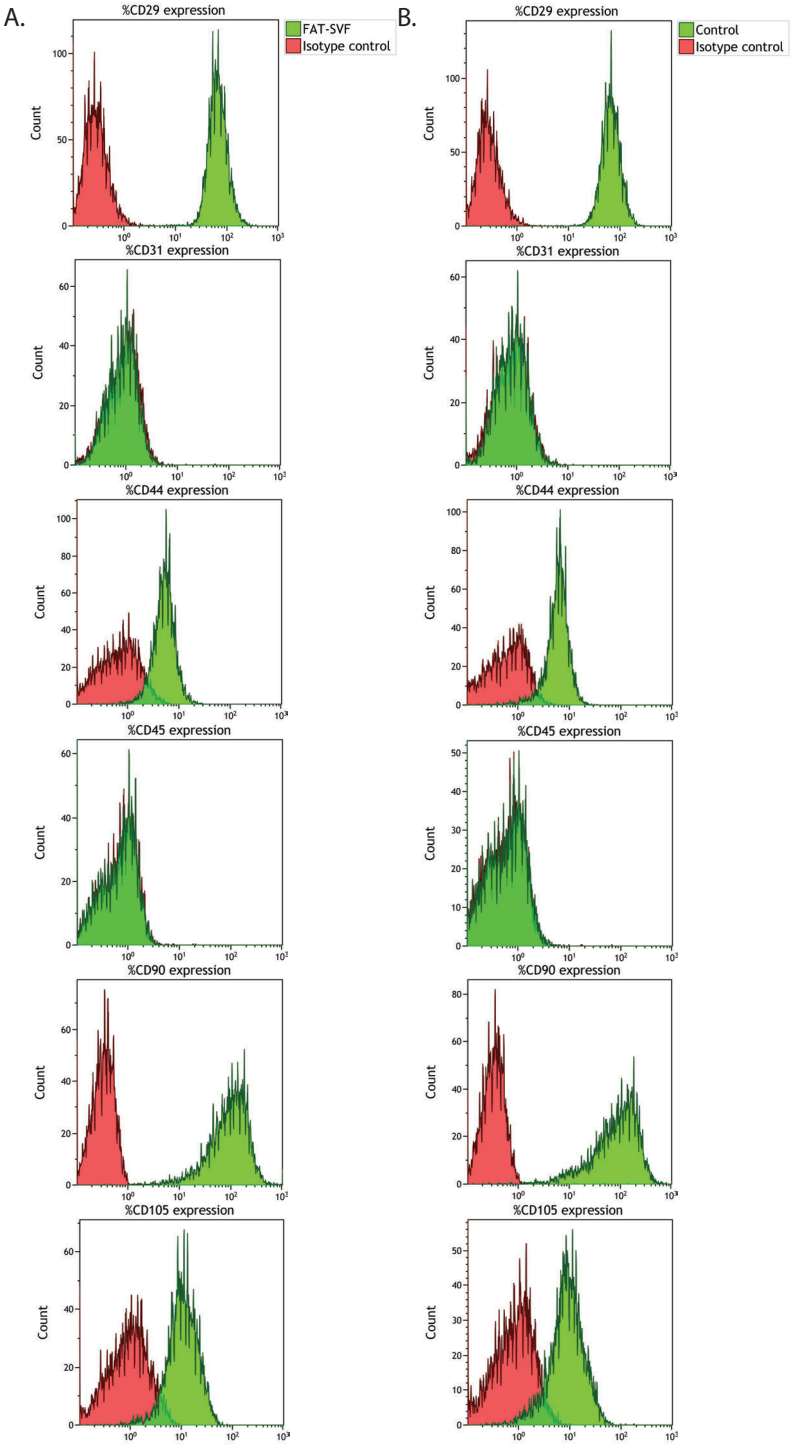

Figure 5. Representative data of CD surface marker expression (CD29, CD31, CD44, CD45, CD90 and CD105) and isotype controls of **(A)** FAT-SVF, and **(B)** Control, after passage 2-4.

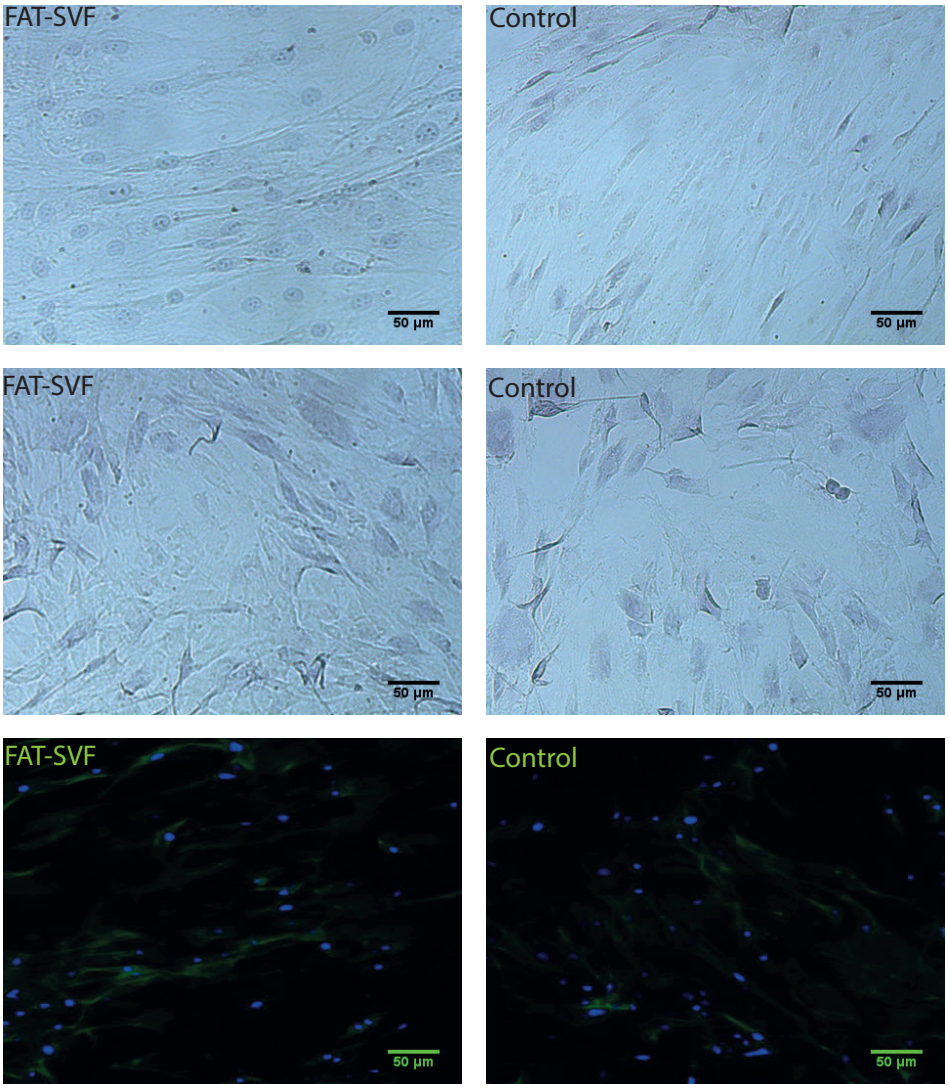

Figure 6. Light microscope and immunofluorescent images of ASC derived from the negative control of FAT-SVF and control. **(A-B)** No visible lipid droplet accumulation and mineralized bone-like noduli formation. **(C)** Slightly expression of F-actin in negative controls as anticipated. After phalloidin-FITC staining, F-actin expression turned green.

04

Fractionation of Adipose Tissue (FAT) procedure with a disposable one-hole fractionator

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ABSTRACT

Background

Adipose tissue has been widely used in regenerative surgery for its therapeutic potential. The therapeutic potential of adipose tissue is often ascribed to the stromal vascular fraction (SVF), which can be mechanically isolated. Mechanical isolation results in SVF containing intact cell-cell communication including extracellular matrix and is therefore named tissue-SVF (tSVF).

Objectives

The Fractionation of Adipose Tissue (FAT) procedure, a procedure to mechanically isolate tSVF, is evaluated using a new disposable one-hole fractionator and compared to the former reusable three-hole fractionator.

Methods

The composition of tSVF obtained by the one-hole fractionator was histologically and histochemically compared to unprocessed adipose tissue. The number of viable nuclear cells in tSVF obtained by the onehole and three-hole fractionator as well as unprocessed adipose tissue were compared after enzymatic isolation and tested for colony forming unit capacity. Flow cytometry was used to compare different cell compositions based on surface marker expression between tSVF isolated by both types of fractionators.

Results

Fractionation of adipose tissue with the one-hole fractionator condenses vasculature and extracellular matrix by disrupting adipocytes. The number of viable nuclear cells in tSVF obtained by both fractionators was comparable and significantly higher than unprocessed lipoaspirate. Furthermore, tSVF isolated by both types of fractionators showed similar cell compositions and comparable colony forming unit capacities.

Conclusion

The FAT procedure with a disposable one-hole fractionator effectively isolates tSVF with a comparable cell count and cell composition in comparison with the three-hole reusable fractionator. A disposable one-hole fractionator, however, is safer and more user friendly.

INTRODUCTION

Adipose tissue has been widely used in regenerative- and reconstructive surgery for its therapeutic potential in the treatment of osteoarthritis, as anti-scarring treatment, to improve wound healing and to compensate for volume loss.[1-4] The therapeutic potential of adipose tissue is often ascribed to the presence of adipose tissue-derived stromal cells (ASCs) in stromal vascular fraction (SVF).[5] SVF of adipose tissue consist of all non-parenchymal *i.e.* non-adipocyte cell types such as ASCs, fibroblasts, vascular cells, immune cells as well as extracellular matrix (ECM).[6-10] Upon culturing, ASCs develop from their *in vivo* precursors *i.e.* pericytes or supra-adventitial cells.[11,12] Cultured ASCs secrete a plethora of growth factors and exosomes that are pro-regenerative and stimulate angiogenesis, promote proliferation of parenchymal cells, suppress apoptosis and modulate immune responses in vivo.[1,13,14] In addition, ASCs secrete ECM components as well as ECM degrading enzymes, which are relevant for tissue remodeling. ASCs are readily isolated from lipoaspirates in high numbers by both enzymatic and mechanical procedures.[15] However, enzymatic isolation is rather costly and time-consuming, because of reagents used such as collagenase that are of non-human origin.[14] Enzymatic isolation procedures result in a single cell suspension of SVF (cSVF) that lacks cell-cell and cell-matrix connections, whereas mechanical isolation procedures result in a tissue-like SVF (tSVF) containing ECM as well as all cell-cell and cell-matrix connections.[15,16] In tSVF, ECM functions as a scaffold for SVF cells and prevents cells from diffusion after injection and therefore might enhances tissue regeneration. Additionally, ECM functions as a slow release reservoir of growth factors.[8,9,17] The interaction between cells and growth factors in tSVF is postulated to increase the regenerative potential as compared to the use of cSVF.

One of the recently published mechanical isolation procedures is the 'Fractionation of Adipose Tissue' (FAT) procedure, which is a fast and low cost intraoperative procedure to obtain tSVF from adipose tissue.[18] It appeared that the original FAT device and procedure would benefit from improvements for several reasons. Firstly, the original fractionator contains an internal disk with three off-centered holes of 1.4 mm. In our experience, the absence of a central hole in the original fractionator leads to congestion in the device, which makes the FAT procedure less practical. Secondly, the original fractionator is a non-disposable device. In view of patient safety, however, sterile disposable devices are preferred over non-disposable devices. For all the aforementioned reasons, a new disposable one-hole fractionator was developed. The purpose of this study is to compare the one-hole fractionator with the original reusable three-hole fractionator based on the composition of the isolated tSVF.

MATERIAL & METHODS

Liposuction and FAT procedures

Liposuction as well as the FAT procedure were executed as described in the study of van Dongen et al. between September 2017 – 2018.[18-20] All included patients gave informed consent according to the Declaration of Helsinki. In short, adipose tissue harvesting was performed with a Sorenson lipoharvesting cannula (Tulip, Medical Products, San Diego, CA) during normal liposuction procedures in three patients. Lipoaspirate was centrifuged after decantation and mechanically dissociated either by the three-hole fractionator or the one-hole fractionator (Tulip, Medical Products, San Diego, CA, fig. 1). After mechanical dissociation, samples from both types of fractionators were centrifuged again yielding a comparable amount of oil (8.4 ml ± 0.4), tSVF (1.1 ml ± 0.4) and infiltration fluid fraction containing a pellet (0.5 ml). One sample of centrifuged lipoaspirate of each patient (n=3) was used as a control.

Immunohistochemistry & Masson's Trichrome

Samples of tSVF obtained by the one-hole fractionator and control lipoaspirate (n=3) were fixed in 10% formalin in phosphate buffered saline (PBS), embedded in paraffin and 4 µm sections were cut. Immunohistochemistry *i.e.* α-Smooth Muscle Actin (SMA) and Perilipin, as well as Masson's Trichrome staining were performed according to the protocol of van Dongen et al.[16] Primary antibodies used in this study were α-SMA (1:200, Abcam, Cambridge, UK) and Perilipin (1:200, Abcam, Cambridge, UK). Secondary antibodies used were polyclonal Rabbit anti-Mouse for α-SMA (1:100, DAKO, Glostrup, Denmark) as well as polyclonal Goat anti-Rabbit for Perilipin (1:100, DAKO, Glostrup, Denmark). A tertiary antibody was only used for α-SMA (polyclonal Swine anti-Rabbit for 1:100, DAKO, Glostrup, Denmark). All samples were visualized and evaluated by light microscopy (Leica Microsystems, DM IL).

Cell Isolation and Culture

Samples of tSVF obtained by the one-hole fractionator and the three-hole fractionator as well as control lipoaspirate (n=3) were enzymatically dissociated in 0.1% collagenase A, 1% bovine serum albumin in PBS and cultured according to our previously published protocol.[16] Cells were counted upon staining with trypan-blue in a Bürker Türk counting chamber.

Flow cytometry

Cells collected from enzymatically dissociated tSVF samples *i.e.* the one-hole fractionator and the three-hole fractionator (n=3) were analyzed for surface marker expression

using flow cytometry. Cells were labelled with the following anti-human monoclonal antibodies: CD31, CD34, CD90, CD105, CD146 (Miltenyi Biotec Bergisch Gladbach, Germany) and CD45 (Biolegend, San Diego, CA, USA) as well as 7-Amino Actinomycin D (Invitrogen, molecular probes, Eugene, OR, USA) to stain for dead cells. Cells were mixed well with the antibodies in FACS buffer (5 mM ethylenediaminetetraacetid acid (EDTA), 1% BSA in PBS) and incubated on ice and in dark for 30 min. Stainings with a single antibody and fluorescence minus one (FMO) were used as controls. A BD FACSCanto II system (BD Biosciences) was used to analyze the samples.

Colony formation assay

Ten thousand viable cells isolated from tSVF obtained by the one-hole fractionator and the three-hole fractionator (n=3) were seeded (six technical replicates) and cultured for twelve days to assess the colony forming capacity of uncultured cells from a single cell. Afterwards, cells were fixed in 4% formalin and stained with 5% Crystal Violet (Sigma-Aldrich, St. Louis, MO). Colony frequency was calculated as the mean number of colonies / total seeded cells x 100%.

Statistical analysis

Immunohistochemistry staining were analyzed with the use of ImageJ, version 1.4.3.67 (NIH, USA).[17] Descriptive statistics were used to evaluate α-Smooth Muscle Actin (SMA), cell numbers with the use of Graphpad Prism, version 5.01 (Graph Pad Software Inc., Los Angeles). Data were expressed as mean ± standard deviation (SD). A paired *t*-test was performed with the use of Graphpad Prism, version 5.01 (Graph Pad Software Inc., Los Angeles).

RESULTS

Fractionation of adipose tissue obtained by the one-hole fractionator condenses tSVF

More α-smooth muscle actin (α-SMA) expression was observed in tSVF (0.83% ± 0.33) as compared to control lipoaspirate (0.094% ± 0.036) for ($p<0.05$), indicating a higher number of small vessels in tSVF obtained by the one-hole fractionator (Fig. 1). Control lipoaspirate was rich in adipocytes (perilipin A expression) while tSVF was essentially devoid of adipocytes. This difference indicates that adipocytes were destructed by the fractionator. More collagen was present in tSVF in comparison to control lipoaspirate, which indicates that the fractionation condensed ECM.

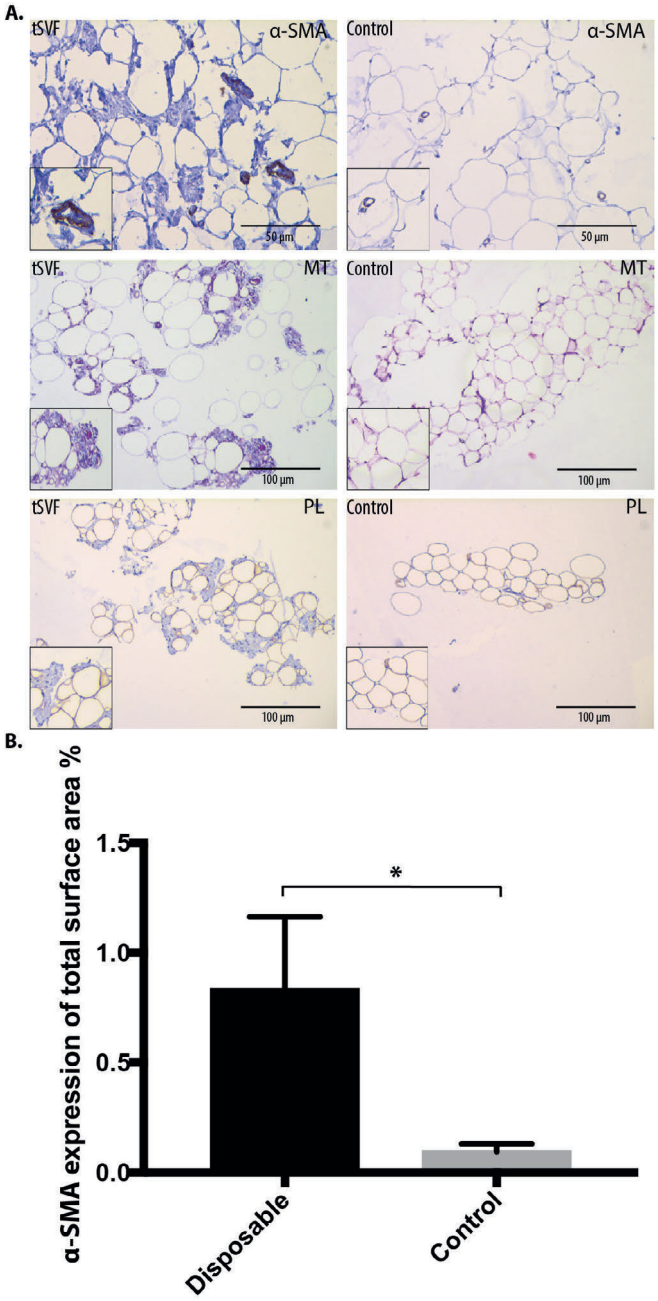

Figure 1. (A) Light microscope images of α-SMA, MT and PL of tSVF obtained by an one-hole disposable fractionator and control lipoaspirate. **(B)** Statistic data of expression of α-SMA in tSVF obtained by an one-hole disposable fractionator and control lipoaspirate. Results are presented as mean ± standard deviation. *Significant more small blood vessels were visible in tSVF as compared to control lipoaspirate ($p<0.05$). α-SMA = alpha-smooth muscle actin; MT = masson's trichrome; PL = perilipin; tSVF = tissue stromal vascular fraction.

Fractionation of adipose tissue obtained with either the three-hole fractionator or the one-hole fractionator condenses cells

Enzymatic isolation of tSVF obtained by the one-hole fractionator and the three-hole fractionator as well as control lipoaspirate resulted in adipocyte-free cell preparation with a mean viable nuclear cell count of respectively $2.35*10^6 \pm 2.98*10^5$, $2.67*10^6 \pm 4.63*10^5$ and $3.12*10^5 \pm 8.99*10^4$ (Fig. 2). No quantitative differences were found for ASCs (CD45-; CD90+; CD105+: reusable 41.4% ± 16.5%, disposable 44.9% ± 18.2%), endothelial cells (CD31+; CD34+: reusable 12.0% ± 4.5%, disposable 19.1% ± 2.3%), leukocytes (CD45+; CD34-: reusable 5.3% ± 3.6%, disposable 5.3% ± 3.6%), pericytes (CD34+/-; CD31-; CD146+: reusable 0.3% ± 0.3%, disposable 0.5% ± 0.5%) and hematopoietic stem cell-like cells (CD45+; CD34+: reusable 0.1% ± 0.2%, disposable 0.2% ± 0.3%) after the FAT procedure with either the three-hole fractionator or the one-hole fractionator (Fig. 3).

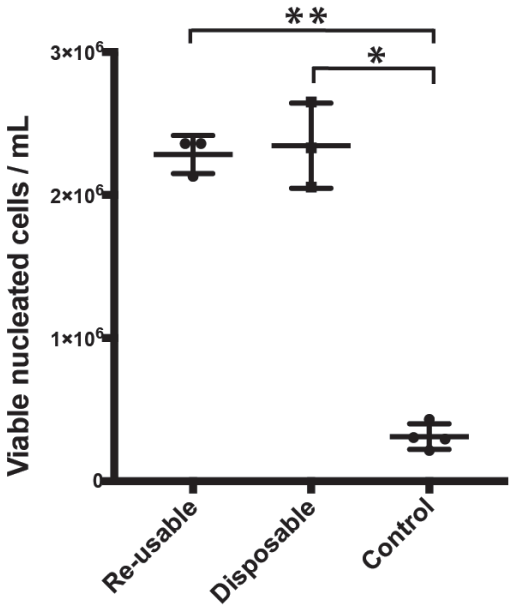

Figure 2. Statistic data of number of viable nucleated cells per 1 mL of tSVF obtained by the reusable fractionator as well as disposable fractionator and control lipoaspirate. Results are presented as mean ± standard deviation. *tSVF obtained by the disposable fractionator contains significant more viable nucleated cells in 1 mL as compared to 1 mL of control (p=0.011).

The number of supra-adventitial cells (CD34bright; CD31-, CD146-) was uncountable low in both types of tSVF. Thus, fractionation by means of the one-hole fractionator as well as the three-hole fractionator reduced the volume of lipoaspirates while the stromal-vascular cells were condensed as compared to control lipoaspirate. No difference in cell count in tSVF was seen between both fractionation procedures.

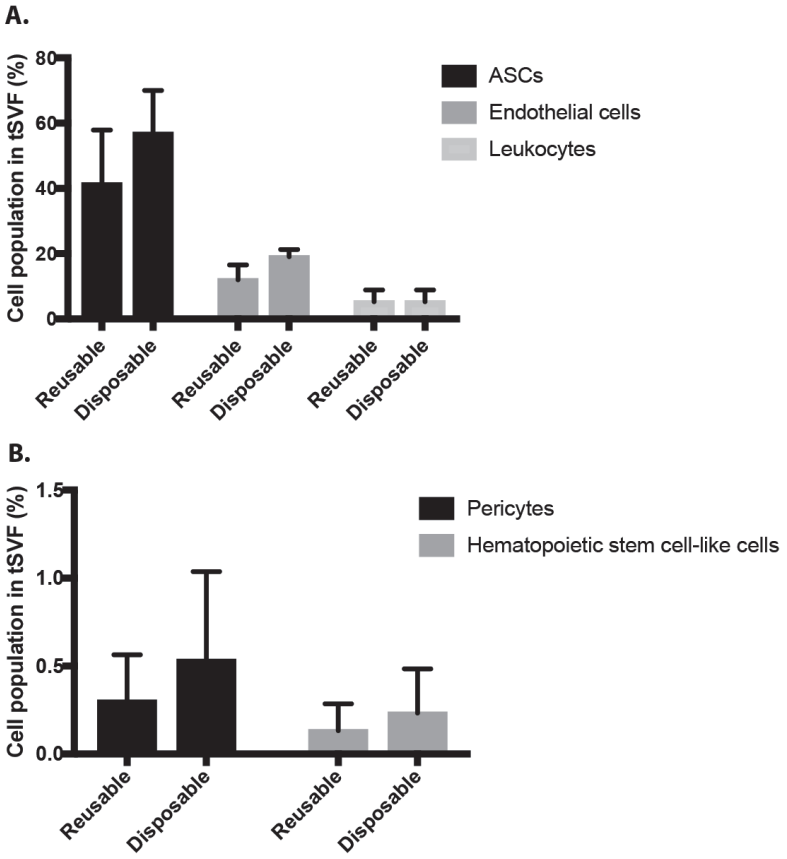

Figure 3. (A) Statistic data of percentage of different cell population in tSVF isolated by means of fractionation with the disposable as well as the reusable fractionator. Results are presented as mean ± standard deviation. No significant differences between all types of cell populations i.e. ASCs (CD45-; CD90+; CD105+), endothelial cells (CD34+; CD31+) and leukocytes (CD45+; CD34-). **(B)** Statistic data of percentage of different cell population in tSVF isolated by means of fractionation with the disposable as well as the reusable fractionator. Results are presented as mean ± standard deviation. No significant differences between all types of cell populations i.e. pericytes (CD34dim; CD31-; CD146+) and hematopoietic stem cell-like cells (CD45+; CD34+). The number of supra-adventitial cells (CD34bright; CD31-; CD146-) were uncountable low in tSVF isolated by the one-hole fractionator and the three-hole fractionator. tSVF = tissue stromal vascular fraction; the one-hole fractionator = disposable fractionator; the three-hole fractionator = reusable fractionator. ASCs = adipose tissue-derived stromal cells.

Fractionation of adipose tissue does not affect colony formation

Colony frequency of uncultured cells derived from tSVF by means of the one-hole fractionator and the three-hole fractionator was similar with respectively 1.29% ± 0.038 and 1.29% ± 0.045 ($p>0.05$) (Fig. 4).

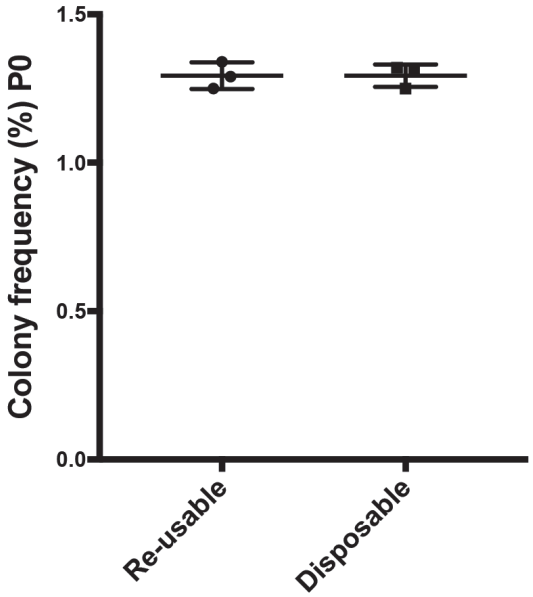

Figure 4. Colony frequency of uncultured cells derived from tSVF isolated by the reusable fractionator as well as disposable fractionator. No significant difference between colony frequency of uncultured cells derived from tSVF. P0 = passage 0; tSVF = tissue stromal vascular fraction; the one-hole fractionator = disposable fractionator; the three-hole fractionator = reusable fractionator.

DISCUSSION

The FAT procedure with the use of the one-hole disposable fractionator showed to be as effective to isolate tSVF with a comparable number of cells and composition of cell types as compared to the former FAT procedure. In our experience, this new system never blocked upon use. The obtained tSVF by means of the one-hole disposable fractionator was composed of condensed vasculature and ECM in comparison with unprocessed adipose tissue. After enzymatic digestion of the tSVF obtained by both types of fractionators, cells were able to attach to tissue culture plastic and form

colonies, indicating the colony forming function has not been disturbed by both systems. However, only low number of donors were used for experiments which might causes a larger standard deviation of the results and is thus a limitation of this study.

Characterization of the composition of cell types in the isolated tSVF can be done by surface marker analysis. In adipose tissue, cells can be divided into two major subpopulations: adipose derived cells (CD45-) and blood derived cells (CD45+).[21] Adipose derived cell populations can be further divided into two main subpopulations: endothelial like cell types (CD31+) and stromal like cell types (CD31-).[21] Three important cell types within the stromal like cell subpopulation (CD45-; CD31-) are the ASCs (CD34+; CD90+; CD105low) and ASCs precursor cells: pericytes (CD34+/-; CD146+) and supra-adventitial cells (CD34+; CD146-).[11,21-23] However, controversy remains about the ASCs precursor cell types and CD surface marker combinations to identify different adipose tissue cell subpopulations.[11,12,23,24] ASCs and its precursor cells are important cell types because of the secretion of many regenerative growth factors and cytokines.[1,13,14] In comparison to several other mechanical and enzymatic isolation procedures *e.g.* Automated isolation system, CHA Biotech Station, Lipokit Medikhan System, PNC's Multistation, Fastem and Lipogems, both types of FAT procedures isolate more stromal cells.[25-28] Enzymatic isolation procedures *e.g.* Cytori, Tissue Genesis Cell Isolation System, Sepax System showed comparable stromal cell populations.[26,29,30] However, differences in cell subpopulations of cSVF and tSVF could be caused by donor dependent variations and different use of CD surface markers and thus comparisons between studies are difficult.[18-21]

The use of the one-hole fractionator seems to offer several advantages over the non-disposable three-hole fractionator. Firstly, any re-usable device could bring a potential risk of contamination and biofilms to grow after sterilization, in particular in the difficult-to-clean small holes.[15,31] Using a disposable fractionator largely eliminates the risk of contamination and thus complies with the most restricted regulations on sterility. Yet, the potential increased risk of contamination in reusable devices in comparison with disposable devices is solely hypothetical instead of based on sterility data, which is another limitation of this study. To even further reduce the risk of contamination during the isolation procedure, a completely closed system could be designed to promote safe clinical use (which has been manufactured in the meantime (ACA-kit, Arthex GmbH, USA). Secondly, in the one-hole fractionator, the opening in the internal disk is situated in the center of the disk resulting in significant fewer blockages. In the original three-hole fractionator, the internal disk contains three holes without a hole in the center of the disk. The tip of the 10 mL syringe faces the center of the three-hole disk in the middle of the fractionator. In this way, blockage of the three-

hole fractionator can occur when lipoaspirate contains substantial amounts of fibrous tissue (*e.g.* in secondary donor sites or in male subjects with limited subcutaneous tissue to harvest from).

Thus far, multiple *in vitro* studies have investigated the composition of the isolated tSVF by means of the FAT procedure or similar mechanical procedures like the Nanofat procedure. [16,18,32] These studies have shown that tSVF contains increased numbers of ASCs as compared to unprocessed lipoaspirates.[16,32] ASCs are important cell types with a high regenerative potential. As mentioned before, ASCs secrete a plethora of growth factors and cytokines which are able to stimulate important regenerative processes such as matrix remodeling and angiogenesis.[1,17,33] Hence, the obtained tSVF is promising for clinical regenerative purposes, such as scar remodeling and wound healing. However, its clinical effectiveness has not been proven yet as no well-designed randomized double-blind placebo-controlled prospective clinical trials investigating the regenerative potential of tSVF have been performed. Well-defined prospective trials with the use of mechanical isolated tSVF are therefore warranted. To date, our research group finished the inclusion of two randomized double-blind placebo-controlled clinical trials using tSVF isolated by means of the FAT procedure to improve scarring after breast reductions and to improve skin quality for the aging face. A safe and easy-to-use disposable system, such as the disposable one-hole fractionator as described in this study, might also pave the way towards a comparative study.

CONCLUSION

The FAT procedure with the one-hole disposable fractionator effectively isolates tSVF with a comparable number of cells and cell composition in comparison with the original described FAT procedure using a three-hole reusable fractionator. However, a disposable one-hole fractionator offers technical and financial advantages over the reusable three-hole fractionator, as it is safer and more user friendly.

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Mechanically derived tissue-stromal vascular fraction acts anti-inflammatory on chondrocytes in vitro

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ABSTRACT

Introduction

Enzymatically isolated stromal vascular fraction as a treatment for osteoarthritis has already shown to be effective. Yet, the use of enzymes for clinical purpose is highly regulated in many countries. Mechanical preparation of SVF results in a tissue-like SVF (tSVF) containing intact cell-cell connections including extracellular matrix and is therefore less regulated. The purpose of this study was to investigate the immunomodulatory and pro-regenerative effect of tSVF on inflammatory chondrocytes *in vitro*.

Materials and Methods

tSVF was mechanically derived using the Fractionation of Adipose Tissue (FAT) procedure. Characterization of tSVF was performed *e.g.* cellular composition based on CD marker expression, colony forming unit and differentiation capacity after enzymatic dissociation (from heron referred to as tSVF-derived cells). Different co-cultures of tSVF-derived cells and inflammatory chondrocytes were analysed based on production of sulphated glycoaminoglycans and anti-inflammatory response of chondrocytes.

Results

Characterization of tSVF-derived cells mainly contained ASCs, endothelial cells, leukocytes and supra-adventitial cells. tSVF-derived cells were able to form colonies and differentiate into multiple cell lineages. Co-cultures with inflammatory chondrocytes resulted in a significant increase of the total number of chondrocytes as compared with cultures of chondrocytes alone ($p<0.05$). IL-1β and COX2 gene expression was upregulated in TNFα-treated chondrocytes. After treatment with (conditioned medium of) tSVF-derived cells, IL-1β and COX2 gene expression was significantly reduced ($p<0.01$).

Conclusion

Mechanically derived tSVF stimulates chondrocyte proliferation while preserving the function of chondrocytes. Moreover, tSVF suppresses chondrocyte inflammation *in vitro*. This pro-regenerative and anti-inflammatory effect shows the potential of tSVF as a treatment for osteoarthritis.

INTRODUCTION

Autologous adipose tissue transplantation is frequently used for a variety of different clinical indications, such as dermal scarring, fat atrophy, body contouring, wound healing, burn wounds, osteoarthritis and perianal fistulas.[1-6] The therapeutic efficacy of adipose tissue is ascribed to the stromal vascular fraction (SVF) containing a heterogeneous mixture of non-adipocyte cell types e.g. immune cells, fibroblasts, endothelial cells, pericytes and adipose derived stromal cells (ASCs).[7,8] ASCs reside in SVF as progenitor cell types attached around vessels as pericytes or supra-adventitial cells.[9,10] In vitro, ASCs secrete a plethora of growth factors, cytokines, chemokines, matrix proteases and extracellular vesicles which stimulate different regenerative processes such as angiogenesis, fibroblast migration and proliferation, matrix remodeling as well as immune modulation.[11-13] These trophic effects illustrate the regenerative potential of ASCs as a source for cell-based therapy to repair different types of damaged tissue.[14]

Nowadays, SVF is often mechanically derived instead of enzymatically isolated because the use of enzymes for medicinal products is highly regulated in many countries. Mechanical preparation of SVF results in a tissue-like SVF (tSVF) containing SVF cells and intact cell-cell communications including ECM. Enzymatically isolated SVF yields a single cell suspension of SVF (cellular SVF or cSVF). ECM acts as a pro-regenerative scaffold to bind and release growth factors, matrix metalloproteinases (MMP), proteins and cytokines in a controlled way.[15,16,17] Several growth factors or enzymes, like transforming growth factor-beta (TGF-β), fibroblast growth factor (FGF) and matrix metalloproteinase 13 (MMP-13), are involved in cartilage homeostasis.[18-20] A disbalance between these growth factors and enzymes play an important role in cartilage degradation, synovitis and osteophyte growth and thus osteoarthritis (OA).[18-20] Osteoarthritis is chronic progressive disease, wherein damaged joints lead to pain, stiffness and disability.[21] ASCs might decrease these clinical symptoms by influencing growth factor stimulated processes involving cartilage degeneration such as blocking TGF-β1-induced fibrosis and reducing collagenase 3 (MMP-13) expression in vitro.[22,23] Furthermore, ASCs can induce immune suppressive effects in OA by exciting macrophages in the synovium to produce interleukin (IL)-10 or induce a switch to M2 macrophages via secretion of prostaglandin-E_2 (PGE2).[24] This could beneficially contribute to the treatment of OA. Hence, tSVF as a source for ASC-cell-based therapy potentially harbors more therapeutic capacity than ASCs alone in the treatment of OA.

Multiple studies have already shown safety and efficacy *e.g.* improved functionality, less instability and reduction of pain of using ASCs and cSVF as a treatment of OA in humans and animals.[25-31] More recently, a case series with tSVF as a treatment for

knee OA in human subjects demonstrated tSVF is also safe to use.[32] The purpose of this study was to investigate the immunomodulatory and pro-regenerative effect of mechanically derived tSVF on inflammatory chondrocytes in vitro.

MATERIAL AND METHODS

Lipoharvesting and the FAT procedure

Lipoaspirate was collected from healthy female patients between 18 and 65 years of age. The FAT procedure was performed in a standardized way as published earlier (Annex).[15,26,33]

Enzymatic Dissociation of tSVF

Samples of tSVF were washed with phosphate buffered saline (PBS) three times. After washing, 0.1% collagenase A in PBS/1% bovine serum albumin (BSA) was added as dissociation medium. Samples were stirred for 1.5 hour in a 37°C water bath. Cells were resuspended in erythrocyte lysisbuffer (thermoFisher) and incubated for 5 min. Samples were centrifuged at 8 °C, 600 *xg* for 10 min. and resuspended in expansion medium consisting of Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Paisley, UK) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT) and 1% Penicillin/Streptomycin). Viable cells were counted with trypan blue in a Bürker Turk counting chamber. Cells collected from enzymatically processed tSVF samples are henceforth referred to as 'tSVF-derived cells'.

Flow cytometry to determine cell composition of tSVF

tSVF-derived cells *i.e.* from the one-hole re-usable fractionator (n=12) were analysed for CD surface marker expression using flow cytometry. Cells were labelled with the following anti-human monoclonal antibodies: CD31, CD34, CD90, CD105, CD146 (Miltenyi Biotec Bergisch Gladbach, Germany) and CD45 (Biolegend, San Diego, CA, USA) as well as 7-Amino Actinomycin D (Invitrogen, molecular probes, Eugene, OR, USxA) to stain for dead cells. Cells were mixed well with the antibodies in FACS buffer (5 mM ethylenediaminetetraacetid acid (EDTA), 1% BSA in PBS) and incubated on ice and in dark for 30 min. Stainings with a single antibody and fluorescence minus one (FMO) were used as controls. A BD FACSCanto II system (BD Biosciences) was used to analyse the samples.

Colony formation unit capacity of ASCs

Ten thousand viable cells enzymatically processed from tSVF passage 0 as well as thousand cells from passage 1 and 2 (n = 11 and six technical replicates) were seeded and cultured for twelve days to assess the colony forming capacity. Afterwards, cells were fixed in 4% formalin and stained with 5% Crystal Violet (Sigma-Aldrich, St. Louis, MO). Colony frequency was calculated as the mean number of colonies / total seeded cells x 100%.

Differentiation capacity of tSVF and ASCs

tSVF-derived cells from passage 0, 1, 2, 3 (n = 11) were collected and used for chondrogenic, adipogenic and osteogenic differentiation assays. For osteogenic differentiation the cells were seeded at a density of 128.000 viable nucleated cells for passage 0 and 32.000 viable nucleated cells for passages 1, 2 and 3 per well of a 12-wells plate in expansion medium. The P0 were cultured for 72 hours and P1, 2 and P3 cells for 24 hours before the medium was changed to osteogenic differentiation medium. The osteogenic differentiation medium consisted of alpha-MEM (Gibco) supplemented with 10% FBS, 10 nM dexamethasone, 0.2 mM ascorbic acid-2-phosphate, 10 mM of β-glycerophosphate and pen/strep. The cells were cultured in osteogenic medium for 3 weeks and medium was changed three times a week. After 3 weeks, the cells were fixed with formalin and washed with PBS and stained for mineralization with 3% Alizarin Red S and washed 6 times with PBS where after microphotographs were taken.

For adipogenic differentiation the cells were seeded at 960.000 for P0 and 240.000 for P1, P2 and P3 cells per well of a 24-wells plate. The P0 were cultured for 72 hours and P1, 2 and P3 cells for 24 hours before the medium was changed to adipogenic differentiation medium that consisted of alpha-MEM supplemented with 10% FBS, 500 µM 3-isobutyl-1-methylxanthine (IBMX), 1 µM dexamethasone, 0.2 mM indomethacin, 1.72 µM insulin and pen/strep. The cells were cultured in osteogenic medium for 3 weeks and medium was changed three times a week. After culture, the cells were fixed in formalin, washed with PBS followed by 60% isopropanol, stained with Oil red O, washed with 60% isopropanol and washed with PBS whereafter they were microphotographed.

For chondrogenic differentiation 1 million P0 or 250.000 P1, P2 and P3 viable nucleated cells were seeded in a 15 mL falcon tube that was centrifuged for 5 minutes at 300xg to pellet the cells. After 3 days, the culture medium was replaced by chondrogenic medium consisting of DMEM supplemented with 1% ITS+ premix (354352; BD Bioscienes), 10–7 M dexamethasone (D8893; Sigma), 0.2 mM L-ascorbic

acid 2-phosphate, 1% Pen / Strep, and 10 ng/mL TGF-β2 (302-B2; R&D Systems). The cells were cultured for 4 weeks in chondrogenic medium and the medium was changed 3 times per week. After 28 days of culture, pellets were formalin-fixed and paraffin embedded. Subsequently, 5 μm sections were stained with 0.125% safranin-O (Merck, counterstained with Weigert's hematoxylin (Klinipath) and 0.4% fast green (Merck)) to stain deposited proteoglycans.

Enzymatic isolation of chondrocytes

Chondrocytes were isolated from donor cartilage of various human patients after total knee arthroplasty allowed by the local ethical committee of the UMC Utrecht (TC-Bio protocolnumber 15-092). Cartilage was minced and tissue fragments were subjected to sequential treatments first with DMEM supplemented with 1% FBS, 100 U/ml of penicillin, 100 m g/ml of streptomycin, and 2.5% (w/v) of pronase E (Sigma, St. Louis, MO) for 1 h, then with DMEM supplemented with 25% FBS, 100 U/ml of penicillin, 100 mg/ ml of streptomycin and 0.125% (w/v) of collagenase (CLS-2; Worthington, Lakewood, NJ) for 16 h at 37° C. Cells were filtered through a 70-mm cell strainer (BD Biosciences, San Diego, CA) and washed. Chondrocytes were expanded to passage 2 in T175 flasks in expansion medium.

Functional analysis of coculture of tSVF-derived cells and chondrocytes

Freshly isolated tSVF-derived cells and passage 2 chondrocytes were cultured in various ratio's in pellets. A total of 250.000 cells, consisting of 0, 25, 50, 75 and 100% nucleated tSVF-derived cells and 100, 75, 50, 25 and 0% chondrocytes, were cultured in 1 mL DMEM (Gibco) supplemented with 2% insulin-transferrin-selenium (ITS)-X (Invitrogen), 50 μg/mL ascorbate-2-phosphate (Sigma), 2% human serum albumin (Sanquin) and penicillin / streptomycin (100 U/mL, 100 μg/mL), which was refreshed every 2-3 days at 37°C at 5% CO2. Immediately after pelleting and after 21 days of culture, RNA was isolated from 3 pellets for RT real-time PCR to investigate the ratio between the cells. After 28 days, 3 pellets were papain digested for biochemical analyses.

Cell ratio analysis of chondrocytes and tSVF-derived cells cocultures

Cocultures of tSVF-derived cells from female donors and chondrocytes from male donors (n = 3 in triplicates) in various ratios were cultured for 21 days. After 21 days, the expression of the Y-chromosomal gene lysine demethylase 5D (KDM5D) gene was measured. 18S was used as housekeeping gene. Total RNA was isolated using Trizol (Invitrogen) as described by the manufacturer. Total RNA (500 ng) was reverse transcribed using the high-capacity cDNA Reverse Transcription kit (Thermo

Fisher Scientific). Real-time polymerase chain reactions were performed using iScript universal SYBR Green (Biorad) in a LightCycler 96 (Roche). Primers used are shown in Table 1.

Sulphated glycosaminoglycans analysis

After 28 days of culture, cocultures of tSVF-derived cells and chondrocytes (n = 3 in triplicates) were digested at 60° C for 18h in a papain enzyme solution consisting of 5 mM L-cysteine, 50 mM Na2 EDTA, 0.1 M NaAc, pH 5.53 with 2% (v/v) papain (Sigma). To measure the concentration of sulphated glycosaminoglycans (GAGs), a dimethylene blue (DMMB) spectrophotometric analysis was performed. The papain digests were 1 : 10 diluted and mixed with the DMMB solution and absorbance was read at 540 nm and 595 nm. Known concentrations of chondroitin sulphate C (sigma) were used as a reference. To correct for the number of cells, DNA amount in the papain digests was measured. Papain digests were diluted 1:20 and Quant-iT Picogreen (Invitrogen) reagent was added. This was incubated for 5 min. at ambient temperature in the dark whereafter the fluorescence was measured at 480 nm excitation and 520 nm emission. Known concentrations of lambda DNA were used as a standard.

In vitro inflammation assay

Chondrocytes (n = 3 in triplicates) were cultured in monolayer at 37°C and 5% CO2 at a seeding density of 25,000 cells/cm^2 in expansion medium. After pre-incubation for 24h, cells were treated with 10 ng/mL tumor necrosis factor (TNF)-alpha (Immunotools). After 24h of treatment, the cells were washed and medium was refreshed with either control medium, conditioned medium (CM) from cultured chondrocytes or cultured tSVF-derived cells or chondrocytes or tSVF-derived cells were added. After 24h, RNA was isolated from the monolayers and gene expression of interleukin (IL)-1β and cyclooxygenase-2 (COX2) were measured as described in 2.7.1. 18S was again used as housekeeping gene. Primers used are shown in table 1.

Table 1. Primers used for real-time PCR and In Vitro Inflammation Assay.

Target gene		Primers
18S	Forward	GTAACCCGTTGAACCCCATT
	Reversed	CCATCCAATCGGTAGTAGCG
KDM5D	Forward	TAACACACACCCGTTTGACAA
	Reversed	GCTGCTGAACTTTGAAGGCTG
IL-1b	Forward	5′-GCTGAGGAAGATGCTGGTTC-3′
	Reversed	5′-TCCATATCCTGTCCCTGGAG-3′
COX2	Forward	5′-GCCCGACTCCCTTGGGTGTC-3′
	Reversed	5′-TTGGTGAAAGCTGGCCCTCGC-3′

KDM5D: lysine demethylase 5D; IL-1b: Interleukin 1 beta; COX2: prostaglandin-endoperoxide synthase 2 (PTGS2)

Statistical analysis

Descriptive statistics were used to evaluate the number of cells in tSVF, cell composition of tSVF, colony forming units as well as the number of cells after co-culture of chondrocytes and tSVF-derived cells, the amount of sGAG and the expression of IL-1β and COX2. Data were expressed as mean with standard deviation. A two-tailed paired t-test was performed to analyze the number of cells after co-culture of chondrocytes and tSVF-derived cells. A two-tailed unpaired t test was performed to analyze the amount of sGAG and the expression of IL-1β and COX2. All data was analyzed using Graphpad Prism, version 5.01 (Graph Pad Software Inc., Los Angeles).

RESULTS

Characterization of tSVF

Enzymatic isolation of tSVF yielded a mean number of viable cells of $2.67 \times 10^6 \pm 4.63 \times 10^5$ per 1 ml (Fig. 1). Composition of tSVF contained a mean number of 48.87% ± 17.59% of ASCs (CD45-; CD90+; CD105+), 39.55% ± 32.38% of endothelial cells (CD31+; CD34+), 3.42% ± 3.18% of leukocytes (CD45+; CD34-), 0.23% ± 0.19% of pericytes (CD34+/-; CD31-; CD146+), 0.32% ± 0.39% of hematopoietic stem cell-like cells (CD45+; CD34+) and 5.95% ± 8.54% of supra-adventitial cells (CD34bright; CD31-, CD146-) (Table 2).

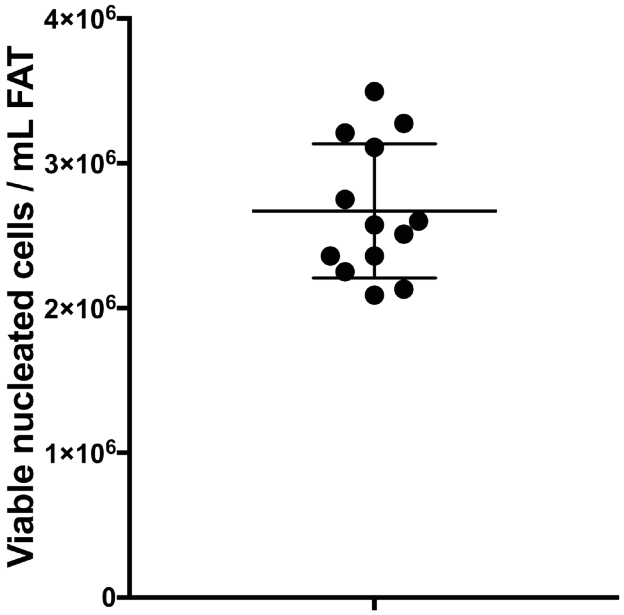

Figure 1. Number of viable nucleated cells per mL tissue-like stromal vascular fraction (tSVF). Each dot represents the value of one donor.

Table 2. Surface marker expression of tSVF.

CD markers	Mean ± SD	Cell population
CD45-; CD90+; CD105+	48.87% ± 17.59%	Mesenchymal stromal cell
CD31+; CD34+	39.55% ± 32.38%	Endothelial cell
CD45+; CD34-	3.42% ± 3.18%	Leukocyte
CD34+/-; CD31-; CD146+	0.23% ± 0.19%	Pericyte
CD45+; CD34+	0.32% ± 0.39%	Hematopoietic stem cell-like cell
CD34bright; CD31-, CD146-	5.95% ± 8.54%	Supra-adventitial cell

After culture, cells present in tSVF were cultured and characterized based on their ability to form colonies which increased each passage (Fig. 2). Moreover, primary freshly isolated and plastic-adherence expanded tSVF-derived cells were able to differentiate into adipogenic, osteogenic and chondrogenic cell lineages (Fig. 3).

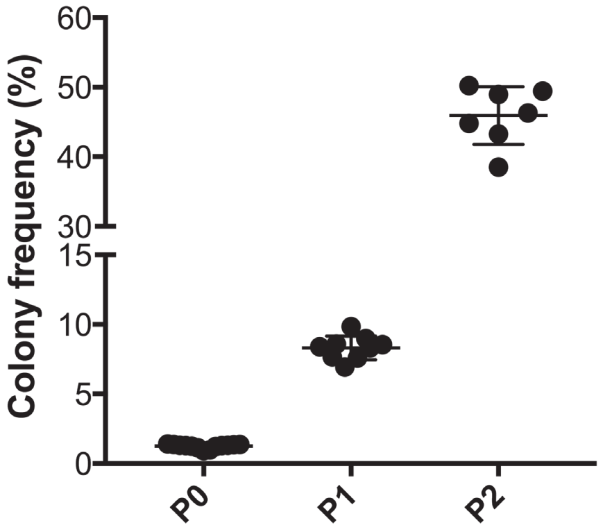

Figure 2. Colony-forming unit assay of tissue-like stromal vascular fraction (tSVF) at P0 (10.000 cells seeded), P1 (1000 cells seeded) and P2 (1000 cells seeded) to examine cell colony formation. The number of violet blue-stained colonies is expressed as a percentage of total seeded nucleated cells. P = passage.

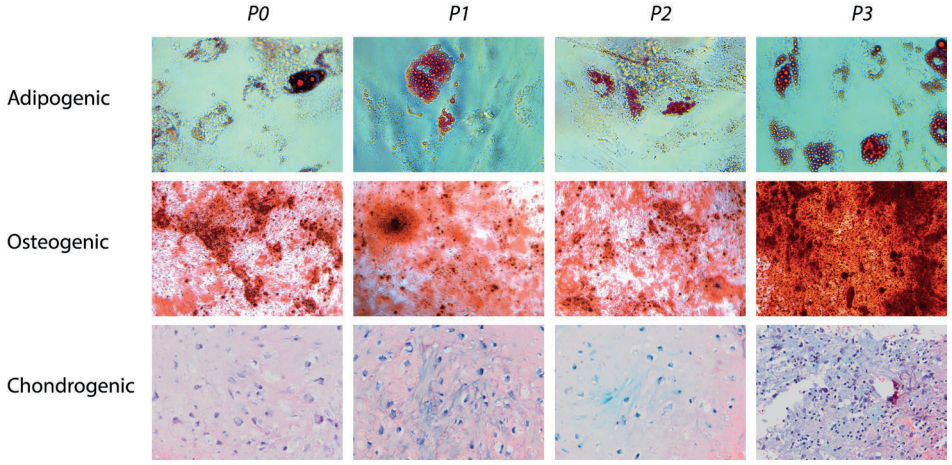

Figure 3. Representative bright-field microscope images of tissue-like stromal vascular fraction (tSVF) at P0, P1, P2 and P3 differentiated into the adipogenic (upper, 40x magnification), osteogenic (mid, 10x magnification) and chondrogenic (lower, 10x magnification) lineages as shown by stainings with oil red O, alizarin red and safranin-O, respectively. P = passage.

Cell ratio analysis of chondrocytes and tSVF-derived cells in co-culture

Chondrocytes co-cultured with tSVF-derived cells in various ratios i.e. 75% / 25%, 50% / 50% and 25% / 75% (chondrocytes / tSVF-derived cells) resulted in an increase of the ratio chondrocytes / tSVF-derived cells after 21 days compared to the baseline (respectively $p<0.05$, $p<0.01$, $p<0.01$, fig. 4). The total number of chondrocytes was significantly higher when co-cultures of chondrocytes and tSVF-derived cells i.e. 75%/25% and 50%/50% were compared with cultures of chondrocytes alone ($p<0.05$, fig. 4).

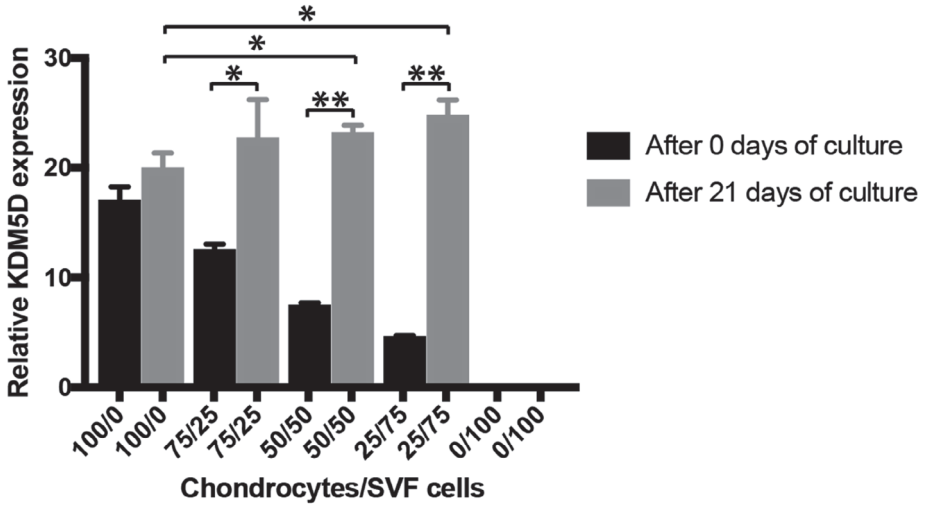

Figure 4. Relative lysine demethylase 5D (KDM5D, y-chromosomal) gene expression of male chondrocytes after 0 days and 21 days of culture. Results are expressed as mean ± SD and analysed with an unpaired t-test when different pellets of co-cultures where compared. A paired t-test was used when the same co-culture was analysed at different timepoints. * p = <0.05. ** p = <0.01.

Sulphated GAGs analysis

No significant difference was observed when mono-cultures of chondrocytes were compared with co-cultures of 25% chondrocytes and 75% tSVF-derived cells ($p>0.05$, fig 5). Significantly more sulphated GAGs were present in mono-cultures of chondrocytes in comparison with co-cultures of chondrocytes and tSVF-derived cells of respectively 75% / 25% and 50% / 50% after 28 days ($p<0.05$, $p<0.01$, fig. 5).

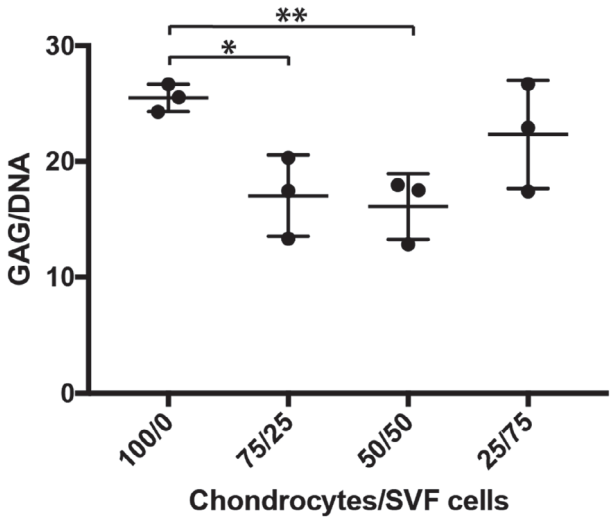

Figure 5. Sulphated glycosaminoglycan (GAG) deposition normalized for DNA content of pellets from co-cultures of chondrocytes and tSVF-derived cells after 28 days of culture at various ratio´s. Results are expressed as mean ± SD and analysed with an unpaired t-test. * p = <0.05. ** p = <0.01.

In Vitro Inflammation Assay

A significant downregulation of IL-1β gene expression was observed in TNFα treated chondrocytes after addition of CM from tSVF-derived cells ($p<0.01$, fig. 6), but not after the addition of CM from chondrocytes (Fig. 6). COX2 gene expression was significantly downregulated in TNFα treated chondrocytes after addition of CM from chondrocytes and CM from tSVF-derived cells ($p<0.05$, $p<0.01$, fig 6). Both IL-1β and COX2 gene expression was downregulated in TNFα treated chondrocytes after addition of tSVF-derived cells or chondrocytes ($p<0.01$, $p<0.0001$, fig. 7), but the gene expressions were more reduced by the addition of tSVF-derived cells compared to chondrocytes ($p<0.01$, fig 7).

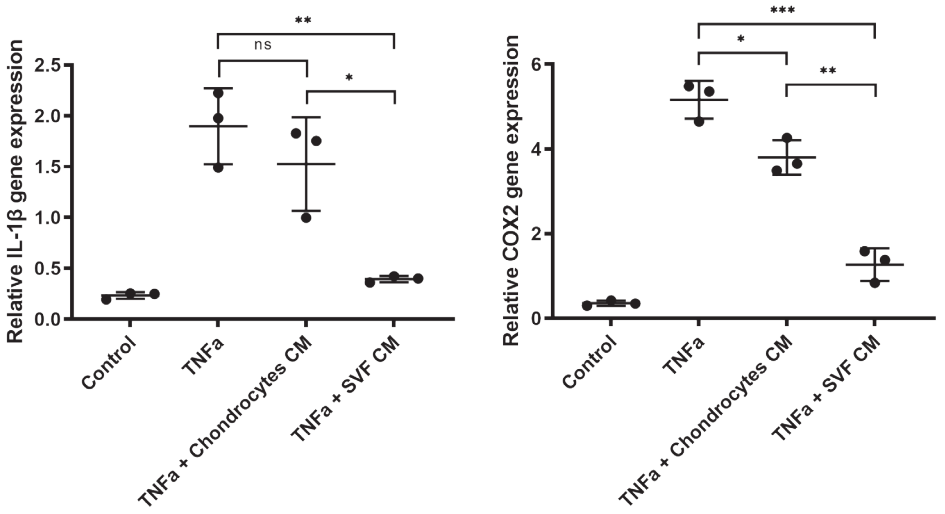

Figure 6. Relative gene expression of interleukin (IL)-β **(A)** and cyclooxygenase 2 (COX2 (prostaglandin-endoperoxide synthase 2 (PTGS2)) **(B)** by tumor necrosis factor alpha (TNFα)-stimulated chondrocytes and subsequently treated with conditioned medium (CM) of chondrocytes or tissue-like stromal vascular fraction (tSVF) for 24h. Results are expressed as mean ± SD and analysed with an unpaired t-test. ns = not significant. * p = <0.05. ** p = <0.01. *** p = <0.001. Control = chondrocytes without TNFα treatment.

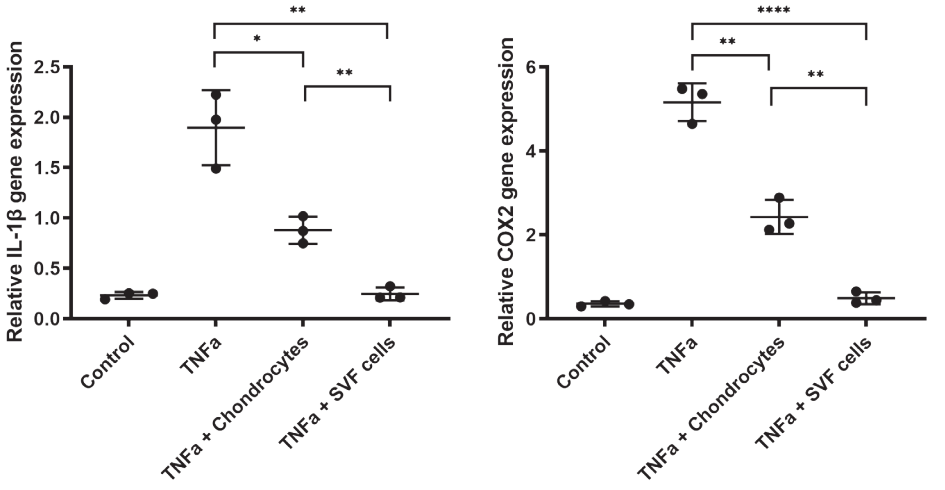

Figure 7. Relative gene expression of interleukin (IL)- 1β **(A)** and cyclooxygenase 2 (COX2 (prostaglandin-endoperoxide synthase 2 (PTGS2)) **(B)** by tumor necrosis factor alpha (TNFα)-stimulated chondrocytes and subsequently treated with chondrocytes or tissue-like stromal vascular fraction (tSVF) cells for 24h. Results are expressed as mean ± SD and analysed with an unpaired t-test. * p = <0.05. ** p = <0.01. *** p = <0.001. **** p = <0.0001. Control = chondrocytes without TNFαtreatment.

DISCUSSION

This study demonstrates that enzymatically processed cells from tSVF – which was mechanically derived by means of the FAT procedure – have a pro-regenerative and anti-inflammatory effect on chondrocytes *in vitro*. Proliferation of chondrocytes was stimulated by tSVF-derived cells as shown in the significant higher ratio of chondrocytes in different co-cultures with tSVF-derived cells as compared to cultures of chondrocytes alone. A pro-regenerative effect of tSVF is postulated as it enables regeneration of functional cartilage by stimulating GAGs formation *in vitro*. Downregulation of IL-1β and COX2 gene expression due to addition of tSVF to inflammatory chondrocytes *in vitro* suggests an anti-inflammatory effect. These aforementioned inflammatory processes are involved in osteoarthritis and can be influenced by the heterogenous cell population of tSVF, Hence, tSVF is a potential effective therapy for the treatment of OA.

Elevated levels of COX2 and IL-1β are found in chronic inflammatory state of joints.[34,35,36] Overexpression of COX2 in osteoarthritis results in an increased production of matrix metalloproteinases (MMPs), reduction of collagen synthesis and stimulation of chondrocyte apoptosis.[34] All of these processes contribute to cartilage degradation leading to more inflammation. This vicious circle of cartilage degradation and inflammation is enhanced by PGE2, a pro-inflammatory mediator produced after stimulation with COX2 and IL-1β.[34] Eventually, cartilage degradation will result in pain. Thus far, several COX inhibitors *e.g.* NSAIDs and selective COX2 inhibitors have been used to treat clinical symptoms of osteoarthritis by blocking synthesis of COX1 and COX2.[37,38] Although, these oral medicaments seem beneficial, there is a serious risk of systemic complications *e.g.* gastro-intestinal ulceration or bleeding and cardiotoxicity.[37,38] Comparable to COX2, IL-1β is a major contributor to inflammatory reactions and catabolic effects to articular cartilage as well.[36,39] In osteoarthritic joints, elevated levels of IL-1β are present in synovial fluid in cartilage and the subchondral bone layer.[36,39] IL-1β inhibits the ability of chondrocytes to repair cartilage by blocking synthesis of type II collagen and aggrecan in ECM.[39,40] IL-1β seems to have a direct adverse effect on chondrocytes as it stimulates the synthesis of MMPs, mainly MMP-1, -3 and -13, which have a deteriorating effect on cartilage.[39,40] Indirectly, IL-1β also degrades cartilage ECM by stimulating the production of the aggrecan molecule proteolytic enzyme ADAMTS metalloproteinases, especially ADAMTS-4.[39,41] Considering the downregulation of IL-1β and COX2 gene expression by tSVF as shown in this current study, local treatment of OA by autologous tSVF might be effective and substantially reduces the risk of systemic complications.[32] Moreover, ASCs have shown to secrete high levels of tissue inhibitors of metalloproteinases TIMP-1 and -2 which

block the degrading effect of MMPs on cartilage.[24] The effects of TIMPs as well as IL-1β, COX2 and other pro-inflammatory signaling molecules might be reversed by tSVF and could possibly slow down the progression of osteoarthritis in the affected joints and thereby reducing pain.

Multiple recently published studies use cSVF as a treatment of osteoarthritis *in vitro*.[42-45] In these studies, however, cSVF is obtained enzymatically by time-consuming isolation procedures.[42-45] These enzymatic procedures disrupt all cell-cell interactions including ECM.[8,16] Although, several studies published about the therapeutic effect of cSVF on osteoarthritis, the regenerative role of ECM is often underestimated.[25-31] In tSVF, the ECM is preserved holding stromal cells *e.g.* ASCs in their local niche.[16] In this way, stromal cells have a higher retention rate after injection and thus probably a prolonged regenerative effect. Besides a cell retaining function, ECM influence cells in a complex mechanical behavioral way.[46] A time-dependent response of cell to loading or deformation, called viscoelasticity, has become widely accepted as a concept wherein mechanical properties of *e.g.* ECM stiffness have an effect on cell proliferation and differentiation.[46] ECM stiffness seems to regulate developmental, homeostatic and regenerative processes.[46] Another important function of ECM is the binding of a plethora of factors and ensuring controlled slow release of these different factors over time.[8,16] A natural slow release scaffold might contribute to a prolonged regenerative effect of tSVF as compared to cSVF.

A limitation of this study is the use of a two-dimensional culture system with a single layer of culture chondrocytes. It is well-known that cells behave significantly different in a two-dimensional culture system as compared to a three-dimensional culture system.[47,48] A three-dimensional culture system mimics processes *in vivo* more accurate.[47,48] In a two-dimensional culture system, the role of synovial inflammation and subchondral bone remodeling and their interaction with chondrocytes cannot be investigated.[47] In 2013, a three-dimensional osteochondral system to mimic the pathogenesis of osteoarthritis was developed which involved mechanical injury, pro-inflammatory cytokine influence and cartilage degeneration.[49] This might be of importance in the translation of results from *in vitro* to *in vivo*.[47] To date, the complex multifactorial pathophysiology of osteoarthritis is not yet fully addressed.[47-50] A second limitation of this study is that it predominantly observes the effect of tSVF on chondrocytes, while subchondral and synovial processes are known to play an important role in OA as well.[20,24] However, both chondrocytes and synovial tissue cells produce pro-inflammatory cytokines with similar underlying mechanisms.[51] Therefore, it is likely that synovial cells will respond the same in terms of downregulation of inflammatory factors in response to tSVF.

Moreover, chondrocytes are the cells that produce cartilage tissue and the co-culture of chondrocytes with tSVF-derived cells resulted in proliferation of chondrocytes and increased GAG deposition.

The results of this study show that mechanically derived tSVF is able to promote chondrocyte proliferation and suppress chondrocyte inflammation *in vitro*. As a result, tSVF has shown to have an anti-inflammatory as well as a pro-regenerative effect on chondrocytes. Consequently, tSVF as a treatment for osteoarthritis seems to be very promising. To further elucidate the beneficial effect of tSVF on osteoarthritis clinically, prospective randomized clinical trials are necessary.

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ANNEX: FAT PROCEDURE

The lipoaspirate was harvested from the abdomen and upper legs with a lipo-harvesting cannula (Tulip, Medical Products, San Diego, CA) after distribution of 500 ml modified Klein's solution (per 500 ml saline, 20 ml of lidocaine, 1% epinephrine 1:100.000 and 2 mL of bicarbonate). The FAT procedure with a re-usable one-hole fractionator in a closed system was performed with the disposable Arthrex Autologous Conditioned Plasma (ACP) Double Syringe Systems (Arthrex, Inc. ABS-10014, Naples, USA). Disposable ACP double syringes were filled with decanted lipoaspirate and centrifuged at 956xg for 2.5 min. with a swing out rotor centrifuge at room temperature (RT) (Hettich Rotofix 32, benchtop, swing out rotor, Tuttlingen, Germany). After centrifugation, the upper oily fraction was discarded by pulling the inner syringe. Then, the lower aqueous fraction was discarded by opening the red closing cap yielding condensed lipoaspirate. Condensed lipoaspirate was used for mechanical dissociation according to the fractionation of adipose tissue (FAT) procedure. After mechanical dissociation, samples were centrifuged again for 2 min. at 760 xg. This yielded an oily fraction that could be easily removed leaving tSVF in the larger outer ACP double syringe.

06

Isolation of stromal vascular fraction by fractionation of adipose tissue

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ABSTRACT

Adipose tissue-derived stromal cells (ASCs) are a promising candidates for cellular therapy in the field of regenerative medicine. ASCs are multipotent mesenchymal stem cell-like and reside in the stromal vascular fraction (SVF) of adipose tissue with the capacity to secrete a plethora of pro-regenerative growth factors. Future applications of ASCs may be restricted through (trans)national governmental policies that do not allow for use of non-human-derived (non-autologous) enzymes to isolate ASC. Besides, enzymatic isolation procedures are also time-consuming. To overcome this issue, non-enzymatic isolation procedures to isolate ASCs or the SVF are being developed, such as the fractionation of adipose tissue procedure (FAT). This standardized procedure to isolate the stromal vascular fraction can be performed within 10 – 12 min. The short procedure time allows for intraoperative isolation of 1 ml of stromal vascular fraction derived from 10 ml of centrifuged adipose tissue. The stromal vascular fraction mostly contains blood vessels, extracellular matrix and ASCs. However, based on the histological stainings an interdonor variation exists which might result in different therapeutic effects. The existing interdonor variations can be addressed by histological stainings and flow cytometry.

INTRODUCTION

Adipose tissue-derived stromal cells (ASCs) are a promising therapeutic cell type for regenerative purposes because of their ability to differentiate in multiple cell lineages and their ability to secrete a plurality of pro-regenerative growth factors.[1,2] ASCs are multipotent stem cell-like stromal cells, which are abundantly present in adipose tissue and easily isolated. In adipose tissue, ASCs are attached around vessels as pericytes and supra-adventitial cells in the stromal vascular fraction (SVF).[3,4] The SVF of adipose tissue contains all non-adipocyte cells (*e.g.* immune cells, fibroblasts, endothelial cells, smooth muscle cells, ASCs).[5]

The therapeutic potential of ASCs is thoroughly investigated clinically for bone and cartilage repair[6], dermal wound healing and fibrosis[7,8] and myocardial infarction[9,10], as well as in non-clinical research for tissue engineering purposes like skin tissue[11] or engineered blood vessels.[12] However, the clinical use of ASCs has become a major challenge because the 'classical' enzyme-based isolation methods are legally restricted in many countries. Enzymatic isolation methods are time-consuming procedures which require non-autologous materials such as enzymes and animal derived products.[13] For those reasons, there is an inherent risk of contamination of the isolated ASCs or SVF cells. Moreover, to generate sufficient numbers of ASCs, culturing and expansion of ASCs is needed. The expansion of ASCs for clinical use requires specialized culture labs (Good Manufacturing Practice facilities (cGMP)) which renders the clinical application of ASCs a costly business.

Therefore, non-enzymatic intraoperative isolation procedures to yield a therapeutic cell fraction from adipose tissue, are being developed to date.[13] Non-enzymatic, mostly mechanical, isolation procedures are faster and less expensive than enzymatic isolation procedures. Furthermore, non-enzymatic isolation procedures do not require non-autologous biological materials and can therefore be used intraoperatively. Non-enzymatic isolation procedures should not be confused with emulsification procedures that are used to increase the injectabillity of adipose tissue.[13,14] In contrast to non-enzymatic isolation procedures, emulsification procedures are not able to disrupt adipocytes. Non-enzymatic isolation procedures often result in a SVF with most of the cell-cell and cell-matrix communications intact (the so-called tissue SVF or tSVF), while enzymatic isolation procedure result in a SVF with only single cells because enzymes disrupt all communication between cells and matrix (the so-called cellular SVF or cSVF).[13] Clinically, the tSVF and cSVF might have a different therapeutic effect as

single cells tend to migrate out of the injection area within the first 24h after injection.[15] In tSVF, the ASCs are still attached around vessels and embedded in the extracellular matrix, which might result in higher retention rates.

The fractionation of adipose tissue procedure (FAT) yields the tSVF in a non-enzymatic manner.[16] This tSVF is an enrichment of blood vessels, extracellular matrix as well as ASCs by the disruption of adipocytes. The ASCs isolated from the tSVF are not affected in their function, phenotype and colony formation capability. Moreover, the high amount of extracellular matrix present in the tSVF may serve as a natural scaffold to deliver and guide cells (*e.g.* ASCs) in their proliferation as well as differentiation. The extracellular matrix in tSVF contains a large number of vessels as well, which can augment vascularization and perfusion. These latter two are important for appropriate wound healing which is frequently impaired in patients suffering from systemic diseases such as diabetes. Therefore, the isolated tSVF by the FAT procedure might be suitable for skin tissue engineering *in vivo* to augment (diabetic) wound or ulcer healing.

Several clinical studies have already shown the beneficial influence of adipose tissue or the stromal vascular fraction on dermal wound healing.[8,17-19] By virtue of the FAT procedure, which is easily standardized, we previously showed that the tSVF composition is subject to interdonor variation. The clinical application of tSVF demands standardized characterization methods. The existing standardized endpoints and methods to validate the isolation procedures and their cellular product are difficult to perform because these methods are time-consuming, complex and expensive. Thus far, no quick intraoperative characterization methods are available. Therefore, we propose easier standardized methods to validate the isolation procedures and their cellular product.

MATERIALS

Liposuction of adipose tissue

1. Human subcutaneous liposuction derived adipose tissue.
2. Scalpel.
3. Modified Klein's solution (per 500 ml of saline, 20 ml of lidocaine, 2% epinephrine 1: 200,000 and 2 ml of bicarbonate).
4. Sorenson type lipoharvesting cannula (Tulip, Medical Products, San Diego, California).
5. 50 ml Luer Lock syringe.

Fractionation of adipose tissue procedure

1. 10 ml Luer Lock syringe.
2. Centrifuge with the capability to go to 956 *xg* (Medilite, Thermo Fisher Scientific, Waltham, MA).
3. Gauge.
4. Fractionator (Luer to Luer connector with three holes of 1.4mm inside) (Tulip, Medical Products, San Diego, CA).

Live/dead assay of tSVF

1. 0.001% carboxyfluorescein diacetate succinimidyl ester (CFDA-SE, ThermoFisher #V12883, Waltham, MA) /serum free Dulbecco's modified eagle's medium (DMEM, Breda, The Netherlands).
2. 0.001% propiumiodide (PI, ThermoFisher #P3566, Waltham, MA)/serum free DMEM.
3. 2% PFA.
4. 4',6-diamidino-2-phenylindole (DAPI) (Life Technologies #D1306, Carlsbad, CA).

Histological characterization of tSVF

1. 10% formalin.
2. 1.8% agarose solution (Select agar, Invitrogen, Life Technologies Corp., Carlsbad, CA, USA).
3. Xylol.
4. 100%, 96%, 70% alcohol.
5. 0.1 M Tris/hydrochloric acid (HCl) buffer pH 9.0.
6. 10 mM Tris/ 1 mM ethylenediaminetetraacetic acid (EDTA) buffer pH 9.0.
7. 3% hydrogen peroxidase/phosphate buffered saline (PBS).
8. α-smooth muscle actin (SMA, Abcam #ab7871, Cambridge, United Kingdom).
9. Perilipin A (Abcam #ab3526, Cambridge, United Kingdom).
10. Von Willebrandfactor (vWF, Dako #A0082, Glostrup, Denmark).
11. 1% Bovine serum albumin (BSA).
12. 1% Human serum.

13. 1% Swine serum.
14. Polyclonal Rabbit anti-Mouse (Dako #P0260, Glostrup, Denmark).
15. Polyclonal Goat anti-Rabbit (Dako #P0448, Glostrup, Denmark).
16. Polyclonal Swine anti-Rabbit (Dako #P0217, Glostrup, Denmark).
17. 3,3'-diaminobenzidine (DAB, Sigma Life Science, St. Louis, MO).
18. Hematoxylin.
19. Mounting solution.

Masson's Trichrome staining of tSVF

1. 4% paraformaldehyde (PFA).
2. 1% triton X-100.
3. Bouin fixative:
    a. 36 ml of picric acid (saturated).
    b. 12 ml of 37% formalin.
    c. 2 ml of acetic acid (glacial).
20. Weighert's iron hematoxylin:
    a. 25 ml of stock solution A.
    b. 25 ml of stock solution B.
    c. Stock solution A:
        i. 0.5 g of hematoxylin.
        ii. 50 ml of 100% alcohol.
    d. Stock solution B:
        i. 0.6 g of iron chloride.
        ii. 49.5 ml of demiwater.
        iii. 0.5 ml of hydrochloric acid.
5. Biebriech scarlet-acid fuchsine:
    a. 0.5 g of Biebriech scarlet.
    b. 0.5 g of acid fuchsine.

   c. 49.5 ml of demiwater.
   d. 0.5 ml of acetic acid (glacial).
6. Phosphomolybdic-phosphotungstic acid:
   a. 2.5 g of phosphomolybdic acid.
   b. 2.5 g of phosphotungstic acid.
   c. 50 ml of demiwwater.
7. Aniline Blue:
   a. 1.3 g of aniline blue.
   b. 1 ml of acetic acid (glacial).
   c. 49 ml of demiwater.
8. 1% Acetic acid:
   a. 0.5 ml of acetic acid (glacial).
   b. 49.5 ml of demiwater.

OPTIONAL: Enzymatic solation of cellular SVF (cSVF) derived from tSVF

1. 0.1% bacterial collagenase A solution:
   a. 50 mg bacterial collagenase A.
   b. 50 ml PBS/1% BSA.
2. 100 μm filters (Greiner Bio-one International GmbH, Kremsmünster, Austria).
3. Lymphoprep (Lucron products).
4. Lysis buffer (Pharmacy University Medical Center Groningen, Groningen, the Netherlands).
5. FACS buffer (PBS/0.5% BSA)

METHODS

Liposuction of adipose tissue

1. Make a small stab incision of 1 cm on the donor site (preferably legs or abdomen).
2. Infiltrate the donor site with a modified Klein's solution (per 500 ml of saline, 20 ml of lidocaine, 2% epinephrine 1: 200,000 and 2 ml of bicarbonate).

3. Place a Sorenson type lipoharvesting cannula into the stab incision.
4. Connect a 50 ml Luer Lock syringe to a Sorenson type harvesting cannula.
5. Pull the plunger backwards to allow for negative pressure and use a surgical clamp or syringe snap lock to maintain the negative pressure.
6. Start harvesting by moving the harvesting cannula forward and backwards.
7. Replace the full 50 ml syringe by an empty 50 ml syringe and start again from step 4.

Fractionation of adipose tissue procedure

1. Divide the harvested adipose tissue in 10 ml Luer Lock syringes without plunger.
2. Decant the adipose tissue for 5 min. at room temperature.
3. Remove infiltration fluid by opening the cap of the 10 ml syringe.
4. Refill syringe to 10 ml and centrifuge the syringe without plunger at 3,000 rpm with a 9.5 cm radius fixed angel rotor or at 960 *xg* for 2.5 min. at room temperature.
5. Remove infiltration fluid by opening the cap of the 10 ml syringe (see Note 1).
6. Remove oil (disrupted adipocytes) by turning the syringe upside down and prevent the adipose tissue from leaking with the use of a gauge.
7. Refill syringe to 10 ml of centrifuged adipose tissue and place the plunger back.
8. Connect the 10 ml syringe with centrifuged adipose tissue to the fractionator and connect an empty 10 ml syringe with plunger to the other side of the fractionator.
9. Push the adipose tissue extensively forward and backwards thirty times (see Note 2).
10. Centrifuge the syringe without plunger at 3,000 rpm with a 9.5 cm radius fixed angle rotor or at 960 *xg* at room temperature for 2.5 min.
11. Remove infiltration fluid by opening the cap of the 10 ml syringe.
12. Remove oil (disrupted adipocytes) by turning the syringe upside down and prevent the stromal vascular fraction (SVF) from leaking with the use of a gauge.

Live/dead assay of SVF

1. SVF is mixed with pre-heated (37°C) 0.001% CFDA-SE and 0.001% (PI) in serum free DMEM and allow for 30 min. of incubation under normal culture conditions (37°C).

2. Wash the SVF with PBS three times.
3. Fix the SVF with 2% PFA for 30 min.
4. Wash the SVF with PBS three times.
5. Stain the nuclei DAPI in the dark for 30 min.
6. Wash the SVF with PBS three times.

Histological characterization of SVF

1. Fix the isolated SVF in 10% formalin overnight at 4°C.
2. Embed the SVF in a pre-heated (60°C) 1.8% agarose solution (1:2).
3. Place the SVF/agarose solution at 4°C for 30 min.
4. Dehydrate samples with the following steps in sequence at room temperature:
    a. 50% alcohol for 30 min.
    b. 70% alcohol for 30 min.
    c. 96% alcohol for 30 min.
    d. Twice in 100% alcohol for 30 min.
    e. Twice in Xylol for 30 min.
5. Embed the samples in paraffin.
6. Cut four mm sections and deparaffinize them in xylol for 15 min.
7. Refresh the xylol and place the samples for another 10 min. in xylol.
8. Move the samples and place them in 100% alcohol for 10 min, then in 96% alcohol for 3 min. and finally in 70% alcohol for 3min at room temperature.
9. Wash the samples in demi water for 3 min.
10. Incubate samples overnight with 0.1 M Tris/HCl buffer (pH 9.0) at 80°C for α-SMA as well as for Perilipin A staining and with 10 mM Tris/1 mM EDTA buffer (pH 9.0) at 80°C for vWF.
11. Cool down to room temperature for 30 min.
12. Wash samples with PBS three times.
13. Endogenous peroxidase activity is blocked with 3% hydrogen peroxidase in PBS at room temperature for 30 min.

14. Wash samples with PBS three times and incubate samples with primary antibodies at room temperature for 60 min.: for α-SMA (1:200) + 1% BSA + 1% Human serum in PBS, for Perilipin A (1:200) + 1% BSA + 1% Human serum in PBS, for vWF (1:200) + 1%BSA + 1% Swine serum in PBS.
15. Wash samples with PBS three times.
16. Incubate samples with secondary antibodies for 30 min. at room temperature: polyclonal Rabbit anti-Mouse (1:100) + 1% BSA + 1% Human serum in PBS for α-SMA, polyclonal Goat anti-Rabbit (1:100) + 1% BSA + 1% Human serum in PBS for Perilipin A and polyclonal Swine anti-Rabbit (1:100) + 1% BSA + 1% Human serum in PBS for vWF.
17. Wash samples with PBS three times
18. Incubate α-SMA sample with a third antibody at room temperature for 30 min. with polyclonal Swine anti-Rabbit (1:100) + 1% BSA + 1% Human serum in PBS.
19. Wash α-SMA samples with PBS three times.
20. Incubate all samples with DAB for 10 min. at room temperature in the dark.
21. Wash all samples in demi water three times for 5 min.
22. Incubate all samples with hematoxylin for 1.5 min.
23. Rinse samples in water for 4 min.
24. Mount all the samples and place a coverslip (see Note 3).

Masson's Trichrome staining of tSVF

1. Follow step 1. till 9. of section 3.3.
2. Fix samples in 4% PFA for 60 min.
3. Permeabilize samples in 1% triton X-100 for 10 min.
4. Wash samples with PBS three times.
5. Fix samples in Bouins fixative at 51 °C. for 5 min.
6. Wash samples in demiwater until color disappears.
7. Incubate samples with Weighert's iron hematoxylin for 20 min.
8. Wash samples in demiwater until color disappears.
9. Incubate samples with Beibrich Scarlet-acid fuchsine for 20 min.
10. Wash samples in demiwater for 2 min.

11. Incubate samples in phosphomolybdic-phosphotungstic acid for 12 min.
12. Wash samples in demiwater until color disappears.
13. Incubate samples in aniline blue for 7 min.
14. Wash samples in demiwater for 2 min.
15. Incubate samples in 1% acetic acid for 5 sec.
16. Wash samples in demiwater until color disappears.
17. Dry samples for 20 min.
18. Mount samples in Permount.

OPTIONAL: Enzymatic solation of cellular SVF (cSVF) derived from tSVF

1. Follow step 1. till 12. of section 3.2.
2. Wash the isolated tSVF with PBS three times.
3. Add 0.1% collagenase A solution 1:1 with tSVF.
4. Stir the collagenase/tSVF mixture in a water bath at 37°C for 1.5h.
5. Centrifuge the sample at 600 *xg* at room temperature for 10 min.
6. Remove supernatant.
7. Collect cell pellet in PBS/1% BSA.
8. Filter the collagenase/tSVF mixture through filters.
9. Centrifuge the sample at 600 *xg* at room temperature for 10 min.
10. Repeat step 6. 7. and 8.
11. Remove supernatant.
12. Collect cell pellet in 30 ml of PBS/1% BSA.
13. Put 15 ml of lymphoprep in a 50 ml tube.
14. Gently add the 30 ml of PBS/1% BSA with the sample.
15. Centrifuge the sample at 1000 *xg* at 4°C for 20 min. and put the brake on 0.
16. Remove the upper layer.
17. Take cells from the interphase carefully.
18. Add PBS/1% BSA to the cells.

19. Centrifuge the sample at 800 *xg* at 8°C for 10 min.
20. Remove supernatant.
21. Resuspend cell pellet in lysis buffer and place the sample on ice for 5 min.
22. Centrifuge the sample at 800 *xg* at 8°C for 10 min.
23. Remove supernatant.
24. Repeat step 19. and 21. till all erythrocytes are disrupted and the red color has disappeared.
25. Resuspend cell pellet in FACS buffer and divide cells in multiple tubes. The number of tubes depends on the number of subset of CD markers used. Additionally, one tube will function as a blanc control and for each fluorophore used, an extra tube will be used for the fluorophore specific isotype control (see Note 4).
26. Centrifuge cells at 300 *xg* at 4°C for 5 min.
27. Resuspend cell pellet in 100 µL FACS buffer.
28. Keep one tube of cells unlabeled and put on ice in the dark for 30 min. This tube functions as a blank control to set up the flow cytometer.
29. Incubate a tube of cells on ice with 5 µL of the preferred fluorophore-conjugated antibodies (1:20) in the dark for 30 min. (Table 1. and see Note 5).
30. Incubate the other tubes of cells on ice with 5 µL of the specific fluorophore isotype control (1:20) in the dark for 30 min.
31. Wash samples with 2 ml FACS buffer.
32. Centrifuge cells at 300 *xg* at 4°C for 5 min.
33. Remove supernatant.
34. Repeat step 10. till 12. three times.
35. Resuspend cell pellet in 300 µL FACS buffer.
36. Proceed to FACS analysis.

NOTES

1. The fractionation of adipose tissue procedure only works when the harvested adipose tissue is separated from all the infiltration fluid and oil by centrifugation at a high speed (960 *xg*). When small amounts of infiltration fluid are left behind, none of the adipocytes will be disrupted and therefore no oil will appear after the final centrifugation step**.** In case of fibrotic adipose tissue is processed by the fractionation of adipose tissue procedure, the fractionator can clog. The fractionator can be cleaned manually with 100% ethanol (Fig. 1).

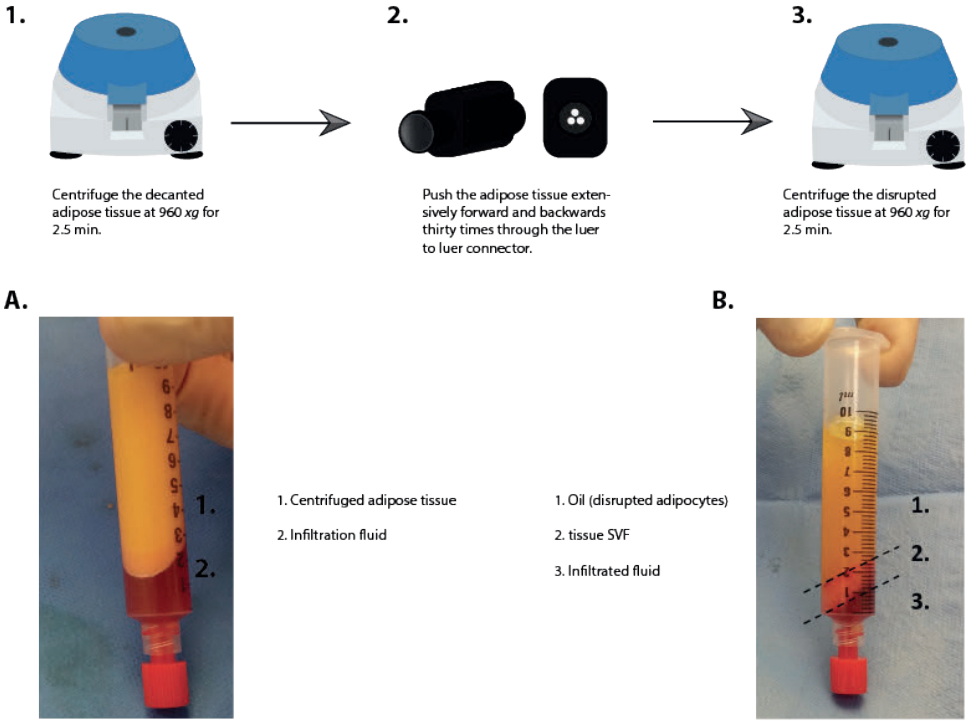

Figure 1. The fractionation of adipose tissue procedure (the FAT procedure). **A)** The composition of one time centrifuged adipose tissue (1. Adipose tissue, 2. Infiltration fluid) at 960 *xg* for 2.5 min. In some cases, oil already appears after the first round of centrifugation. **B)** The composition of adipose tissue (1. Oil (disrupted adipocytes), 2. Tissue SVF, 3. Infiltration fluid) after centrifugation at 960 *xg* and disruption by means of the fractionator and centrifugation at 960 *xg*. SVF = stromal vascular fraction.

2. In case some infiltration fluid is left behind, the harvested adipose tissue will turn white when the harvested adipose tissue is pushed forward and backwards through the Luer-to-Luer connector. After the second round of centrifugation, no oil will appear and therefore the stromal vascular fraction cannot be isolated. This is called emulsified adipose tissue; the liquid content is mixed with the adipose tissue content and allows for a better injectable fraction (Fig. 2).

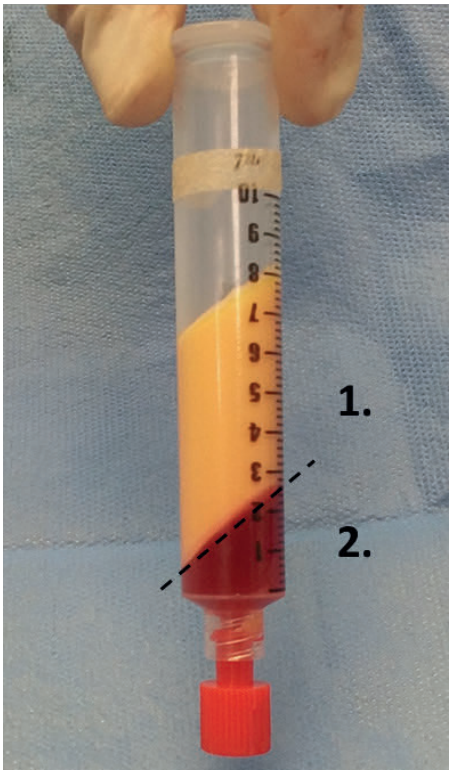

Figure 2. Emulsified adipose tissue with the use of the Nanofat procedure. **1)** Adipose tissue and **2)** Infiltration fluid.

3. Results of the immunohistochemistry images can be analyzed with the use of ImageJ software (freeware, NIH). α-SMA and vWF stainings are measured by drawing a line around the tissue sample of interest and set a threshold to separate positive cells from negative cells. Perilipin A staining can be analysed by manual counting of the positive adipocytes (Fig 3).

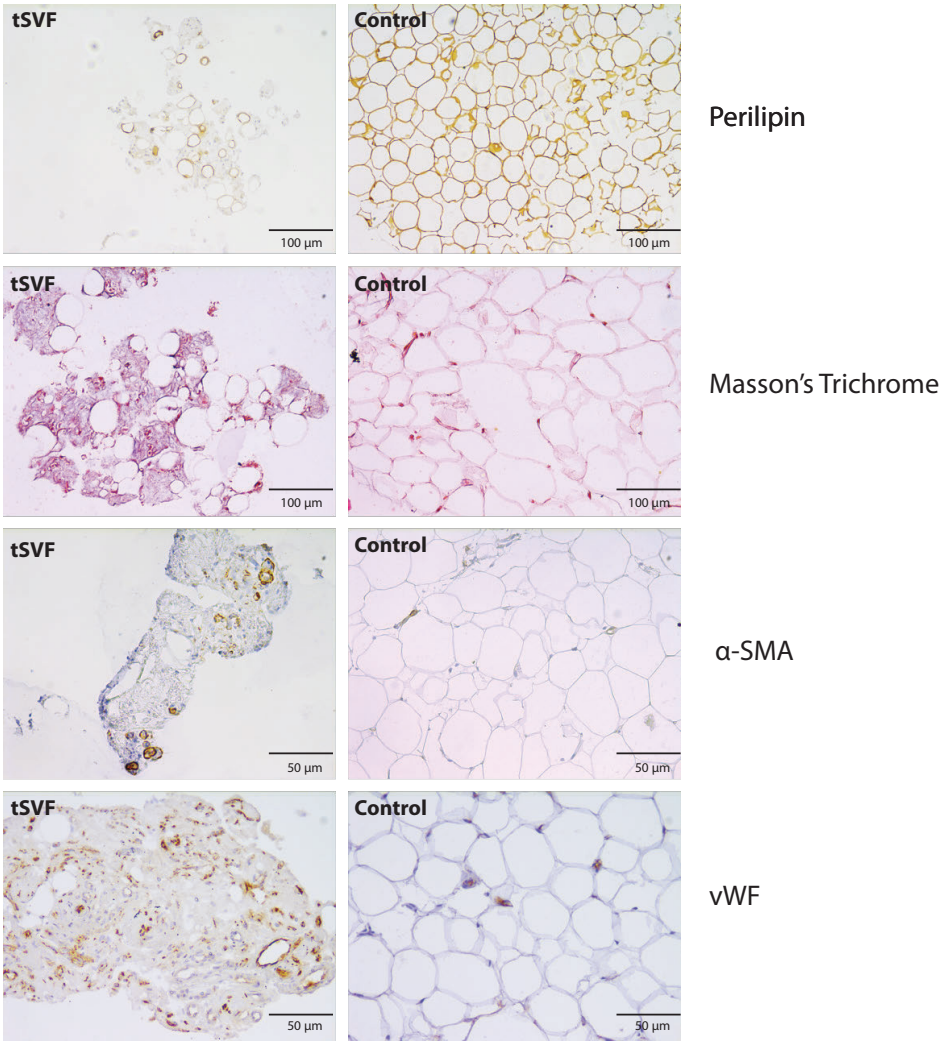

Figure 3. Immunohistochemistry staining examples of how tSVF and unprocessed adipose tissue (control) should look like when a perilipin, masson's trichrome, α-smooth muscle actin (α-SMA) and von Willebrandfactor (vWF) staining are performed.

4. For a complete characterization of the tSVF, flow cytometry analysis of enzymatic isolated tSVF is advised. Each tube contains all the CD markers used to analyse the preferred cell type present in the tSVF (regardless a positive or negative expression of the CD marker on the surface of the cell type). It is possible to analyse multiple cell types with the same CD markers in one tube. The maximum number

of CD markers used in a single tube depends on the chosen fluorophore (*e.g.* allophycocyanin (APC) and fluorescein isothiocyanate (FITC)). Each CD marker used in a single tube should contain a different fluorophore.

5. Different types of fluorophore-conjugated antibodies can be combined and used to analyze different cell types in the tSVF (Table 1). Table 1 contains a recommended set of CD markers to analyze the most important adipose derived cell types in the tSVF.[13] However, there is no consensus regarding the correct subset of CD markers for each cell type.[3,4,13,20-22]

Table 1. The phenotype of the most important adipose derived ($CD45^{neg}$) cell types based on CD marker expression in freshly isolated tSVF.

Cell type	CD31	CD34	CD45	CD90	CD105	CD146
Progenitor pericyte	Negative	Positive	Negative	Positive	Negative	Positive
Pericyte	Negative	Negative	Negative	Positive	Negative	Positive
Supra-adventitial cell	Negative	Positive	Negative	Positive	Negative	Negative
Fibroblast	Negative	Negative	Negative	Positive	Negative	Negative
Adipose derived stromal cell	Negative	Positive	Negative	Positive	Low percentage positive	Negative
Vascular endothelial cell	Positive	Positive	Negative	Positive	Low percentage positive	Positive
Endothelial cell	Low percentage positive	Positive	Negative	Positive	Low percentage positive	Positive

Low percentage positivity means that there is a small subpopulation that expresses this CD marker. CD45 is used to distinguish between two large populations in tSVF: adipose derived population ($CD45^{neg}$) and blood derived population ($CD45^{pos}$).

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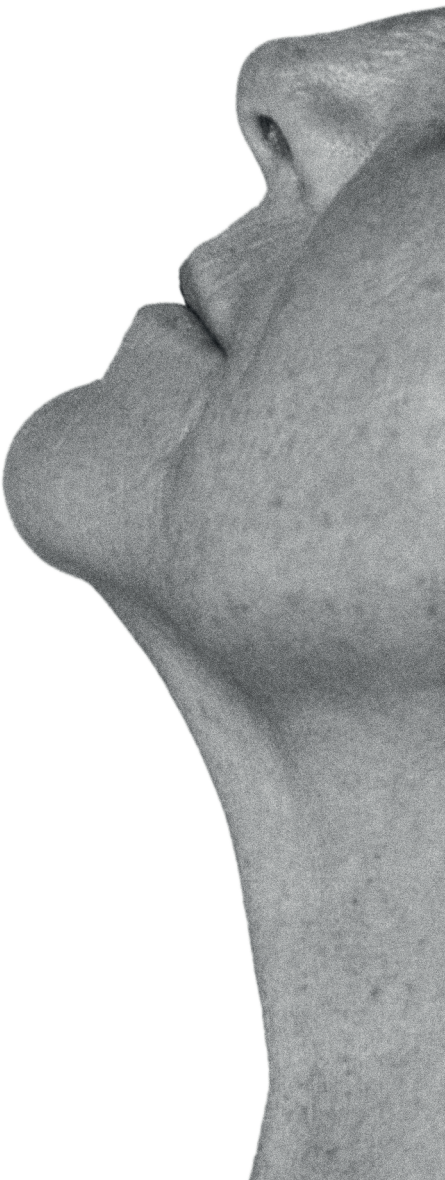
PART II

Adipose tissue and skin rejuvenation



07

The power of fat and its adipose-derived stromal cells: emerging concepts for fibrotic scar treatment

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ABSTRACT

Lipofilling or lipografting is a novel and promising treatment method for reduction or prevention of dermal scars after injury. Ample anecdotal evidence from case reports supports the scar-reducing properties of adipose tissue grafts. However, only a few properly controlled and designed clinical trials have been conducted thus far on this topic. Also the underlying mechanism, by which lipofilling improves scar aspect and reduces neuropathic scar pain, remains largely undiscovered. Adipose-derived stromal or stem cells are often described to be responsible for this therapeutic effect of lipofilling. We review the recent literature and discuss anticipated mechanisms that govern anti-scarring capacity of adipose tissue and its adipose-derived stem/stromal cells. Both clinical and animal studies clearly demonstrated that lipofilling and ADSC influence processes associated with wound healing including extracellular matrix remodeling, angiogenesis and modulation of inflammation in dermal scars. However, randomized clinical trials, providing sufficient level of evidence for lipofilling and/or ADSC as an anti-scarring treatment, are lacking yet warranted in the near future.

DEVELOPMENT OF LIPOFILLING PROCEDURES

Transfer of adipose tissue, also known as fat grafting, lipografting or lipofilling is recognized as a promising and novel technique for correction of volume deficiency, skin rejuvenation and as treatment for scars. This is strongly supported by evidence-based clinical trials as well as fundamental studies in animals and *in vitro*. The first case of lipofilling in literature dates from 1893, when Gustav Neuber described the first free fat transfer for a scar which had left a young man with a soft tissue defect of the face [1].

As soon as liposuction was further developed in the mid 1980's, also interest developed of re-using the lipoaspirated subcutaneous adipose tissue. Liposuction pioneers such as Illouz and co-workers [2] developed the first clinical applications and methods for lipofilling to restore or gain volume. The real breakthrough in lipofilling came with fat harvesting, subsequent processing and subcutaneous administration as described by Coleman [3], which allowed better survival of the lipograft. Centrifugation was the first successful attempt to improve fat graft survival by removing oil, fluid and dead cells from the harvested fat tissue. This method also inspired clinical trials to assess volumetric augmentation of the breast and buttocks [4,5].

Initially, introduced by Coleman in the early nineties, the use of small liposuction and lipofilling cannulas also opened the door for lipofilling of the face and hands for both reconstructive and aesthetic purposes. Especially in these applications with rather superficial lipofilling, effects described as 'more than volume alone' were often observed [3,6]. This included an improved appearance and quality of the skin and has subsequently been described in many case reports. Yet a mechanistic underpinning was still lacking. These clinical observations initiated a wide range of clinical applications for lipofilling other than just volume adjustment [7]. This novel idea to use lipofilling for treatment of (the consequences of) tissue damage, has led to the use of lipofilling to treat burn scars [8] and even to alleviate scar-associated pain as occurring e.g. after mastectomy [9].

In 2001, Zuk and colleagues [10] demonstrated that adipose tissue had a source of endogenous mesenchymal stem cells, which were named adipose-derived stem or stromal cells (ADSC). This discovery significantly advanced the use of lipofilling as a regenerative therapy, as it had been shown that at least one of the components of adipose tissue had therapeutic potential. Since then, many of the beneficial effects observed after lipofilling have been attributed to ADSC.

In this review the authors, both clinicians and biologist, try to bridge the gap between both worlds, provide a review of recent literature and summarize possible mechanism behind the anti-scarring effect of adipose tissue and its adipose-derived stem/stromal cells.

LIPOFILLING ON A CELLULAR LEVEL

Liposuction simply implicates the harvest of adipose tissue under negative pressure with small-bore suction cannulas. By this, the architecture of the fat tissue is disrupted and small lumps of adipose tissue are harvested and collected in a sterile environment (bag or collector), which can then be used for lipofilling subsequently. Inevitably, some degree of hypoxia occurs around the grafting of the lipoaspirate. In the recipient, the integration of the graft requires extensive (re)vascularization, which is primed by the occurring hypoxia as well as by the pre-existing microvasculature in the graft. Too large 'lumps' of lipograft obviously develop necrotic cores due to diffusion insufficiency, as a result of which the graft 'take' may be reduced [11-13]. Adipocytes are sensitive to hypoxia and as a consequence prone to apoptosis [11,12,14]. Depending on the technique and time that is required for harvesting and lipofilling [15,16], 40-90% of the injected lipograft volume will remain [17], while the rest is resorbed within months after grafting. Oily cysts may remain in the grafted area as a consequence of this fat necrosis. To improve fat graft survival, different processing techniques are used (e.g. centrifugation, decantation, gauze-towel technique). In a systematic review, these techniques are compared for viability of the fat graft as a whole [18] in terms of number of viable cells and in terms of graft volume survival in human and animal models. For fat graft survival, the gauze-towel processing technique is found to be superior to centrifugation or decantation. However, if the focus lies on the number of ADSC in adipose grafts, centrifugation improves the number of ADSC that can be isolated, compared to a non-centrifuged fat [19]. Thus, depending on the goal of lipofilling, different fat processing techniques need to be considered carefully.

Adipose tissue, the energy storehouse of the human body, consists of a parenchymal mass of adipocytes that is structurally supported by connective tissue and perfused by blood vessels. All non-adipocyte tissue is called stroma or stromal tissue. Adipocytes are the main volumetric component of adipose tissue although they only comprise up to 20% of all cells [20]. Adipocytes consist of a thin layer of cytoplasm with an eccentric nucleus, while most of the volume is made up by the large central vacuole in which triglycerides predominantly are stored [21].

During development, adipose tissue is derived from the mesodermal germ layer. The mesenchymal stem cells (MSC) that reside in the mesoderm differentiate into adipocytes to form adipose tissue. However, after the embryogenic formation of adipose tissue, some of the mesenchymal stem or stromal cells remain. In the adult situation, these MSC are the previously mentioned ADSC. In the adipose tissue, ADSC reside around the vasculature [22-25]. Furthermore, ADSC retain the ability to differentiate into adipocytes, thus functioning as a source to regenerate adipose tissue [26].

LIPOFILLING AS A METHOD TO TREAT SCARS

As stated above, lipofilling is beneficial for skin and scar treatment. In recent years, a limited number of retrospective and prospective supported previous anecdotal clinical observation (Table1).

Clinical studies

Clinical efficacy of lipofilling in scar areas is determined either by improvement of the appearance of a scar, such as size, thickness, stiffness, discoloration of the scar. In the case of painful scars, this effect can also be measured by a decrease in pain. In the first subsection of this summary of clinical studies, the focus lies on the ability of lipografts to improve several of the above mentioned appearance of scars, whereas in the second subsection focus lies on the ability to reduce pain.

Scar appearance

Macroscopically, scars are characterized by different appearance than the surrounding skin: discoloration, stiffness and roughness are features of scarring. In clinical studies, different outcome measures are used to quantify the degree of scarring on a macroscopic level. The first method often used to assess scar severity are patient or observer rated grading scales, in which several aspects of scarring (e.g. color, stiffness, thickness, irregularity) are rated. A second method is to use measuring devices for skin elasticity or dermal pigmentation.

The efficacy of lipofilling to improve scar appearance has been investigated in sixteen case reports or clinical trials [6,8,27-38] (Table 1a).

Table 1a. Clinical studies on lipofilling to improve scar appearance.

Reference	Study type	Study population	Intervention
Balkin et al. 2014	Retrospective, controlled	Patients with cleft lip repair (n=30, 37 sides). Immediately treated.	Intervention: submucosal, subcutaneous, intra-muscular and periosteal lipofilling (n=20) Control: no lipofilling treatment (n=10)
Benjamin et al. 2015	Case-report	1 Patient with scarring of the lower extremity after trauma.	Intervention: subcutaneous lipofilling (2 interventions)
Bollero et al. 2014	Prospective, non-controlled, non-blinded, non-randomized	Patients with scars after trauma (n=19).	Intervention: subscar lipofilling (28 interventions)
Bruno et al. 2013	Prospective, controlled, non-blinded, non-randomized	Patients with burn wound scars (n=93 scars). Mean scar age of 2.3 years.	Intervention: intra- and subscar lipofilling (n=93) Control: saline injection (n=93)
Byrne et al. 2015	Retrospective, non-controlled	Patients with burn wounds scars of hand (n=13). Mean scar age of 2.3 years.	Intervention: subdermal lipofilling
Coleman 2006	Case-report	1 patient with chronic acne scars.	Intervention: subdermal lipofilling
Guisantes et al. 2012	Cases-report	Patients with retractile and dystrophic scars (n=8)	Intervention: intrascar lipofilling depending on treated area (11 interventions)
Klinger et al. 2008	Cases-report	Patients with scars as a result of hemifacial 2nd and 3d degree burns (n=3). Scar age of 2, 3 and 13 years.	Intervention: dermal-hypodermal junction lipofilling (2 interventions per patient)
Maione et al. 2014	Prospective, controlled, non-blinded, non-randomized	Patients with short-limb deformity syndrome presented retractile and painful scars (age >1 year) caused by surgical procedures (n=36).	Intervention: dermal-hypodermal junction lipofilling (n=36) Control: saline injection (n=36)

Follow up	Results	Complications
Photographic analysis by 3 independent observers using a visual 5-grade scale (mean follow-up of 24.7 months).	Less cleft lip related deformity in overall facial, upper lip, nose and midface appearance in treated group.*	No complications reported
Visual evaluation of the lower extremity.	Patient noted improvement in mobility and appearance, less neuralgic pain,	No complications reported
Visual evaluation of photographs (pre-operative, 1 month and 3 months postoperative).	Among 28 interventions, 24 showed visual improvement in skin quality. 1 case showed improvement initially, but not after 3 months.	No complications reported
Immunohistochemical analysis of scar biopsies, subjective evaluation using a questionnaire, photographic analysis by independent observers using the VSS (preoperative, 3 months and 6 months postoperative).	After 6 months, a decrease in Langerhans cells and increase in P53 and Ki67.** No difference in P67 count. Improvements in VSS scores from 41 (preoperative) to 15 (6 months postoperative) and questionnaire scores from 31 (pre-operative) to 95 (6 months postoperative) compared to untreated group.	Not mentioned
Aesthetic, functional and satisfaction scores were measured using a TAM (Goniometer), GSM (Dynamometer), DASH, MHQ and POSAS after 9.1 months [range 3 months – 1.3 years]	The mobility improved*, but there was no grip strength and DASH improvement. A trend towards significant improvement in MHQ scores was noticed. A significant improvement in the POSAS scores was visible, except the scores for pain and itch.	No complications reported
Visual evaluation of photographs (pre-operative, 11 months and 3 years and 7 months postoperative).	Visual improvement in skin quality.	Not mentioned
Photographic analysis by 2 independent observers using a visual 4-grade scale (mean follow-up of 18 months).	Improvement in skin quality, 5 cases obtained a score of 4 and 3 cases obtained a score of 3.	No complications reported
Histological evaluation of scar biopsies and MRS (preoperative, 13 months postoperative during operation 2, 3 months postoperative).	Histological improvement: patterns of new collagen deposition and more dermal hyperplasia and neoangiogenesis. Presence of annexial structures is nearly normal. MRS revealed similar signal enhancement of sof tissue between affected and unaffected facial sides.	Not mentioned
A modified POSAS and durometer measurements to measure skin hardness were performed (preoperative and 3 months postoperative).	Reduction of scar hardness after treatment*, while no significant reduction occurred in the control group. Reduction of all POSAS parameters, except itching in the treatment group.* No POSAS scores in control group reported.	Not mentioned

Table 1a. Continued.

Reference	Study type	Study population	Intervention
Mazzola et al. 2013	Retrospective, non-controlled	Patients who underwent tracheostomy healed by secondary intention resulting in a retracting scar (n=11). Scar age of 4-10 years.	Intervention: lipofilling in the plane between skin and subcutaneous tissue. (2 interventions, interval of 6-12 months)
Pallua et al. 2014.	Prospective, non-controlled, non-blinded, non-randomized	Patients with facial scars of different causes (n=35).	Intervention: subcutaneous lipofilling
Phulpin et al. 2009	Retrospective, non-controlled	Patients with aesthetic subcutaneous or submucous head and neck reconstruction after radiotherapy (n=11).	Intervention: deep and superficial subcutaneous lipofilling
Ribuffo et al. 2013	Retrospective, controlled	Patients underwent MRM and IIBR + PMRT (n=32). Lipofilling performed 6 weeks after PMRT.	Intervention: deep and superficial subcutaneous lipofilling (n=16) Control: no lipofilling treatment (n=16)
Sardesai et al. 2007	Prospective, non-controlled, non-blinded, non-randomized	Patients with various scar types (n=14). Scar age of >1 year, 8.5 years on average.	Intervention: subcutaneous lipofilling
Wang et al. 2013	Retrospective, non-controlled	Patients with bilateral gluteal concave deformities associated with intragluteal injections. (n=12)	Intervention: deep, intermediate and superficial layer lipofilling of the gluteal
Zellner et al. 2014	Retrospective, controlled	Patients with cleft lip repair (n=35, 44 sides). Immediately treated with lipofilling.	Intervention: submucosal, subcutaneous, intra-muscular and periosteal lipofilling (n=19) Control: no lipofilling treatment (n=16)

Follow up	Results	Complications
Evaluation of patient satisfaction (mean follow up of 21.3 months)	Patients described functional and aesthetical improvement and were all satisfied. 2 cases with severe retraction needed 1 additional lipofilling procedure.	No complications reported
A POSAS, tissue oxygen saturation, hemoglobin levels and microcirculation (Doppler spectrometry) measurements performed (pre-operative, 1 month, 3 months, 6 months and 12 months follow-up).	Improvement in overall POSAS scores, both patient score as observer score.** Only 12 months scores mentioned. Early postoperative measurements revealed increased hemoglobin levels and reduced microcirculation, but both normalized after 7-90 days.	No complications reported
Aesthetic and functional scores were measured using a 5-grade scale (mean follow-up of 39.9 months).	Skin scoring tests revealed more softness, more pliability and improvement of skin quality of the irradiated skin. No scores mentioned.	No complications reported
Capsular contracture was measured using Bakers' classification. Patients' satisfaction was evaluated using a 3-grade scale. (Mean follow-up of 18 months).	7 complications reported in the control group compared to none in the lipofillinggroup.* Higher capsular contracture rates in the control group compared to the lipofilling group. Patient satisfaction increased, but no scores were mentioned.	No complications reported
Dermal elasticity (Cutometer), vascularity and pigmentation (Derma- Spectrometer) measured. Patients' perception (POSAS) and observers' perceptions (POSAS and VSS) evaluated. (preoperative and 12-16 months postoperative)	Increase of dermal elasticity** and no difference in vascularization and pigmentation. Decrease of scar stiffness and thickness in patients' perception.** Less relief and pliability in observers' perception using a POSAS, pliability decrease was confirmed using a VSS.**No differences in vascularization and pigmentation (POSAS and VSS).	Not mentioned
Effect of fat grafting on the skin was evaluated by severity of irregularity, quality of skin patterns and visual impact. Overall satisfaction was evaluated using a 5-grade scale. (Follow up of 3-44 months).	9 cases scored 4-5 and 3 cases scored 3 on the satisfaction scale after treatment. Improvement in skin texture was observed in all cases. Softening of hypertrophic scars was observed, started 1 month postoperative and continued to 12 months postoperative. No preoperative scores mentioned.	1 case with cellulitis in the feet and calves
Photographic analysis by 3 independent observers using a visual 5-grade scale (mean follow-up of 266 days).	Less cleft lip related deformity in overall facial, upper lip, nose and midface appearance(<6 months) and in upper lip appearance (>6 months).* No significant improvement in cleft lip related deformity in the overall nose area (<6 months) and overall facial and midface area (>6 months).	Not mentioned

In ten studies of these publications, comprising of a total of 156 patients, complications were recorded: in nine of these ten studies, no complications were recorded whereas in one study with 12 patients there was a case of cellulitis reported as a complication. Hence, it seems that risks of lipofilling in scar areas is rather low. All fourteen case reports or clinical trials reported some degree of amelioration in scar appearance after lipofilling: in other words, scars became less different from normal skin and/or became less visible. However, overall result of these clinical studies is not unequivocal. Firstly, not all studies use the same outcome measurements to report scar appearance: most studies used patient satisfaction or patient and observer rated grading scales for scar severity to report the effect of lipofilling, whereas other studies used measuring devices for skin elasticity or dermal pigmentation. Secondly, whether or not there is improvement in scar appearance varies within these studies: some studies report improvement in most patients, contrasted by no effect in a few other patients. Lastly, also, within the same study, improvement after lipofilling in one outcome measure (e.g. less stiffness of the scar) is reported, but there is no improvement in other outcome measures (e.g. no improvement in discoloration). Thus, the overall trend is that lipofilling improves scar appearance in several different outcome measures, which is confirmed by two systematic reviews [39,40]. However, due to lack of uniformity in intervention and follow up, no definitive conclusions can be drawn.

Only five well designed controlled studies had well-defined objectives and outcome parameters and had included both non-treated [27,34,37] or placebo [29,31] controls. Four of these studies focused on clinical outcomes [27,31,34,37] and are discussed below and one addresses histological changes [29] and is discussed in the next section.

In two studies, performed under supervision of the same senior researcher [27,37], the effect of lipofilling as adjuvant procedure to reduce formation of new scars after surgery is evaluated. During primary cleft lip repair surgery, efficacy of lipofilling is examined by comparison of pre- and post-operative pictures for residual cleft stigmata by a blinded reviewer panel. Compared to primary cleft lip repair without lipofilling, it resulted in significantly less residual cleft stigmata and thus in better scar appearance. Apparently lipofilling led to reduction of scar formation. Also already existing scars can be treated by means of lipofilling: in prosthetic breast reconstruction in the setting of post mastectomy radiotherapy, post-radiotherapy lipofilling can reduce the degree of capsular contracture as measured by the Baker classification [34]. Here, lipofilling apparently is able to prevent or even (partially) revert the fibrotic process of capsular contracture. Another example is the treatment of post-surgical scars in patients with achondroplasia that require surgical limb lengthening [31]. In this study, lipofilling was

compared to saline injection: lipofilling significantly increased skin pliability and all but one parameter of the patient and observer scar assessment scale improved. Thus, lipofilling apparently improves appearance of existing scars.

Pain reduction

Efficacy of lipofilling as a means for pain reduction was investigated in six case reports or studies [7,9,41-45] (Table 1b). No complications were recorded in six of seven studies with a total of 204 patients; one study did not mention any complications. All studies reported a significant reduction of pain after treatment of painful scars: only in two of these studies there was no difference found in one [7] and in two [41] patients out of the entire population. Three studies included control groups, where lipofilling was compared to no treatment [9,42,43]. Two of these studies, performed at the same institute, focused on lipofilling as treatment for neuropathic pain after total mastectomy [9] or breast conserving surgery [42]. In both studies, it was shown that lipofilling can reduce pain as measured on a visual analogue scale by approximately 3 points in the lipofilling group, compared to about 1 point in the control group. The third study compared results with a representative patient cohort: women who have undergone breast reconstruction and irradiation after mastectomy [43]. In the lipofilling group there was a significant improvement of all parameters of the LENT-SOMA classification (pain, telangiectasias, breast edema, atrophy and fibrosis) after treatment. For unknown reasons, the authors did not compare and analyze the treatment group with a control group, still but they concluded that lipofilling leads to pain relief as well as amelioration of scar appearance.

Table 1b. Clinical studies on lipofilling to reduce pain.

Reference	Study type	Study population	Intervention
Caviggioli et al. 2011	Retrospective, controlled	Patients with severe scar retraction and PMPS after mastectomy with axillary dissection and radiotherapy (n=113).	Intervention: dermal-hypodermal junction lipofilling (n=72) Control: no lipofilling treatment (n=41).
Huang et al. 2015	Prospective, non-controlled, non-blinded, non-randomized	Patients with painful neuropathic scars with persistent symptoms (n=13). [range 3 months - 13 months]	Intervention: dermal-hypodermal junction and subcutaneous lipofilling.
Klinger et al. 2013	Retrospective, semi-controlled non-blinded, non-randomized	Patients with retractile and painful scars compromising daily activity (n=20). Scar age of > 2 years.	Intervention: dermo-hypodermic junction lipofilling. Control: saline injection.
Maione et al. 2014	Prospective, controlled, non-blinded, non-randomized	Patients with PMPS after lumpectomy and radiotherapy (n=96). Lipofilling performed >1 year after radiotherapy.	Intervention: dermal-hypodermal junction lipofilling (n=59). Control: no lipofilling treatment (n=37).
Panettiere et al. 2009	Prospective, controlled, non-blinded, non-randomized	Patients with irradiated reconstructed breasts after mastectomy for carcinomas (n=61, 62 breasts).	Intervention: subscar lipofilling (serial interventions till patient was satisfied or result was stable). (n=20) Control: no lipofilling treatment (n=41).
Rigotti et al. 2007	Prospective, non-controlled, non-blinded, non-randomized	Patients with side effects of radiotherapy with severe symptoms and irreversible function damage (LENT- SOMA scale grade 3 and 4)(n=20). Scar age 1 -30 years.	Intervention: purified lipofilling.
Ulrich et al. 2012	Prospective, non-controlled, non-blinded, non-randomized	Patients with painful episiotomy scars (n=20). Mean time after episiotomy was 10.3 months.	Intervention: subscar lipofilling

Abbreviations: VSS = Vancouver Scar Scale, TAM = Total Active Movement, GSM = Grip Strength Measurement, DASH = The Disabilities of the Arm, Shoulder and Hand, MHQ = Michigan Hand outcome Questionnaire, POSAS = Patient and Observer Scar Assessment Scale, MRS = Magnetic Resonance Scan, MRM = Modified Radical Mastectomy, IIBR = Immediate Implant-Based Reconstruction, PMRT = Post-

Follow up	Results	Complications
Pain evaluation using a VAS (mean follow-up of 13 months).	Decrease of pain in treated group compared to untreated group.**	No complications reported
Pain evaluation using VAS and NPSI scores (pre-operative, 1 week, 4 weeks and 24 weeks postoperative).	Decrease of VAS and VSS scores after 1, 4 and 24 weeks compared to preoperative scores.** No other comparisons between postoperative measurements performed. No effect in 2 cases.	No complications reported
Pain and skin quality of the scar was evaluated using the POSAS questionnaire (without control group). Scar hardness was measured using the durometer (with control group). Both after 3 months.	All POSAS scores (patient and observer scores) decreased significantly except for itching. Scars hardness decreased postoperative compared to preoperative in the treated group.* No significant decrease of scar hardness	No complications mentioned
Evaluation of spontaneous pain using a VAS (preoperative and 1 year postoperative).	A mean decrease of pain of 3.1 in the treated group and 0.9 in the control group. More decrease of pain in the treated group compared to the control group.**	No complications reported
Functional results were evaluated using the LENT-SOMA scoring system, 3 months after the last treatment. Aesthetic results were evaluated using a 5-grade scale.	Scores for pain, telangiectasia, breast edema, atrophy and fibrosis decreased in the intervention group after 3 months.** No significant difference for above mentioned scores compared to the control group after 3 months. Aesthetic outcome improved in the intervention group compared to the control group.*	No significant complications reported
LENT-SOMA grading scale scores evaluation (mean follow-up of 30 months).	Reduction of LENT-SOMA grading scale scores.** Improvement observed in all patients, except 1 case.	No complications reported
Perineal pain evaluation using a MGPQ, a PPI and VAS. The SSSRS was used to evaluate the sexual satisfaction of the patients. (preoperative, 1, 3 and 6 months).	Reduction of pain after 1, 3 and 6 months in all pain questionnaires.* Improvement in sexual satisfaction after 1, 3 and 6 months.* No comparison performed between postoperative time points.	No major complications reported

Mastectomy Radiotherapy, PMPS = Post-Mastectomy Pain Syndrome, VAS = Visual Analogue Scale, NPSI = Neuropathic Pain Symptom Inventory, MGPQ = McGill Pain Questionnaire, PPI = Present Pain Intensity index, SSSRS = Sabbatsberg Sexual Self-Rating Scale

** Significant difference ($p<0.05$)*

*** Significant difference ($p<0.001$)*

Influence of lipofilling in scars at the tissue level

Microscopically, scars display a loss of rete ridges, sebaceous glands and hair follicles. Also, they are characterized by increased dermal and epidermal thickness [46,47]. The epidermal thickening is caused by excessive proliferation of keratinocytes. In the dermis, the thickening is caused by excessive ECM production by myofibroblasts, mainly consisting of collagen type I [48]. Not only is there an increase in the amount of collagens, but also in the collagen fiber thickness, maturation and degree of disorganization [46,47]. Even though there is an increase in the amount of ECM in scarring, some components of normal skin (e.g. elastin, decorin) are less abundant in scars [49].

In two patient studies, skin biopsies have been acquired before and after treatment of scars with lipofilling [8,29], one study evaluating a complete series of biopsies from a single patient [8]. After lipofilling, the general structure of the skin improved, collagen was remodeled, and there was an increase in vascularization.

In a large, placebo-controlled study, lipofilling in large burn scars was compared to saline injection [29]. In 96 patients, half of the scar was injected with saline (placebo or sham treated group), the other half was injected with lipoaspirate. Skin biopsies were taken and analyzed after three and six months. Overall, the histological structure of the scars returned near to that of normal skin: a better organization and alignment of collagen fibrils, better vascularization of the dermal papillae, less melanocytic activity in the epidermis and an increase of the amount of elastin fibers. On cellular level, there was an increase in cell divisions in the basal layer of the epidermis and Langerhans cells migrated downwards into this basal layer. Also, levels of pro-fibrotic factor Transforming Growth Factor beta 1 (TGF-β1) and pro-angiogenic factors Vascular Endothelial Growth Factor (VEGF) decreased.

In summary, histological improvement in scar appearance was noted in both studies, expressed as a plethora of changes on both histological as well as cellular level. However, why and how lipofilling results in the improvement of all these aforementioned aspects of scarring including pain reduction, remains to be elucidated.

Animal studies

In contrast to clinical studies thus far, experimental animal models have been able to demonstrate the mechanisms and influence of lipofilling on dermal scars, scar exterior and scar pain (Table 2).

Table 2a. Animal studies on lipofilling to improve scar appearance.

Reference	Animal model	Intervention	Follow up	Results
Garza et al. 2014	Mouse Radiation of scalp skin	Treatment: lipofilling (human adipose tissue) 4 weeks after irradiation. Control: no lipofilling and/or no radiation.	Histology of skin for epidermal thickness (H&E), collagen arrangement (picrosirius red) and vessel density (CD31). CT for fat graft retention. Histology of fat graft. Assessments 2 and/or 8 weeks after lipofilling.	Return of dermal thickness to normal level. Decrease in collagen level to normal level. Increase of vascular density. All for irradiated skin treated with lipofilling, compared to non-treated irradiated skin. Less fat graft retention in irradiated group compared to non-irradiated group.
Sultan et al. 2011	Mouse Full thickness burn wound on dorsum	Treatment: lipofilling (human adipose tissue) 2 weeks after injury. Control: saline injection	Blood flow measurement by Laser-Doppler. Photographs. Histology for collagen arrangement (picrosirius red) and vessel density (CD31). Gene and protein expression analysis of skin. Assessment 4 and/or 8 weeks after lipofilling.	Improvement in color and texture of wound area. Increased blood flow in wound area at 4 but not at 8 weeks. Increase in pro-angiogenic proteins and decrease of pro-fibrotic proteins. Increased vessel density at 4 weeks. Better collagen alignment at 8 week. All for lipofilling versus control group.
Sultan et al. 2011	Mouse Radiation of dorsum skin	Treatment: lipofilling (human adipose tissue) 4 weeks after irradiation. Control: saline injection and/or no irradiation	Photographs. Histology for epidermal thickness (H&E), collagen arrangement (picrosirius red), vessel density (CD31) and pro-fibrotic marker (Smad3). All at 4 and/or 8 weeks after lipofilling.	Decrease in radiation ulcer size and less hyperpigmentation. Less epidermal thickening. Normalization of vascular density. Decrease in amount of Smad3 (activation not measured). All outcomes for lipofilling treated irradiated animals compared to saline treated irradiated animals.

Table 2b. Animal studies on lipofilling to reduce pain.

Reference	Animal model	Intervention	Follow up	Results
Huang et al. 2014	Rat Full thickness burn wound of hind paw	Treatment: lipofilling (rat adipose tissue) 4 weeks after injury Controls: saline injection or no treatment, and/or sham burn wound.	Behavioral testing for neuropathic pain: paw withdrawal test with mechanical and heat stimuli. Histology of hind paw skin (H&E, MTC) and of spinal cord (microglial activation). All at 4 weeks after lipofilling.	Reduction of burn induced allodynia. Improvement of skin histology in burn wound treated with lipofilling: decrease in collagen deposition, increased cellularity. Less microglial activation in spinal cord. All observations for burn wounds treated with lipofilling, compared to saline injection.
Huang et al. 2015	Rat Full thickness burn wound of hind paw	Treatment: lipofilling (rat adipose tissue) 4 weeks after injury Controls: saline injection and/or sham burn wound	Behavioral testing for neuropathic pain: paw withdrawal tests. Assessment of inflammatory markers in hind paw skin (COX-2, iNOS, nNOS) and spinal cord (IL-1β, TNFα, p-IkB and p-NFkB). All at 4 weeks lipofilling.	Reduction of burn induced allodynia. Decrease of inflammatory markers in hind paw skin and in spinal cord. Decrease in inflammatory pathway activation (p-IkB and p-NFkB) and in pro-apoptotic pathway activation (p-JNK) in spinal cord. All for burn wounds treated with lipofilling, compared to saline injection.

Abbreviations: H&E = hematoxilin and eosin, MTC = Masson's trichrome, IL-1β = interleukin 1 beta, COX-2 = cyclo-ogygenase 2, TNFα = tumor necrosis factor alpha, CD31 = cluster of differentiation 31, iNOS = inducible nitric oxide synthase, nNOS = neuronal nitric oxide synthase

Scar histology has been investigated in two studies using irradiation skin damage models in rodents [50,51] (Table 2a). Skin fibrosis after radiation in general is a clinical relevant problem, which can easily be reproduced in rodents. After radiation, dermatitis develops, which eventually gives rise to fibrotic skin characterized by epidermal thickening and irregular deposition of collagen in the dermis. Also, compared to normal skin, irradiated skin areas have an increased vessel density. In two studies in mice, it has been shown that treatment with lipofilling can reduce all these hallmark features of radiation-damaged skin [50,51]. Decrease in SMAD3 protein levels, a key protein in the pro-fibrotic pathway TGF-β/Smad signal transduction pathway, partly explains the mechanism of scar improvement [50]. In a slightly different model in mice with full thickness burn wounds, it has been shown that lipofilling leads to better scar appearance by increasing pro-angiogenic factors VEGF and stromal cell-derived factor 1 (SDF-1) and decreasing pro-fibrotic factor TGF-β1 [52].

Reduction of neuropathic pain has been reported in two studies of Huang and co-workers [53,54] Ttable 2b). Allodynia, painful perception of a normally non-painful stimulus, after burn wound injury was tested in rats by means of behavioral testing. After burn injury, lipofilling reduced burn induced allodynia. On the one hand, lipofilling reduces skin fibrosis and scarring after burn injury [53,54] and lowers expression of pro-inflammatory mediators in the skin [54]. On the other hand, lipofilling induces changes in the spinal cord as well decreases microglial activation and by lessens activation of the pro-inflammatory NFkB signal transduction pathway in spinal cord cells [54].

It can be concluded that lipofilling in rodent models for skin injury and fibrosis, reduces adverse fibrotic changes. This appears to be mediated by factors from the lipograft that can inhibit activation of both fibrotic and inflammatory signal transduction pathways. All changes caused by lipofilling in a dermal scar have been drawn schematically in Figure 1.

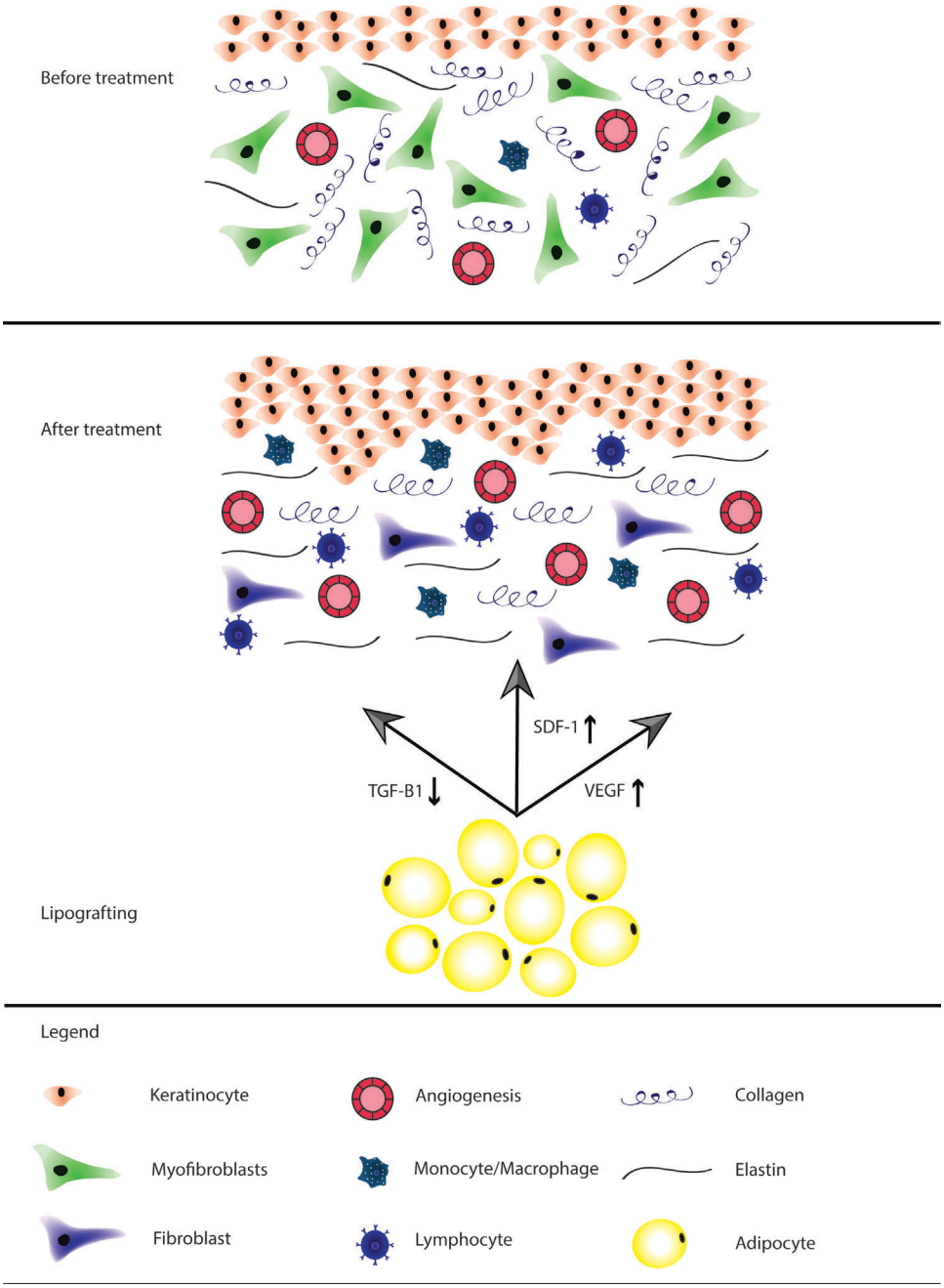

Figure 1. Schematic overview of dermal scar on tissue level, before and after treatment with lipofilling.

THERAPEUTIC MODE OF ACTION OF ADSC

ADSC: stem or stromal cells?

Because of their ability to differentiate into different cell types, ADSC are sometimes referred to as adipose stem cells. However, a true stem cell has the potential to differentiate into other cell types, while maintaining a stable population of stem cells by the process of self-renewal [55] with indefinite proliferation capability due to telomerase activity [56]. Embryonic stem cells are an example of such pluripotent stem cells: they can undergo an infinite number of cell divisions and can differentiate into all cell types of the three germ layers during embryonic development [57]. ADSC, on the other hand, are a type of adult stem cell that have no telomerase activity and therefore have a limited capacity of proliferation [58]. ADSC can only differentiate into a limited number of cell types, which makes them multipotent progenitor cells. Hence, in the case of ADSC, the authors prefer to speak of adipose-derived stromal cells instead of adipose-derived stem cells.

Isolation

ADSC can be isolated either from intact adipose tissue or from lipoaspirates. The adipose tissue or lipoaspirate is subjected to enzymatic digestion using proteases such as collagenase, dispase or trypsin [10,59-61]. After digestion, the Stromal Vascular Fraction (SVF) that contains ADSC as well as several other cell types, is separated from the mature adipocytes by differential or density gradient centrifugation [10,59-61]. For cell culture, the SVF is then seeded into cell culture dishes. Only ADSC adhere to the tissue culture plastic, whereas other, non-adherent cell types such as erythrocytes, endothelial cells and immune cells, are removed by washing [60]. Then, the remaining ADSC are culture-expanded or cryopreserved until further use.

ADSC in vivo versus in vitro

Adipose tissue contains two major components: SVF and adipocytes. SVF is a heterogeneous mix of cells of eleven main subpopulations based on CD-surface marker expression: seven adipose derived populations (CD45-) and four blood derived populations ($CD45^{pos}$) [61]. Three important subpopulations of $CD45^{min}$ cells are pericytes (*in vivo*: ($CD34^{pos}$)/$CD34^{min}$/$CD146^{pos}$/$CD31^{min}$), supra adventitial cells (*in vivo*: $CD34^{pos}$/$CD146^{min}$/$CD31^{min}$) and ADSC (*in vivo*: $CD34^{pos}$/$CD90^{pos}$/$CD31^{min}$/$CD105^{low}$) in a very low number [61-64]. Pericytes and supra-adventitial cells are both identified as precursor cells of ADSC, but there remains controversy [22,23,62,63].

Enzymatic isolation and culture of those precursor cells or ADSC results in a large series of cells that can be used in regenerative medicine. After several days of culture the *in vivo* phenotype of precursor cells changes into an in vitro specific phenotype. Most of the cells will lose their CD34 expression and almost all of the cells gain expression of CD105 [61,62]. The CD105 marker is also known as endoglin and is a TGF-β type III receptor, which is expressed on virtually all cells of mesenchymal origin, but also on *e.g.* endothelial cells. Ten to twenty percent of the subpopulations remain $CD34^{pos}$, but their proliferation rate and adipogenic differentiation ability is significantly lower as compared to the $CD34^{min}$ subpopulation [61,63]. This suggests that 80%-90% of the so-called ADSC, characterized by their phenotype *in vitro* ($CD34^{min}/CD105^{pos}$), are not present *in vivo:* in other words: the majority of ADSC acquire their phenotype through culturing. Culturing of ADSC also causes dramatic shifts in secretome, as will be discussed within a few sentences below. The different components and cell types of all fractions of adipose tissue are summarized in Figure 2.

Some studies have described that regenerative potencies of ADSC is caused by secretion of trophic factors or differentiation into other cells [65]. *In vivo*, little is known about the secretion of trophic factors by ADSC. *In vitro*, secretion of trophic factors by ADSC in medium (called ADSC conditioned medium) is affected by many aspects: differences in culture conditions, donors, methods and medium and cell counts results in different expression of growth factors. For instance, hypoxia culture upregulates VEGF, platelet derived growth factor, placental growth factor and insulin-like growth factor II [66]. A 3D culture structure results in thousands of genes with a significant higher mRNA expression related to extracellular matrix (ECM), cell adhesion, wound healing and growth factors as compared to a 2D structure [67]. Concentrations of proteins related to angiogenesis, ECM remodeling and regeneration increase as well [67].

The regenerative potency of SVF might be caused by the interaction between cells and growth factors. For example, angiogenesis is significant greater when pericytes and endothelial cells are combined rather than the use of pericytes or endothelial cells alone [68]. Growth factors like VEGF, hepatocyte growth factor and TGF-β and extracellular matrix (ECM) stimulate angiogenesis [69].

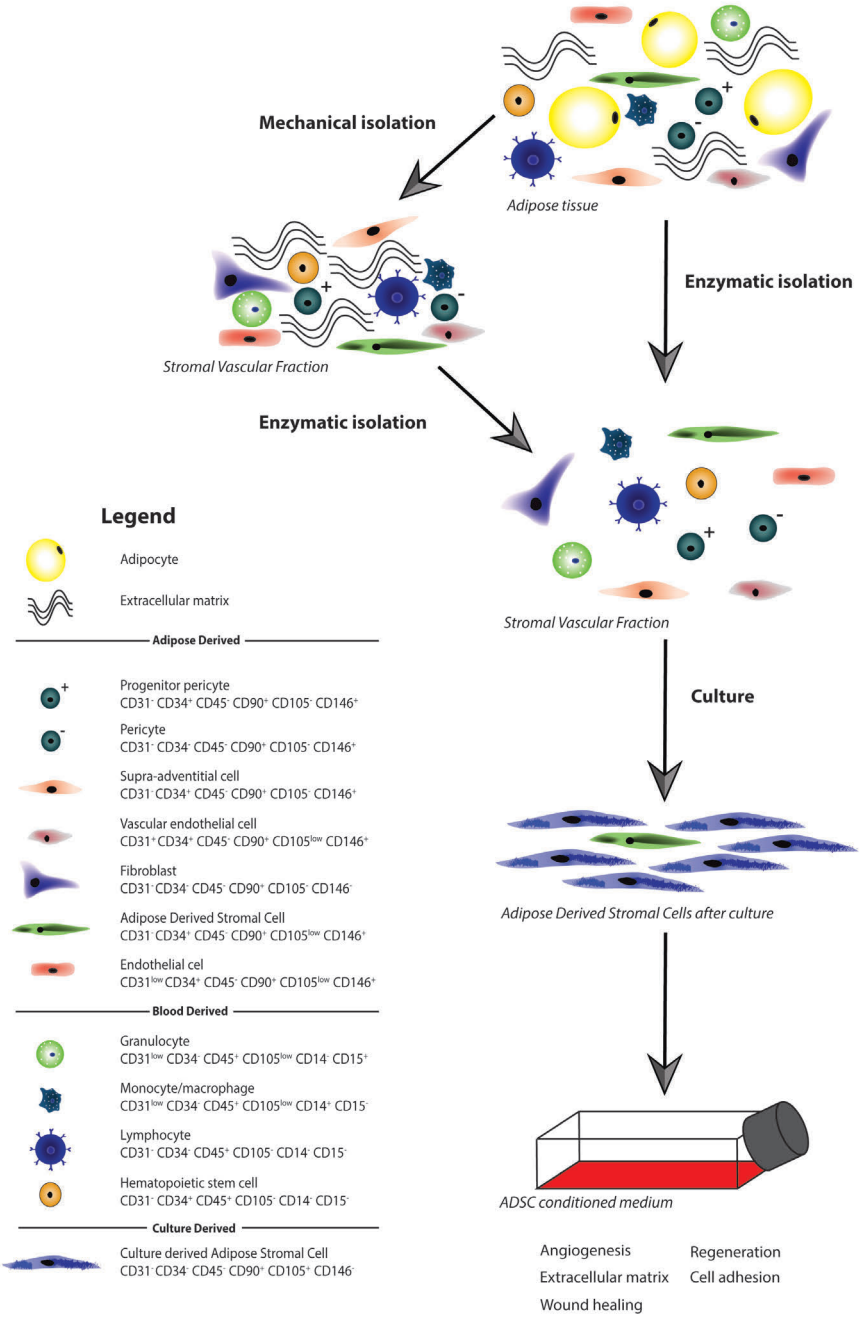

Figure 2. Summary of different fractions of adipose tissue before, during and after mechanical or enzymatic isolation of ADSC. Cell types and their cell surface markers are represented for all different fractions.

ECM influences morphogenesis and migration speed depends on ECM density during angiogenesis [70]. Furthermore, ECM functions as a scaffold for other cell types at the site of injection. The interaction of cellular integrins, i.e. matrix receptors, suppresses pro-apoptotic signaling. Thus, applications that include intact, non-enzymatic, generated SVF might favor graft survival. However, only mechanical isolation of SVF preserves ECM, while enzymatic isolation of SVF disrupts all communicative connections between cells. As compared to cultured ADSC and in vitro studied growth factors, freshly isolated SVF contain cells with still their *in vivo* phenotype and growth factor secretion respectively. As compared to lipofilling, the use SVF might avoid possible complications like cyst formation or overfilling [71]: because only small volumes (less than ten milliliters) of SVF are injected. Thus, since injected volume is limited, there is no risk of overfilling. Since no adipocytes are injected, there is also no risk of oily cyst formation.

ADSC AS AN ANTI-SCARRING TREATMENT

Clinical studies

To date, the use of ADSC as a cell therapy for treatment for fibrosis has not been thoroughly investigated in clinical studies. ADSC have been applied in two non-controlled, non-randomized studies investigating the effect of ADSC-enriched lipografts on healing of chronic, intractable radiation ulcera in 10 patients [72] and for correction of soft tissue defects in 29 patients [73]. It was concluded that ADSC improve wound healing [72] and fat graft take [73] and concomitantly decrease deep tissue fibrosis and dermal scarring. However, fundamentally, there is ample evidence for these effects: ADSC increase angiogenesis, can induce mitosis in resident tissue cells and are able to remodel ECM. Based on the design of both studies, no definitive conclusions can be drawn on the effectiveness of the use of ADSC as scar treatment.

On the other hand, studies in the field of cell-assisted lipotransfer (CAL), where lipografts are combined with ADSC in order to improve fat graft survival, there have been several properly designed, controlled clinical trials [74-76] to demonstrate the efficacy of CAL for improvement of lipograft survival over lipofilling alone. In these studies no serious adverse events were reported after injection of autologous freshly isolated [74,75] or culture expanded [76] ADSC. It can be concluded that use of autologous ADSC in patients is safe. These clinical trials warrant the dissection of the underlying mechanism via animal models and in vitro investigations of underlying molecular pathways.

Animal studies

In animal wound healing models, where ADSC were used to speed up wound healing [77-80] it was observed that ADSC reduce severity of scarring after wound closure (Table 3). ADSC improved the wound healing rate in three out of four studies and smaller fibrotic areas remained after wound healing [77]. Yet, the epidermal thickness increased [79,80], and the gene expression of the pro-fibrotic markers α-smooth muscle actin and TGF-β1 decreased [79,80] while the gene expression of anti-fibrotic fibroblast growth factor and pro-angiogenic VEGF [79] increased. Together, this indicates that *in vivo* administered ADSC, suppress the formation of dermal scar, through augmented wound healing. The comparison with clinical treatment of pre-existing scars is hampered, because these animal studies more prevent scar formation than revert pre-existing scars.

In animal models specifically designed to study scarring [81,82] and to study the fibrotic disorder of Peyronie's disease [83] (Table 3), it was noted that deposition of extracellular matrix components, such as collagen type I and III and elastin, was decreased after treatment of scars with ADSC. Also, collagen fiber alignment improved in the treated scar areas [81,82]. Functionally, treatment of scars with ADSC lead to smaller scars [81] and less scar elevation [81]. Together, we surmise that the remodeling of the fibrotic matrix in a scar by ADSC is one of the components that governs scar reduction. Interestingly, ADSC are derived from connective tissue (SVF of fat), but appear to act as 'good guys' in contrast to the scar myofibroblasts, which are connective tissue cells too, but 'bad guys'. The ADSC are capable of tilting the balance between ECM deposition and ECM degradation in favor of degradation. Whether this depends solely on matrix influence or also on direct influence on the scar-resident myofibroblast remains to be investigated.

In conclusion, treatment of wounds or mature scars with ADSC in different animal models have shown to result in faster wound healing and reduction of scar tissue on both macroscopic and microscopic level. Thus, use of autologous ADSC to improve wound healing and to prevent or diminish scar tissue in patients, seems to be a very exciting and promising way to go.

Table 3. Animal studies on ADSC as a treatment for wound healing and scar prevention or reduction.

Reference	Animal model	Biomaterial	Intervention	Follow up	Results
Castiglione et al. 2013	Rat Peyronie's disease (TGF-β1 induced)	No	$1x10^6$ labeled human ADSC Control: PB Local injection	Protein expression and histomorphometric analysis of the penis, Erectile function measurements 5 weeks after ADSC-treatment.	Decrease in collagen III and elastin deposition (immunofluorescence). Improved erectile function. Both in ADSC-treated vs. control group.
Lam et al. 2012	Mouse Splinted excisional wound healing model	Small intestinal submucosa (SIS)	$1x10^6$ mouse ADSC on SIS patch Control: patch alone or Topical application of ADSC	Wound healing speed, fibrosis (H&E and MTC staining) after wound healing. Measured at day 14 after wounding.	Wound healing improved slightly with ADSC on SIS. Decreased fibrotic area with topical ADCS and with ADSC on SIS Both compared ADSC on SIS to untreated or SIS alone.
Lee et al. 2011	Nude mouse Splinted excisional wound healing model	Collagen gel	$1x10^6$ human ADSC in collagen gel Control: human dermal fibroblast in collagen gel, or collagen gel alone	Photographs of wound area size 10 days after wounding. Scar size 28 days after wounding (H&E staining).	ADSC collagen gel group had a faster wound closure rate than control, but slower than DF collagen gels. Scar size increased in ADSC and DF collagen gel groups compared to control (based on H&E staining alone).
Uysal et al. 2014	Rat Full thickness excisional wound	No	$1x10^7$ labeled rat ADSC Control: $1x10^7$ rat BMSC or PBS Local injection	Wound healing speed. Histology for neovascularization, epithelial thickness (both H&E). Immunostaining for cytokeratin, αSMA, FGF, VEGF, TGF-β1, β2 and β3. All at day 56 after wounding.	Increased wound healing speed, neovascularization and epithelial thickness. Lower αSMA, TGF-β1, β2 and β3 and higher FGF and VEGF expression. All outcomes for ADSC and BMSC treated groups vs. control group
Yun et al. 2012	Pig Scarring model, after full thickness wound	No	$1x10^6$ labeled human ADSC Control: PBS Three consecutive local injections	Area, color and flexibility of scar. Histological assessment of collagen arrangement (MTC), number of mast cells. Gene expression analysis of scar tissue. All until 50 days after ADSC injection.	Slightly smaller scar area and slightly higher pliability. Higher amount of mature collagen. Lower mast cell count. Lower gene expression of αSMA and TIMP1, higher expression of MMP1. All outcomes for ADSC treated group vs. control group.

Table 3. Continued.

Reference	Animal model	Biomaterial	Intervention	Follow up	Results
Zhang et al. 2015	Rabbit Hypertrophic scar model, after full thickness wound	No	$4x10^6$ labeled rabbit ADSC Control: ADSC CM, culture medium, or untreated. Local injection	Histology for scar size and collagen arrangement (H&E and MTC). Gene expression analysis of scar tissue. All until 35 days after ADSC injection.	Less scar elevation. Less deposition and better alignment of collagen. Lower gene expression of αSMA and collagen I. All outcomes for ADSC or ADSC CM treated groups vs. culture medium or untreated groups.
Zonari et al. 2015	Rat Full thickness excisional wound	PHBV scaffold	$1x10^6$ labeled rat ADSC in PHBV scaffold Control: PHBV scaffold or untreated	Wound healing speed, skin thickness (H&E), vessel density, collagen arrangement (MTC) and gene expression analysis. All until 28 days after wounding.	No difference in wound healing speed. Improved skin thickness and collagen fiber organization. Lower αSMA and TGF-β1, higher TGF-β3 gene expression. No difference in vessel density at 28 days. All these outcomes for ADSC in scaffold vs. scaffold alone.

Abbreviations: ADSC = Adipose Derived Stem/Stromal Cell, SIS = Small Intestinal Submucosa, H&E = Hematoxillin and Eosin, MTC = Masson's Trichrome, DF = Dermal Fibroblast, αSMA = alpha Smooth Muscle Actin, FGF = Fibroblast Growth Factor, VEGF = Vascular Endothelial Growth Factor, TGF-β = Transforming Growth Factor beta, BMSC = Bone Marrow Mesenchymal Stem/Stromal Cell, PBS = Phosphate Buffered Saline, TIMP1 = Tissue Inhibitor of Metalloproteinase, MMP = Matrix Metalloproteinase, ADSC CM = ADSC Conditioned Medium, PHBV = Polyhydroxybutyrate-co-Hydroxyvalerate

In vitro studies

Myofibroblasts play a major role in wound healing and scarring: activated myofibroblasts proliferate, produce extracellular matrix like collagens and have the ability to contract. After wound healing, myofibroblasts normally are resolved via apoptosis. However, if myofibroblasts persist, scarring will be the end result [84]. In two in vitro studies, it has been shown that trophic factors, produced by ADSC, can inhibit the myofibroblast phenotype of dermal fibroblasts after stimulation with the pro-fibrotic cytokine TGF-β1 [85] and can inhibit that of fibroblasts derived from Dupuytren's nodules [86]. Proliferation, extracellular matrix production and contraction of these fibroblasts were reduced, which indicates that growth factors and cytokines of ADSC have the ability to prevent or even to reverse dermal scarring.

FUTURE PERSPECTIVES

As discussed throughout, harnessing the power of fat for fibrotic scar treatment, is an emerging concept in regenerative medicine. Fat can however be used in several fashions: as whole adipose tissue in lipofilling, or in loose components such as SVF, ADSC or even ADSC conditioned medium. In our opinion, each of these forms has its own ideal application in regenerative medicine (Figure 3). The use of whole adipose tissue in lipofilling is optimal when there is a soft tissue defect which needs filling. Besides the 'volumizing' effect, scar reduction is a beneficent side effect of this treatment. Though, when extra volume is not a requirement or even a contraindication, the use of SVF offers an excellent alternative. In the setting of fibrotic dermal scars in areas where addition of extra volume is not aesthetically desirable, SVF is a good alternative for whole adipose tissue. Besides for use in dermal fibrotic scars, use of SVF opens the door for other clinical applications. Whole adipose tissue is not fit for use in fibrotic disorders in organs, such as cardiac or liver fibrosis. SVF however, would be a suitable alternative to combat organ fibrosis. SVF has all the requirements to act as a scaffold for repair, since it contains ready-to-use microvasculature, ECM and ADSC to orchestrate the repair process. For example acceleration of wound healing or alteration of early scar formation would be exemplary candidates for use of SVF. Nonetheless, in case of a pre-existing scars, a more rigorous remodeling of the mature scar tissue is necessary. Here, the microvasculature and ECM components of SVF are not a prerequisite. Thus, the application of ADSC would suffice. ADSC could orchestrate the remodeling, for example by immunomodulation or by instruction of the resident tissue cells from a synthetic to a proteolytic or a non-contractile phenotype. Last but not least, ADSC conditioned medium offers the ultimate solution when only instructive (growth) factors

are required. In this way, use of allogeneic cells or xenogenic cell culture products can be circumvented, resulting in an off-the-shelf product. ADSC conditioned medium would be ideal for topical application or injection in wounds or developing scars.

CONCLUSION

Since Neuber's first report in 1893, the use of adipose tissue, has gradually developed into an exciting new way to be used in the treatment and prevention of scar tissue. After lipofilling or after application of ADSC, improvement of scar appearance or reduction in scar related pain has been reported in many case reports and clinical studies. Lipofilling and ADSC seem promising to lessen the severity of developing as well as pre-existent fibrotic scarring. A factor which complicates definitive conclusions in the efficacy of lipofilling and ADSC, is the wide variety in experimental design of the studies. Each study uses different outcome measurements, at different time points in pre-existent as well as in developing scarring. Up to date, large randomized controlled clinical trials using lipofilling, ADSC, SVF or ADSC conditioned medium for fibrotic scar treatment, are still lacking. For future randomized controlled clinical trials, we recommend researchers to carefully select their source of stromal cells depending on their goal.

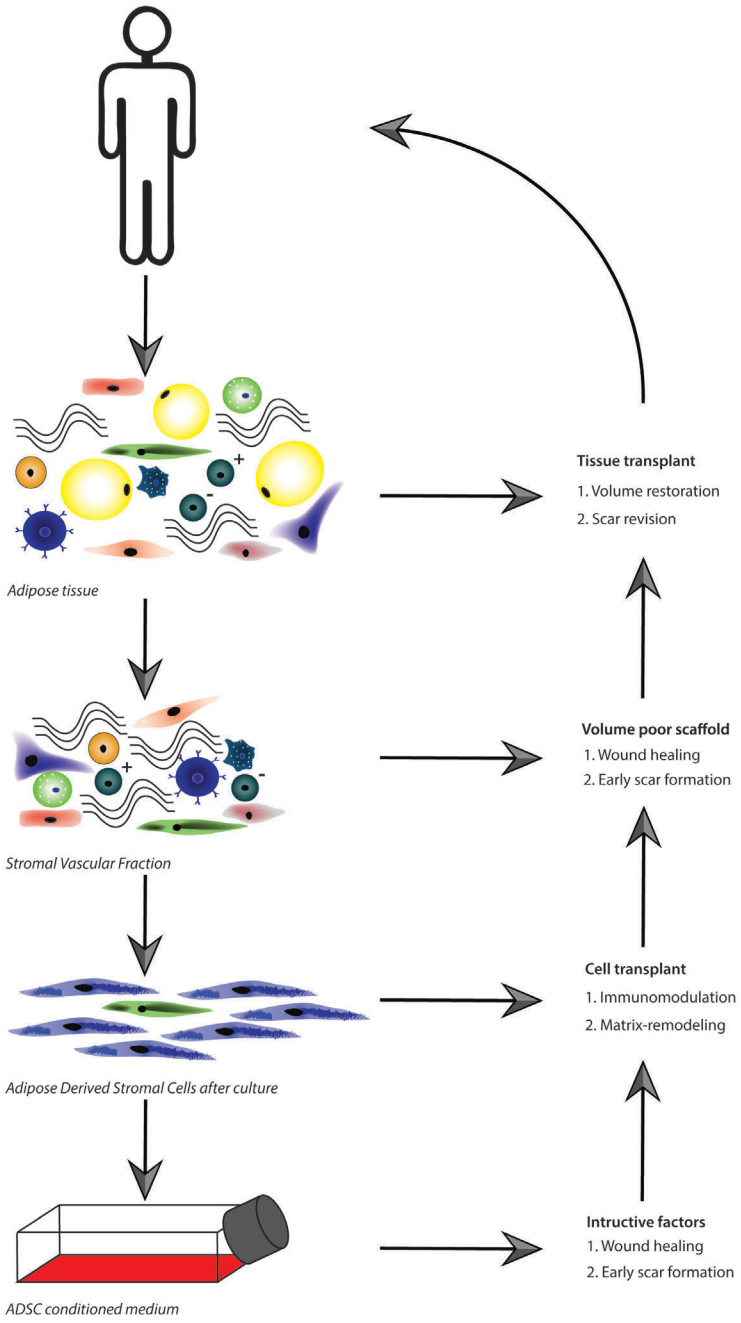

Figure 3. Harnessing the power of fat for fibrotic scar treatment: as whole adipose tissue in lipofilling, or in loose components such as SVF, ADSC or ADSC conditioned medium. As listed, we propose each form has its own ideal application.

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08

Tissue stromal vascular fraction to prevent dermal scarring: a prospective randomized multicenter clinical trial

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In preparation

ABSTRACT

Introduction

The skin is the first physical barrier of the human body to pathogens. After skin defects occur, the wound starts to heal in consecutive and overlapping phases. Ideally, a wound heals without a scar, however multiple factors contribute to pathological scar formation *e.g.* chronic inflammation or large wound size. Till now, no evidence-based treatment is available to stimulate wound healing that regenerates damaged skin without a scar. Tissue stromal vascular fraction (tSVF) of adipose tissue is a heterogenous mixture of cell types embedded in an extracellular matrix loaded with a large number of growth factors and cytokines that stimulate and modulate all wound healing-related processes including parenchymal proliferation, angiogenesis, matrix remodeling and inflammation. In this study, we hypothesized that tSVF increases dermal wound healing of post-surgical wounds and thus suppress subsequent scar formation.

Material & methods

This prospective, double-blinded, placebo-controlled, randomized trial was conducted between 2016 and 2020. In total, 40 female mammoplasty patients were enrolled with 34 completing the follow-up. All patients received tSVF in the lateral 5 cm of the horizontal scar of one breast directly postoperative after a bilateral reduction mammaplasty. The other lateral 5 cm of the horizontal scar of the other breast received a placebo injection comprising 0.9% NaCl. tSVF was isolated by means of the fractionation of adipose tissue (FAT) procedure. Results were obtained using the patient and observer scar assessment scale (POSAS), photograph evaluation using a visual analogue scale (VAS) by blinded observers and number of complications up to 1 year postoperative.

Results

Injection of tSVF improved postoperative scar appearance as compared to a placebo injection evaluated using the POSAS questionnaire six months postoperative. This difference in scar appearance lost significance after twelve months. In both groups, postoperative scars were barely visible after twelve months. No improvement was seen based on the evaluation of photographs of postoperative scars between both groups.

Conclusion

Immediate postoperative injection of tSVF suppresses scar formation up to six months postoperative, although this effect seems to disappear after twelve months. This study indicates that tSVF accelerates early wound healing and coincides with barely visual scar formation.

INTRODUCTION

Wound healing is the primary response of the body to a tissue or skin defect in order to regenerate a first mechanical barrier for pathogens. Initial, wound healing is a physiological reaction following consecutive phases: hemostasis, inflammation, proliferation and remodeling.[1] During hemostasis, a fibrin clot is formed to stop bleeding and retain cytokines and growth factors released from platelets *e.g.* platelet derived growth factors (PDGF), transforming growth factor-β (TGF-β), fibroblast growth factors (FGF), vascular endothelial growth factor (VEGF) and insulin-like growth factor (IGF).[2-4] These factors initiate the transition from hemostasis to inflammation by attracting macrophages and neutrophils. During the inflammation phase, granulocytes and macrophages phagocytose debris and invading pathogens and thus from a barriers to protect the wound. When all contaminating bacteria are removed, neutrophils start to disintegrate and macrophages shift from type 1 to type 2 phenotype allowing to initiate the proliferative phase, which is characterized by fibroblast proliferation, migration and differentiation into myofibroblasts.[5,6] Then, these (myo)fibroblasts start to synthesize collagen and play a key role in wound contraction.[7] In the final remodeling or fibrotic phase, the ratio of type I and type III collagen is reversed from more type III to more type I with stronger crosslinking of collagen fibers.[8]

Ideally, these consecutive and overlapping phases result in scarless regeneration of the skin. However, several factors contribute to pathological scar formation after wound healing *e.g.* chronic inflammation or large wound size causing excessive myofibroblast invasion over-producing large amounts of collagen. This excessive collagen deposition yields stronger cross-linking density which stimulates myofibroblasts to further contract and secrete more collagen. In physiological wound healing, fibrotic tissue is replaced by healthy tissue before fibrotic initiates such a positive feedback loop.[9,10] Significant pathological wound healing is often caused by diseases and disorders *e.g.* diabetes mellitus, peripheral arterial disease or burn wounds. Although, inflammation is important for proper wound healing, a chronic state of inflammation decrease vascularization and causes necrosis such as seen in diabetic ulcers.

Till now, no standard evidence-based treatment is available to increase normal ideal wound healing by stimulating simultaneously multiple wound healing related processes *e.g.* angiogenesis, immunomodulation and matrix remodeling. However, recent developments have shown that stromal vascular fraction (SVF) of adipose tissue, which is a heterogenous mixture of cell types containing a plethora of growth factors and cytokines, should be able to stimulate the aforementioned wound healing related processes.[11-13] Traditionally, enzymatically, prepared SVF consist of adipose

tissue-derived stromal cells (ASCs), fibroblasts, endothelial cells, smooth muscle cells and pericytes.[14] If prepared by mechanical fractionation, SVF also contains micronized adipose tissue devoid of adipocytes *i.e.* extracellular matrix maintains all relevant adhesions and is then referred as tissue-SVF (tSVF).[15] The extracellular matrix is an important and often underestimated therapeutic component in SVF and functions as a natural slow release scaffold of cytokines and growth factors.[12] Besides, extracellular matrix affects cell behavior via biochemical signaling as well as via mechanical signaling such as topography and stiffness. These latter instructive cues lack in enzymatically prepared SVF.[16]

In this prospective clinical study, tSVF is evaluated for its potential to increase dermal wound healing of a post-surgical wound to subsequently suppress scar formation.[17-19] A bilateral reduction mammoplasty was used as a clinical model to test both the safety and efficacy of tSVF in wound healing in a healthy female population.

MATERIAL & METHODS

Study overview

This study was conducted as a multi-center, double-blind, randomized, placebo-controlled clinical trial in the Netherlands. The study was carried out in compliance with the Declaration of Helsinki and approved by the national medical ethics committee (CCMO) of the Netherlands (National legislator trial code: NL55651.000.16, Dutch trial register code: NTR5719). All subjects provided written informed consent prior to start of the study.

Patient population and randomization

A sample size calculation estimated the number of subjects required for this study at 34. Due to a higher number of drop-outs than expected, 6 subjects were additionally included with a total number of participating subjects of 40. In- and exclusion criteria are summarized in table 1. Subjects who underwent a bilateral wise pattern reduction mammoplasty were included and each breast was randomly divided in one of the following groups: tSVF injection or saline injection. Patients were followed for 1 year and blinded during the entire study. Patient enrollment started at June 2016 until the study ended at June 2020.

Table 1. In- and exclusion criteria of subjects participating in this study.

Inclusion criteria	Exclusion criteria
Females	Breast surgical intervention 1 year prior start of the study
Age 18-60	Any oncological event in history
	Known psychiatric condition
	Known systemic disease that will impair wound healing
	Smoking
	Pregnancy or active child wish
	Frequent exposure to known carcinogenic substances (*e.g.* work related)
	Active or previous use of hormone replacement therapy

Harvesting, preparation and injection of tissue-stromal vascular fraction

Forty ml of adipose tissue was harvested from abdominal region with very fine Tonnard harvesting cannulas (Tulip, Medical Products, San Diego, CA) to obtain two times 1 ml of tSVF by means of the fractionation of adipose tissue procedure (FAT).[20] Briefly, harvested lipoaspirate was decanted and centrifuged at 3,000 rpm with a radius fixed angle rotor (Medilite, Thermo Fisher Scientific, Waltham, MA) for 2.5 min. at room temperature (RT). Then, two times 10 ml of centrifuged lipoaspirate was mechanical dissociated using a fractionator (a luer to luer connector hub with three holes of 1.4mm inside) by pushing lipoaspirate 30 times forward and backwards. After fractionation, lipoaspirate was centrifuged again using identical settings. One ml of tSVF was injected into the lateral 5 cm of the horizontal scar of one breast, while the lateral 5 cm of the other breast received 1 ml of saline. Injection was performed into the wound edges using a 18G needle after suturing intracutaneously with monocryl 4.0.

Patient and observer scar assessment scale

Scar appearance was assessed with the use of the patient and observer scar assessment scale (POSAS) by both the patient and plastic surgeon 6 months and 12 months postoperative. As primary outcome, the total average score of the patient part of the POSAS questionnaire was used, while the observer part served as secondary outcome. Each scar characteristics of both the patient and observer part of the POSAS questionnaire was analysed separately. A lower score represents an aesthetically better scar than a higher score. Only the lateral 5 cm of the horizontal scar was assessed.

Photographic evaluation

Scar appearance was assessed using photographic evaluation of postoperative photographs 6 months and 12 months after surgery. All photographs were digitally taken and white balanced using a color chart with Adobe Lightroom CC Version 4.0. All photographs were cropped to only display the lateral five cm of the horizontal scar using Adobe Photoshop CC Version 19.1.9. Photographs were randomly divided into three online surveys using SurveyMonkey (SurveyMonkey, Palo Alto, CA). Three observer groups of three plastic surgeons rated each blinded photograph of one survey from 1 – 10 on a visual analogue scale (VAS). In total, three surveys containing postoperative photographs were analyzed. After at least 72 hours, the same survey in a random sequence was scored again to determine the inter- and intra-observer agreement.

Statistical Analysis

Statistical analysis was performed by an independent statistician (KMV) that received blinded data from the first author (JAD). A paired samples *t*-test was used to analyze the outcomes of the POSAS questionnaire. A two-sided $p<0.05$ was considered statistically significant. Inter- and intra-observer agreements were determined based on a weighted kappa coefficient in SPSS.[21] All analysis and designing of the graphs was done with the use of prism 8 (GraphPad software, La Jolla, CA, USA).

RESULTS

Demographics

A total of thirty-four subjects were enrolled in this study. Six subjects were lost to follow-up before the first postoperative visit and three subjects did not complete the entire study. All subjects lost to follow-up did not specify reasons and were compensated by including six additional subjects. Mean age of subjects was 40 ± 13 years old. No complications related to tSVF injections occurred.

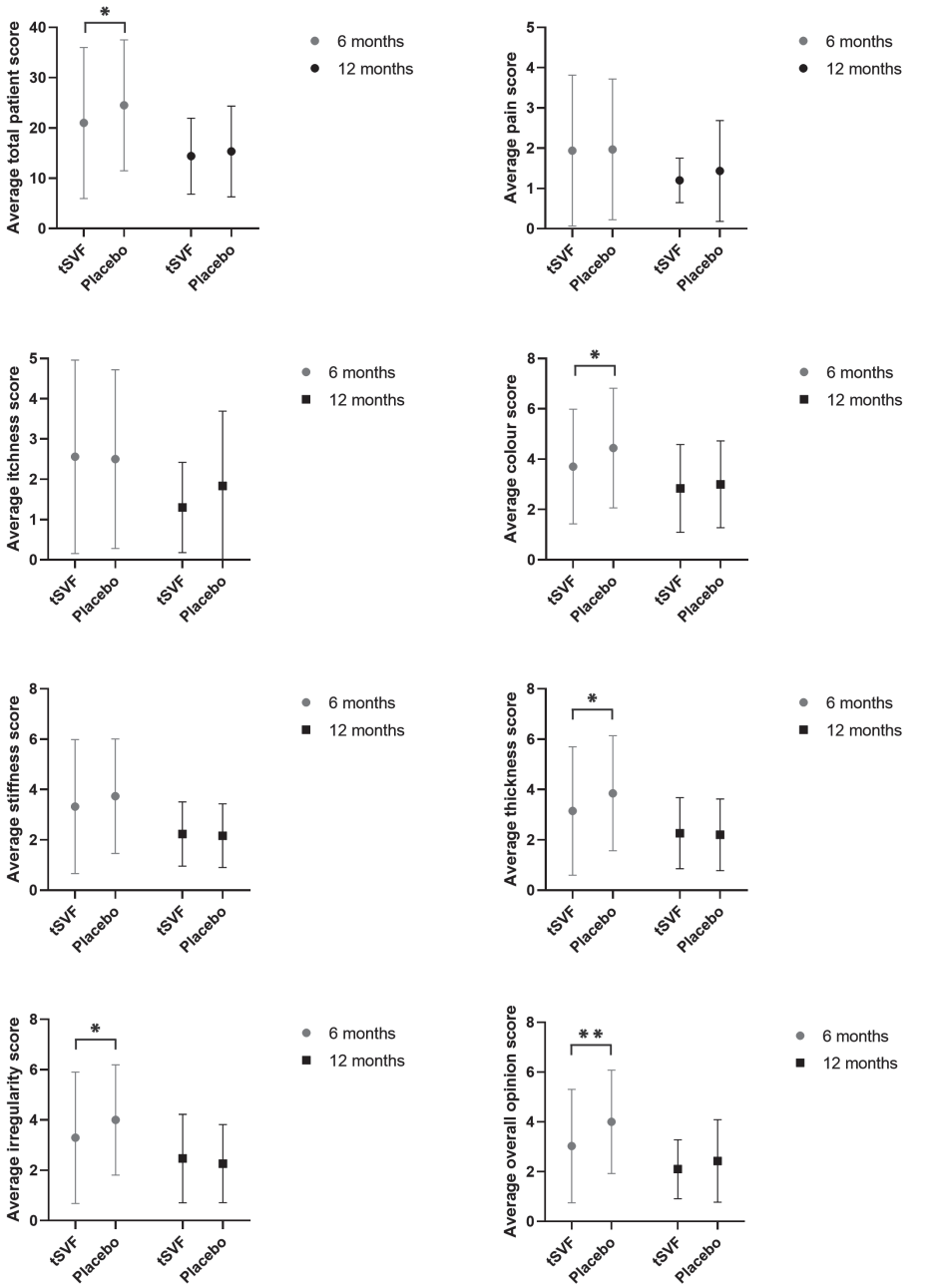

Figure 1. Changes in patient scores of the POSAS questionnaire *i.e.* total score, thickness, stiffness, pain, itchiness, irregularity, color and overall opinion are presented between both the tSVF and control group for all timepoints. ** A lower score for overall opinion was shown in the tSVF group in comparison with the control group six months postoperative ($p<0.01$). * A lower total score as well as score for thickness, irregularity and color was shown in the tSVF group in comparison with the control group sixth months postoperative ($p<0.05$). No significant difference was seen in scar characteristics twelve months postoperative. tSVF = tissue stromal vascular fraction.

tSVF suppresses scar formation up to six months postoperative

Injection of tSVF improved postoperative scar appearance significantly as compared to a placebo injection as evaluated at six months postoperative (Fig. 1). However, this significant difference in scar appearance between the tSVF and placebo group disappeared after twelve months. The average total score of the patient part of the POSAS questionnaire was 21 ± 15.0 versus 24.5 ± 13.0 for respectively tSVF and the placebo group after six months ($p<0.05$, fig. 2). A sub-analysis of scar characteristics revealed a difference in color, thickness, irregularity ($p<0.05$) as well as the overall opinion ($p<0.01$). The average total score of the observer part of the POSAS questionnaire was 18.8 ± 11.3 versus 23.6 ± 11.2 for respectively tSVF and the placebo group after six months ($p<0.01$, fig. 2). A sub-analysis of scar characteristics revealed a difference in vascularity, relief ($p<0.05$) as well as thickness, pliability, surface and overall opinion ($p<0.01$, fig. 2).

3.3 Photographic evaluation

Post-operative scar appearance between tSVF treated and placebo treated wound areas did not differ irrespective of the moment of assessment (Fig. 3). The intra-observer agreement was fair (0.21 - 0.40) for four observers, while the intra-observer agreement was poor (<0.2) for five observers (Table 2). All inter-observer agreements within each observer group were poor. The low intra- and interobserver agreements highlight the subjective nature of a photographic evaluation of postoperative scar appearance.

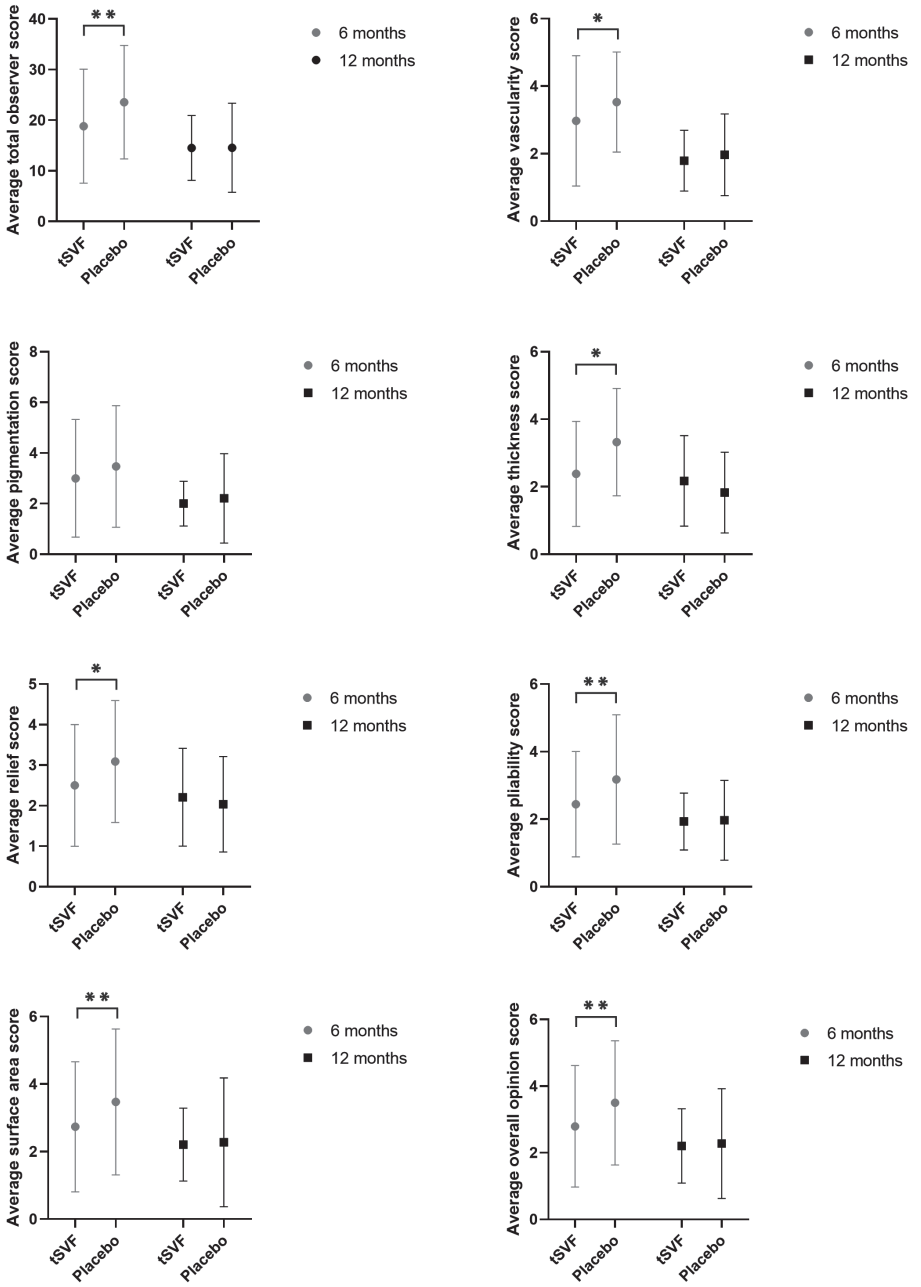

Figure 2. Changes in observer scores of the POSAS questionnaire *i.e.* total score, thickness, pliability, relief, surface, pigmentation, vascularization and overall opinion are presented between both the tSVF and control group for all timepoints. ** A lower total score as well as score for pliability, surface and overall opinion was shown in the tSVF group in comparison with the control group six months postoperative ($p<0.01$). * A lower score for thickness, relief and vascularization was shown in the tSVF group in comparison with the control group six months postoperative ($p<0.05$). No significant difference was seen in scar characteristics twelve months postoperative. tSVF = tissue stromal vascular fraction.

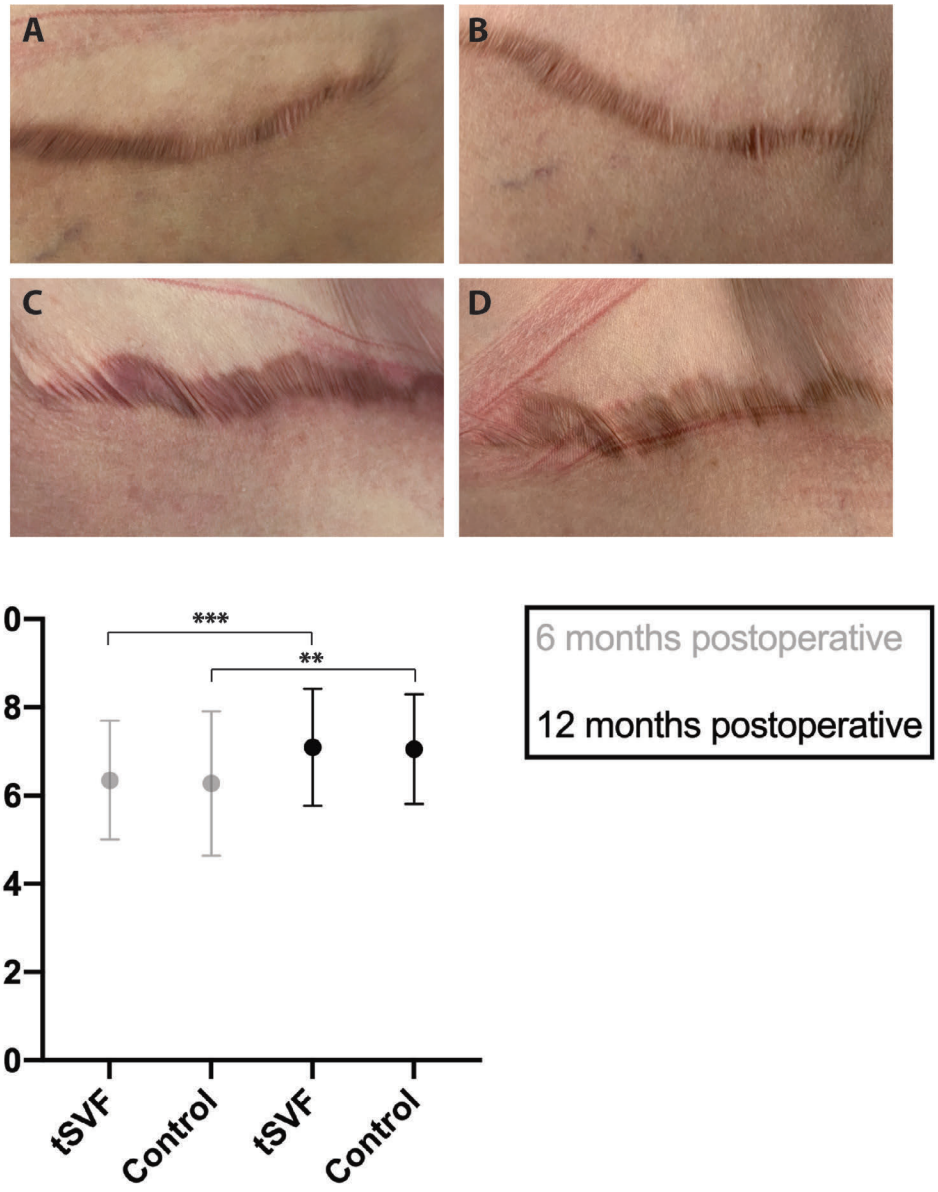

Figure 3. Photograph of a scar after treatment with tSVF **(A)** six months postoperative and **(B)** twelve months postoperative. Photograph of a scar after treatment with placebo **(C)** six months postoperative and **(D)** twelve months postoperative. **(E)** Total scores of postoperative scars after six and twelve months evaluated by blinded plastic surgeon. tSVF = tissue stromal vascular fraction. No significant difference was seen in scar appearance between both groups six and twelve months postoperative. **/*** In both groups scar appearance was significant better after twelve months as compared to six months.

Table 2. Intra- and interobserver agreements based on Fleiss weighted kappa coefficient.

Observers	Fleiss weighted kappa intra-observer
1	0.277
2	0.212
3	0.146
4	0.150
5	0.143
6	0.120
7	0.212
8	0.153
9	0.249
Observer groups	**Fleiss weighted kappa inter-observer**
1	0.028
2	0.101
3	0041

DISCUSSION

This prospective randomized multicenter clinical trial testing the effect of tSVF on wound healing demonstrated a significant effect of tSVF on wound healing; immediate postoperative injection of tSVF significantly suppresses scar formation after a reduction mammoplasty as evaluated after six months. The scars were significantly improved in color and a decrease of thickness as well as irregularity was seen. This clearly indicates an accelerated early wound healing resulting in a faster transition from wound to final scar. After twelve months, the saline treated scars show a comparable aesthetic appearance with the tSVF treated scars indicating a slower wound healing. Apparently, during the final phase of wound healing *i.e.* remodeling phase, the initial advantage of tSVF is overtaken. One can speculate, that the disappearing effect of tSVF indicate a faster transition from the inflammation to remodeling phase during wound healing without creating structural differences in finalized scars. A faster transition might be caused by a larger influx of immune cells such as macrophages and subsequently increased shift from M1 to M2 phenotype under the influence of ASCs (under revision ASJ). M2 macrophages are largely responsible for initiating tissue repair following immune suppression at the end of the inflammation phase of wound healing.[22] Once the remodeling phase has started after several weeks, the injected tSVF cells might not be therapeutic anymore to improve scar appearance.

While we report on tSVF *i.e.* a fraction of fat, others reported the immediate effect of fat grafting after reduction mammoplasty or cleft-lip repair.[23-25] Kemaloğlu et al. treated thirty patients with either fat grafting or nanofat-enriched fat grafting immediate after a reduction mammoplasty while fifteen patients did not receive additional treatment.[25] This study also found a significant scar appearance improvement as evaluated by a VAS and Vancouver Scar Scale (VSS) after treatment with fat grafting or nanofat-enriched fat grafting as compared to the control group at six months. Their VAS and VSS subscores were comparable for both the fat grafting and the nanofat-enriched fat grafting groups, except for the amount of pigmentation. The level of pigmentation was reduced in the nanofat-enriched fat grafting group. These results are thus fully in line with the six months results in this study. This shows that adipocytes are not necessary to initiate a faster wound healing because tSVF deprived of adipocytes shows comparable results with nanofat-enriched fat grafting. The study of Kemaloğlu et al. did not evaluate their results after 1 year.[25] It is well-known that scar maturation takes at least one year and therefore, no definite conclusions regarding final scar appearance after immediate fat grafting can be drawn within one year period.

In contrast to scar prevention therapy, scar remodeling therapy focuses on reducing the amount of already deposit collagen instead of preventing collagen deposition. In scar remodeling, ASCs injections in mature fibrotic areas might stimulate matrix metalloproteinase release causing matrix remodeling as seen in *in vitro* studies.[26,27] Matrix remodeling contains replacement of thick and aligned collagen bundles by smaller, randomly aligned collagen bundles. These changes might occur under influence of ASCs in highly vascularized tissue with influx of immune cells *e.g.* T-lymphocytes, mast cells and M2 macrophages (under revision ASJ). Several studies investigating fat grafting or any therapeutic component of adipose tissue *e.g.* ASCs or SVF for modulation of matured scars show promising results: improvement of the general appearance of scars occurs by normalizing the color, reducing irregularities and fibrotic area.[28-30]

Multiple clinical studies regarding fat grafting and dermal wound healing have shown encouraging results with increased wound healing rates.[31] Though, controversy remains because some clinical trials report a high rate of only partially closed wounds.[32-34] Two of these studies used enzymatically isolated SVF comprising of a heterogeneous mixture of a single cell suspension devoted from cell-cell connections including extracellular matrix (cSVF).[32,33] After application of cSVF, the vast majority of cells will probably diffuse away from the site of injections within hours. Hence, the regenerative effect of cSVF at the wound bed might be limited. In this study, tSVF containing intact cell-cell interactions as well as extracellular matrix is injected in a freshly created wound bed. These cell-cell interactions prevent cells *e.g.* ASCs to emigrate directly after injection.

Moreover, the extracellular matrix is able to bind and release growth factors and cytokines as well as to bind and initiate proliferation or differentiation of cells. Hence, the extracellular matrix is able to control and guide the regenerative potential of SVF cells for a longer period of time. The regenerative effect *i.e.* increased wound closure time might last longer after application of tSVF instead of cSVF due to the presence of the extracellular matrix components.

No conclusions regarding the effect of tSVF on the first weeks of wound healing can be drawn from this study due to the intended study design. Yet, this was also not the goal of this clinical study; the goal of this study was scar prevention by simulating the early phase of wound healing. Hence, data of early wound healing is missing: for that aspect, another new designed prospective randomized controlled trial will be initiated. Another limitation of this study is the relatively high loss to follow-up rate, although loss of subjects was compensated. The high loss might be caused by the study design in which healthy subjects had to undergo a punch biopsy of the reduction mammoplasty scar. Although unknown, one might speculate that especially those subjects that could not detect any difference in scar appearance between both scars could have more easily left during follow-up. These subjects might be replaced by subjects with a more visible effect of tSVF on scar appearance which positively interferes with the results.

In conclusion, an immediate injection of tSVF significantly improves scar appearance after six months, an effect that is no longer apparent after 1-year follow-up. These results indicate that tSVF definitely accelerates the early phase of wound healing. Future new designed prospective studies are needed to study the exact effect of tSVF on earlier stages of wound healing.

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09

The addition of platelet-rich plasma to facial lipofilling: a double-blinded, placebo-controlled, randomized trial

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ABSTRACT

Background

Lipofilling is a treatment modality to restore tissue volume, but may also rejuvenate the aging skin. Platelet-rich plasma has been reported to augment the efficacy of lipofilling, both on graft take and rejuvenation by altering the ADSC. Authors hypothesized that PRP addition would increase the rejuvenating effect while shortening recovery time.

Methods

The study conducted was a single-centre, double blinded, placebo-controlled randomized trial (2012-2015). In total, a well-defined cohort of 32 healthy females enrolled in the study, with 25 completing the follow-up. All patients underwent aesthetic facial lipofilling with either saline or PRP added. Outcome was determined by changes in skin elasticity, volumetric changes of the nasolabial fold, recovery time and patient satisfaction during follow-up (1 year).

Results

PRP did not improve the outcome of facial lipofilling when looking at skin elasticity improvement, graft volume maintenance in the nasolabial fold or patient satisfaction. Patient recovery after surgery however, dropped significantly. Furthermore, no skin rejuvenation effects from lipofilling could be observed.

Conclusion

This study clearly demonstrates that the addition of PRP to the lipograft significantly reduces patient's reported recovery time, but does not significantly improve skin elasticity, volume retention nor overall patient satisfaction as compared to lipofilling alone. Moreover, reported effects of 'normal' (not SVF/ADSC enriched) lipofilling on skin rejuvenation, as has been reported and suggested to be seen in clinical studies when used in combination with facelift surgery, could also not be addressed.

INTRODUCTION

Lipofilling, i.e. autologous fat transplantation or fat grafting, has become an important treatment modality in facial rejuvenation procedures: it is a safe procedure that requires only limited additional operating time. The presence of ASCs (Adipose stem Cells) in the lipograft[1] could result in tissue regeneration[2-4]. This has resulted in a paradigm shift towards the combination of facial rejuvenation by using both surgical lifting techniques as well as lipofilling procedures to restore both volume[5, 6] and tissue damage on a cellular level. By this combination of both surgical lifting and lipofilling, effects of gravity, loss of skin elasticity due to elastin degradation, loss of volume due to fat atrophy and bone resorption[7, 8] are all well addressed.

Fat grafting not only restores volume; it is also attributes to regeneration processes that become apparent by improved surface structure and tissue elasticity [9]. Nature's own regenerative source of wound healing is clot formation after platelet aggregation, homing of the cells involved in repair and fibronogenesis. Another reliable manner to produce injectable clots is the generation of platelet-rich plasma (PRP) and to use that to augment wound healing. PRP both serves as an instant scaffold for regeneration as well as a rich source of pro-regenerative growth factors [10].

With extensive experience of the use of PRP as an additive to facial lipofilling procedures in our clinic dating back to 2005, retrospective analysis revealed several significant beneficial effects when adding PRP to the lipograft [11]. We hypothesized that the addition of PRP to lipografts would augment tissue regeneration. This hypothesis was subsequently tested in this double-blind randomized placebo-controlled clinical trial for facial lipofilling.

METHODS

Study overview

The study conducted was a single-centre, patient and investigator blinded, placebo-controlled trial undertaken at Bergman Clinics The Hague, the Netherlands. A flow-chart overview of the study is shown in Figure 1. Patients' follow-up was 12 months in order to obtain long-term lasting results.

The study protocol complied with the Declaration of Helsinki and was approved by local medical ethics committee Zuid-west Holland (National legislator trial code: NL35142.098.11, local METC code: 12-014). All patients provided written informed consent.

Patient population and randomization

Prior to inclusion, a power calculation was performed based on the limited available published data at that time. Following this calculation, aim was to include 32 subjects that would receive facial lipofilling in this study, with one half of the population receiving PRP, the other half a placebo (sterile saline), serving as control group. A detailed description of the randomization process in available online. Inclusion-exclusion criteria were strict, and are listed in Figure 1.

The primary outcome of the study was skin elasticity improvement (R7 parameter measured by the MPA580 device) on predetermined fixed measurement locations (Figure 2) overlaying the area of intervention. Secondary outcome parameters of the study were: other changes in skin characteristics (R5-R6 parameters, MPA850, same locations), graft take (nasolabial fold decrease) and patient questionnaires regarding recovery time and satisfaction.

Patient enrollment in the study started in 2012 and ended mid 2015. During enrollment in this study, patients were prohibited to undergo further subsequent facial rejuvenating procedures. If a patient still did, the patient was excluded from the study.

Inclusion criteria:
- Female, age 35-65 years
- Stable normal BMI (20-25, 1-year stable)

Exclusion criteria:
- Smoking
- Pregnancy or active child wish
- Prior operations in the mid-face
- Active or previous use of hormone replacement therapy.
- A known systemic disease that will impair wound healing (e.g. diabetes mellitus, known atherosclerosis with an event that required hospitalization, collagen diseases, diseases of the skin)
- A known psychiatric condition
- History of cancer

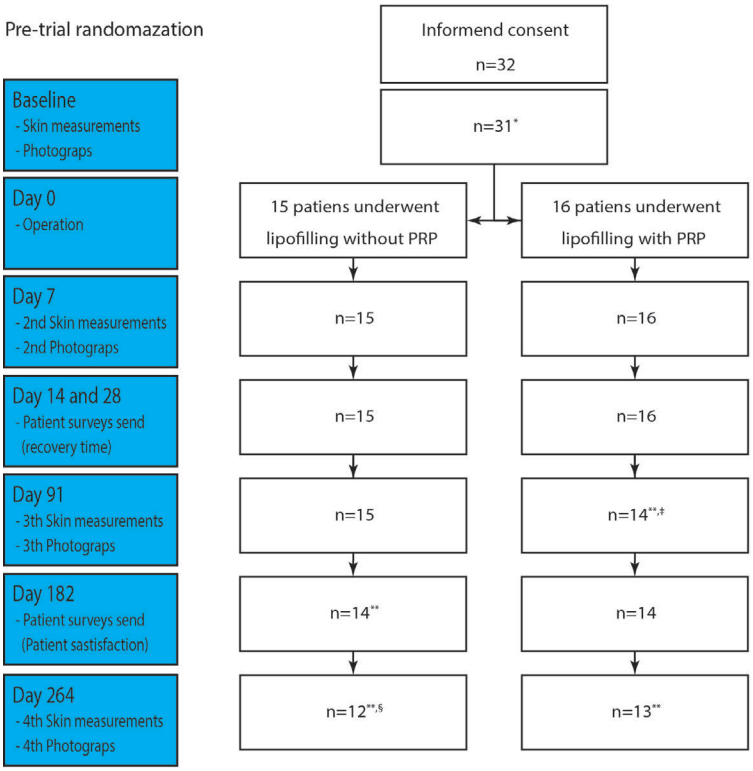

Figure 1. Study design with a breakdown of enrolled subjects that completed the study, and inclusion/exclusion criteria. Excluded: *patient was diagnosed with a gastro-intestinal oncological disease; **patients failed to complete all follow-up moments by not showing up; ‡patient chose to leave the study due to personal circumstances; §patient underwent aesthetic facial surgery during the follow-up.

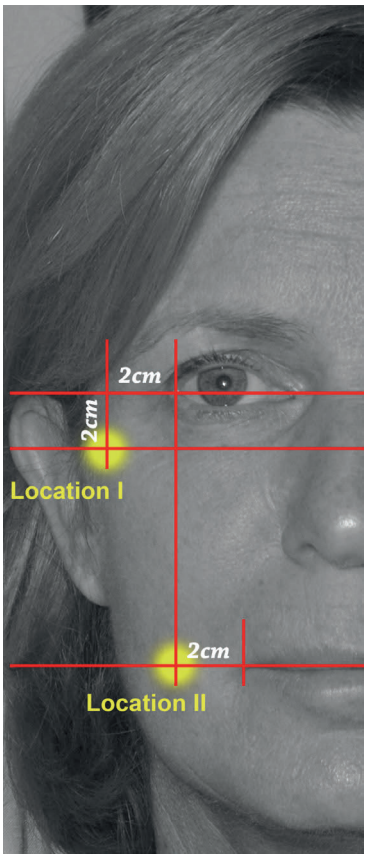

Figure 2. Locations of skin measurements, shown locations were marked before each measurement. Patients lay down on an examination table to enable correct Cutometer probe placement. Location I: 2cm lateral and 2 cm caudal from the lateral canthus. Locations II: 2 cm lateral from the lateral commisure.

Procedures

At the operating theater, with the patient mildly sedated, 30cc of whole blood was drawn from the patient, with an additional 2cc for platelet analysis. Following the pre-trial randomization, opening of the envelope determined whether the whole blood was either discarded, or introduced into the Biomet GPSIII device for PRP isolation (3cc PRP output) following the manufactures protocol. 3cc of sterile saline was used as placebo control.

Lipoharvesting, processing and lipofilling were performed following the standard Coleman method: however, both the lipoharvesting - and lipofilling cannulas were

significant smaller (harvester: 2.4mm x 22cm, injector: 0.9mm x 5cm). The upper legs served as donor site in all patients. Location and applied lipofilling volume is presented in Figure 3. All procedures have been performed by the same surgeon (HPS), who at that time had already experience with more then 2000 lipofilling procedures. A detailed, step-by-step description of the lipofilling procedure is available online as video supplement[5].

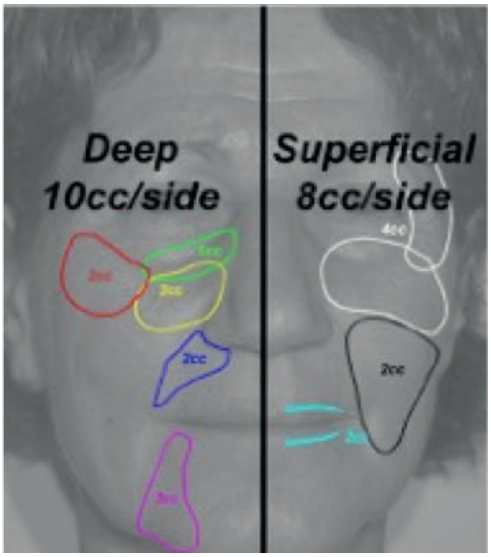

Figure 3. Lipofilling locations and applied volume. Both superficial and deep lipofilling was performed on both sides of the face. In total 18cc per side, 36cc in total. Within the PRP group, 3cc of PRP was added into the lipofilling planes.

Legend: ***Deep:*** *Temporal projection (red), Nasojugal groove (green), Central midface (yellow), Nasolabial-fold (blue), Marionette line, Pre-jowling area and chin (pink).* ***Superficial:*** *Temporal and central midface area (white), Lower midface-cheek area (black), White rolls (cyan).*

Skin measurements

Local skin quality was measured with the Multi Probe Adapter system (C&K Colone Germany) containing the Cutometer MPA580 (elasticity) probe. The cutometer is a valid method in objectifying elasticity of the skin [12-16]. Measurements were done on fixed locations for every patient (Fig. 2) at every follow-up moment. Before each measurement, the probes were calibrated and tested for correct function. Also local temperature and humidity were logged. True skin elasticity was defined by the

Cutometer MPA850 R7 output parameter (the ratio of elastic recovery to the total deformation, elaborated by the R5 (the net elasticity) and R6 (the ratio of viscoelastic to elastic extension) parameters.

Volumetric changes of the nasolabial fold

Standardized photographs were captured in three views with a professional 3D camera system (AP, 3Q left and right) at every follow-up moment. Primarily, 3D reconstructions were used to determine volumetric facial changes over time, but were abandoned due to data inconsistency, variation and reproducibility of the measured area. Instead, the pre-operative, 3 months and 1 year post-operative AP-views were used to determine changes in the nasolabial fold depth using a validated grading method (Merz Scale) [17-20], that consists of five options (I=minimal fold expression to V=most prominent fold expression) In total, four independent plastic surgeons served as expert panel. The nasolabial fold was chosen because alteration in depth would implicate relevant external changes of facial appearance.

Patient reported recovery time and satisfaction

Recovery after the procedure was assessed by means of two patient questionnaires send at 2 and 4 weeks after the operation. Questions included the number of days required to return to work and or resume social activities without using camouflaging agents, and notable changes in facial volume and skin expressed on a visual analogue scale (VAS 1: no changes -10: most significant changes).

Patient reported satisfaction was recorded by means of a questionnaire send 6 months after surgery: questions included overall satisfaction, changes in volume effect, skin changes and whether or not they would recommend the procedure to a peer (VAS 1-10).

Statistical analysis

Statistical analyses were performed by an independent statistician that received all blinded data by the principal investigator, along with the original randomization from the surgeon.

All analyses were done using SPSS 20 (IBM, Chicago, IL, USA). Data Fig.s were generated using Prism 6 (GraphPad Software, La Jolla, CA, USA). The paired samples t-test, ANOVA analysis of covariance and standard linear regression were used. All data fulfilled the requirements for normality and equal variances. A two-sided $p<0{\cdot}05$ was considered statistically significant.

RESULTS

In total 32 patients, that met inclusion criteria, were enrolled in this study, with finally 25 patients completing the study. Seven patients were excluded from the study: four patients failed to complete all follow-up moments by not showing up, one patient was diagnosed with a gastro-intestinal oncological disease (ruled as undiagnosed pre-existent, and unconnected with the study ruled by the independent physician), one patient underwent aesthetic facial surgery during the follow-up, and one patient chose to leave the study due to personal circumstances. Excluded patients, unfortunately, could not be replaced due to limited study duration as allowed by the Ethical board.

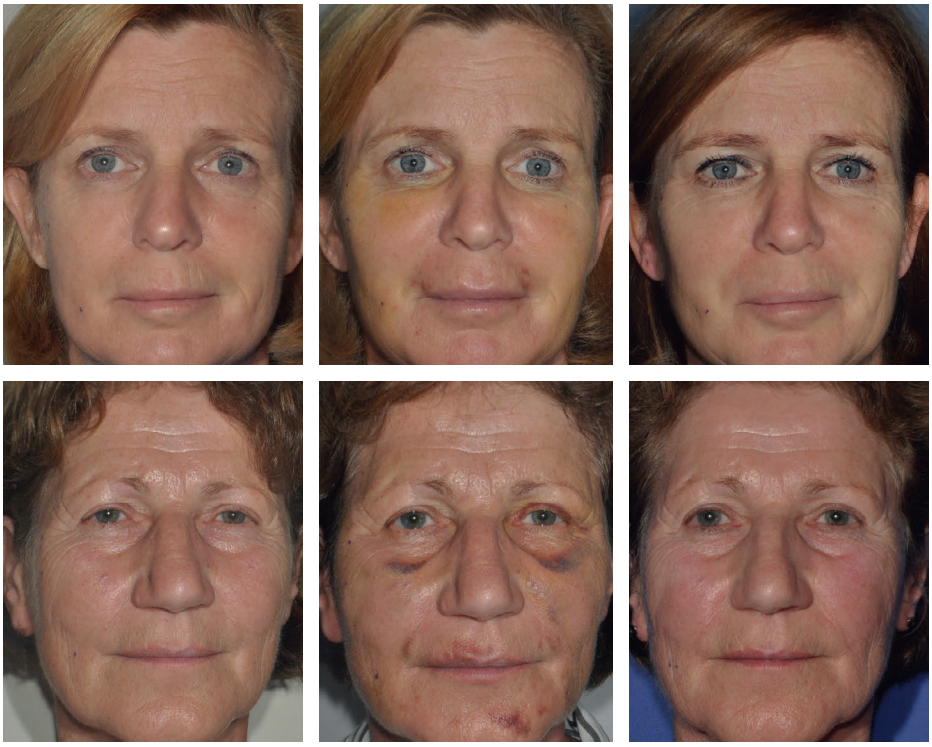

Figure 4. Average results: pre-operative (left column), 1 week (center column) and 1 year (right column AP photographs. Upper row: PRP +, second row: PRP -.

Of all patients that completed the study, 13 received lipofilling with PRP (PRP+) and 12 lipofilling with saline (placebo, PRP-). Average photographic results are presented in Fig 4. Mean patient age at time of operation was 52 years (±6.75, [38-63]), with

no significant age difference between both groups. Whole blood platelet counts were within normal range for all patients (Table 1). Of all patients that completed the study, 13 received lipofilling with PRP (PRP+) and 12 lipofilling with saline (placebo, PRP-). Average photographic results are presented in Fig 3. Mean patient age at time of operation was 52 years (±6.75, [38-63]), with no significant age difference between both groups. Whole blood platelet counts were within normal range for all patients (Table 1).

Table 1. Study population characteristics.

	Group I			Group II				Overall		
	No PRP(n=12)			**PRP(n=13)**				**(n=25)**		
	Mean	(SD)	[Range]	Mean	(SD)	[Range]	*p**	Mean	(SD)	[Range]
- Age at time of operation	52.5	(7.1)	[42-63]	51.73	(6.7))	[38-62]	ns	52.10	(6.8)	[38-63]
- Platelet count at time of operation	234.2	(47.9)	[153-299]	250.1	(37.5)	[168-312]	ns	242.8	(42.9)	[153-312]
	n			n			*p**	n		
- Recorded complications (major or minor)	0			0			-	0		

** students' t-test*

Lipofilling with or without PRP does not significantly change overlying skin elasticity in this study

Analyzed R7 parameter data (representing true elasticity) from both groups, showed no significant difference before intervention (Fig. 5). The PRP+ group did not differ significantly from the placebo group at any moment. Data correction for age, room temperature, humidity conditions and baseline (pre-operative) measurements resulted in similar findings. Analyzed R5 and R6 data showed comparable values in both groups, at every follow-up moment.

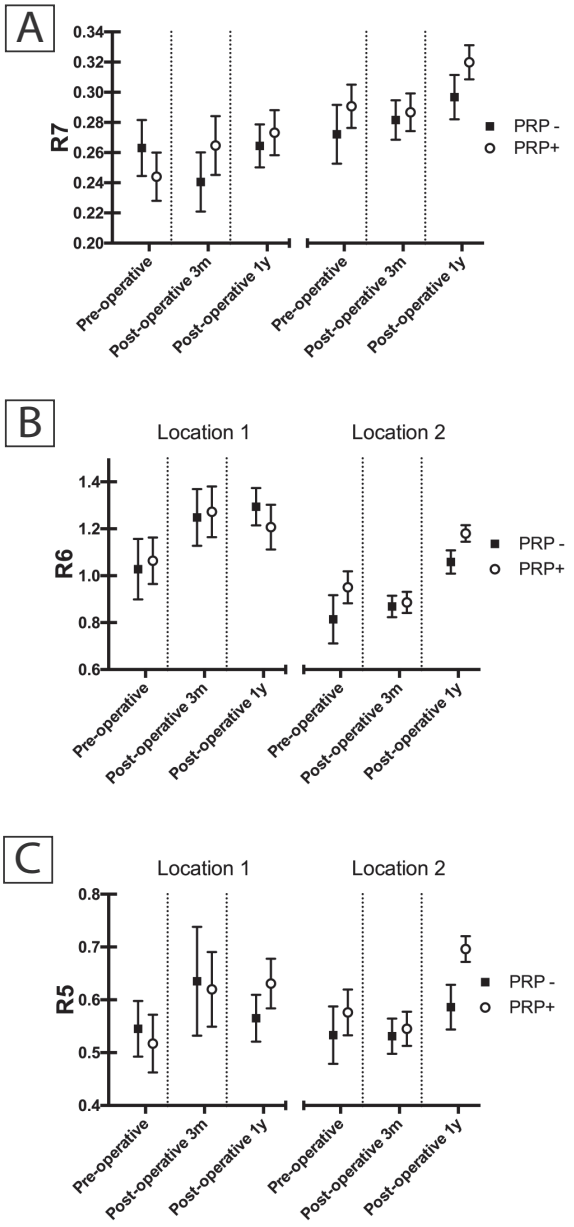

Figure 5. Changes in average true skin elasticity (R7) and R5-6 parameter, for both groups preoperative and during follow-up measured with Cutometer MPA850 at both locations. Data represents group means with SEM. **A:** R7 parameter location 1 and 2: true skin elasticity, higher values represent an increase in skin elasticity and a positive effect. There is a marginal increase in both groups, with no significance. **B:** R6 parameter location 1 and 2: the ratio of viscoelastic to elastic extension. Lower values represent a positive effect. **C:** R5 parameter location 1 and 2: the net elasticity: Higher values represent a positive effect. Again, a minimal gain in both groups, with no significant differences, between both groups or within each group at every follow-up moment.

Regression analysis of pre-operative R7 parameter as a function of age showed a negative correlation in both groups, comparable to Enzure et al.[21]. However, after intervention, the correlation reverses (Fig. 6), which could be a sign of facial rejuvenation. Changes were most noticeable in the PRP+ group: the high prediction value of the regression line (R=0.542, p=0.055) could suggest that sample size in this study was not adequate. Interestingly this reversal was only notable on Location 1 R7, not on location 2 nor with the R5-R6 parameters.

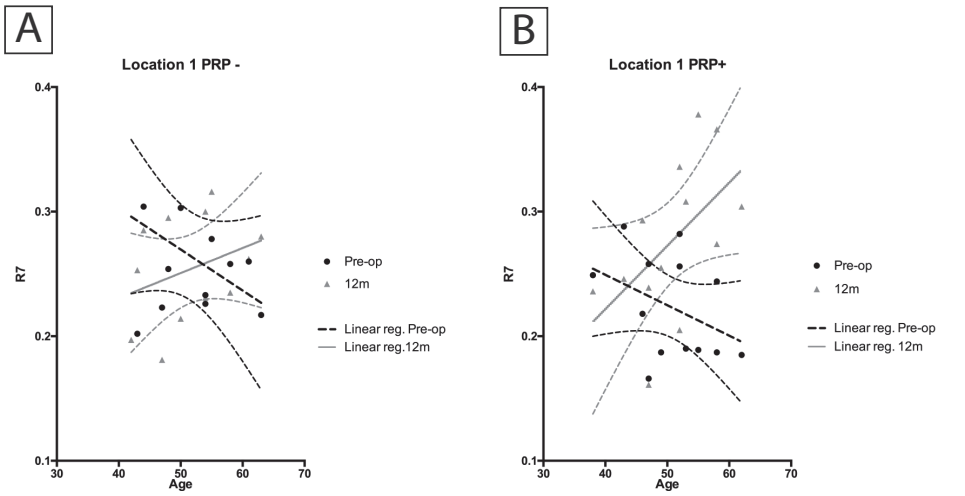

Figure 6. Regression analysis of true skin elasticity as a function of age, before and 12 months after intervention. All measurements and the calculated regression curve with 95% C.I. are presented **A:** PRP-. Pre-operatively age correlates negatively with true elasticity (y= -0.003293*x+0.4343 R=0.402 p=0.195), but this correlation reverses 12 months post-operatively (y= 0.002005*x+0.1507" R=0.326 p=0.299). **B**: PRP +. Again, a negative correlation before operation (y= -0.002444*x+0.3471" R=0.392 p=0.184), with a stronger reversal after intervention (y= 0.005078*x+0.01921" R=0.542 p=0.055) compared to PRP -.

Volumetric changes of the nasolabial fold

Summarized data from both groups, at every follow-up moment are presented in Figure 7, lower scores represent a less prominent nasolabial fold. Grading scores showed a high level of agreement between each expert (all Spearman ICC r >0.576, p<0.001).

Pre-operative scores were comparable in both groups (PRP-: μ:2.359 ±0.1531, PRP+: μ:2.622 ±0.2388, p>0.05). Data after 3 months and 1 year also showed comparable

results, with no significant differences between both groups at any moment. Furthermore, no changes between pre- and postoperative scores within each group were found.

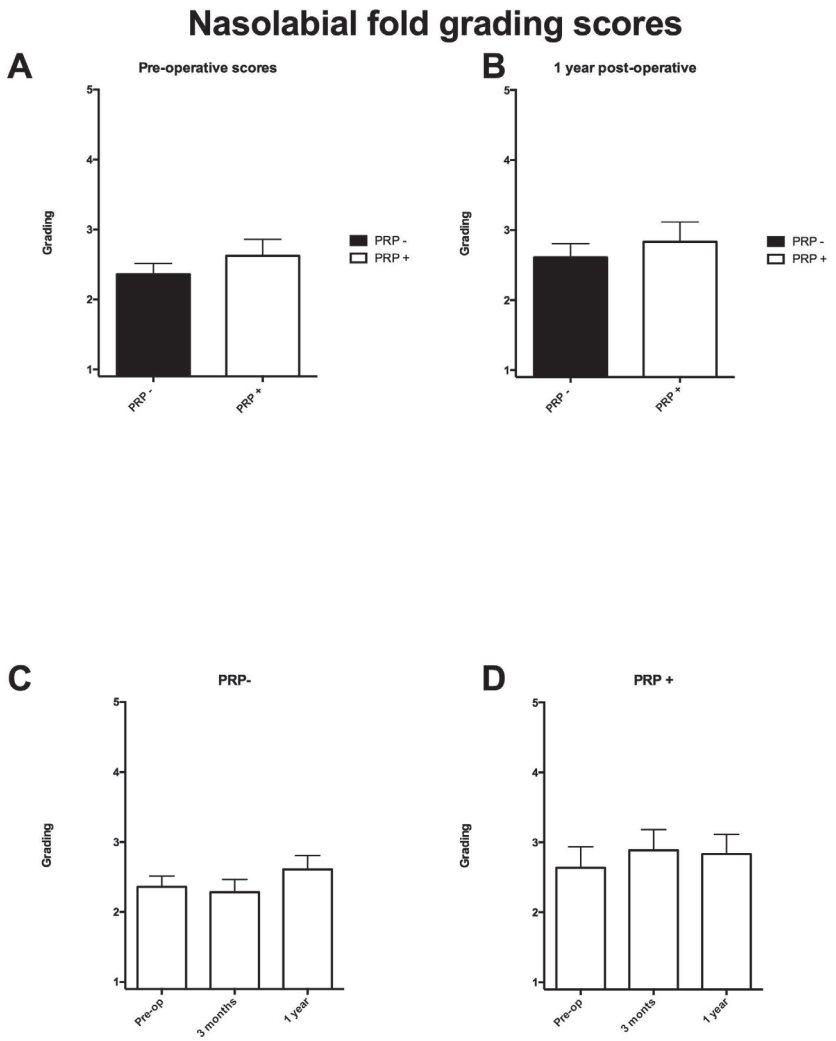

Figure 7. Result of nasolabial fold grading preoperative and during follow-up. Data represents group means with SEM from grading's by four experts. Lower scores represent a less prominent nasolabial fold. No significant differences, between both groups or within each group at every follow-up moment could be calculated. **A:** Pre-operative scores for both groups **B:** Post-operative scores for both groups **C-D:** Changes in grading's during follow-up for both groups. No effect was observed during follow-up.

Addition of PRP speeds up recovery, but does not increase patient satisfaction

Patient questionnaire reported recovery time, derived from the number of days returning to work/social activities with or without camouflaging agents, showed a significant faster recovery in the PRP+ group (Table 2). Mean number of days returning to work/Social activities with camouflaging agents was 9 days (μ=9,133 σ=3,701, p<0.01) in the PRP+ group and 15 days in the control group (μ=15,43 σ=4,949). Return to work/social actives without camouflaging agents supported this finding (PRP +: μ=14,87 σ=4,604 vs PRP -: μ=20.57 σ=6.61 p<0.05). Questions regarding noticeable differences in facial volume and skin quality after 2 and 4 weeks showed no differences (p > 0.05).

Patient satisfaction, changes in volume and skin quality, reported after 6 months proved to be similar in both groups (data not presented). Overall satisfaction was reported as 'moderate'. Positive skin changes were reported by several patients in both groups, contradicted by patients that did not notice any skin changes at all. Overall, the level of recommendation of the procedure to peers was negative for both groups, mainly as told by them because of higher expectations of the effect of the procedure.

Table 2. Recovery time.*

	Group I		Group II		
Facial Lipofilling	**No PRP(n=12)**		**PRP(n=13)**		
	Mean	(SD)	Mean	(SD)	*p***
- Return to work/social activities with camouflaging agents	*15.4*	*(9.1)*	*9.1*	*(3,7)*	*0.0101*
- Return to work/social activities without camouflaging agents	*20.6*	*(6.6)*	*14.9*	*(4.6)*	*0.0112*

** Recovery time was defined as the patient reported number of days after surgery returning to work/social activities*

*** Independent samples t-test*

DISCUSSION

This randomized placebo controlled double blinded study was undertaken to investigate the possible beneficiary effects of adding PRP to aesthetic facial lipofilling in a well-defined healthy patient cohort. The results clearly demonstrate that the addition of PRP to the lipograft significantly reduces patient's reported recovery time. However, the addition of PRP to the lipograft does not significantly improve skin elasticity, changing in nasolalial fold depth nor overall patient satisfaction as compared to lipofilling alone. The reversal in the correlation of net elasticity as a function of patient age could suggests some form of rejuvenation by lipofilling that is enhanced by PRP, but lacked significance with the number of patients in this study.

Reported in vitro effects of PRP [10, 22-24] thus could not be reproduced in our clinical study setting, possibly by incontrollable patient related confounding factors combined with a small therapeutic window for effect. Moreover, reported effects of 'normal' (not SVF/ASC enriched) lipofilling on skin rejuvenation, as has been reported and suggested to be seen in clinical studies when used in combination with facelift surgery[2, 9] could also not be addressed and forces us to question what the additional effect (next to some volume enhancement) of normal lipofilling is when used during facelift surgery.

Lipofilling does not increase skin elasticity in the aging face, even with added PRP.

Since the comeback of lipofilling, suggestions were made that it is 'more than a filler' [2] and may induce rejuvenation of the skin. However, this ASC induced effect, is only well studied after deep dermal injury (e.g. thermal- radiation damage, excessive scarring [4, 25-27]). Surprisingly skin rejuvenation of the normal aging skin has only be described, and studied histologically by Rigotti et al. [9]In this study, an increase in dermal elastin deposition was reported in biopsies after normal lipofilling of the aging skin. However, to this date, no controlled studies were done to verify the clinical relevancy of their finding. In our study, skin elasticity was determined with the Cutometer since it is a reliable and validated method of measuring skin age, and the mostly likely candidate to show changes, supported by the findings of Rigotti et al. Nevertheless, there remains minor controversy regarding the reliability of the Cutometer. A study of Nedelec et al. presented low intraclass correlation coefficients of skin elasticity measurements of dermal scars. The intraclass correlation coefficients found for normal skin elasticity measurements were, however, acceptable for the R0 (0.81), R6 (0.81) and R7 (0.78) parameter [28]. We found that normal (not SVF/ASC boosted) lipofilling with or without PRP did not alter skin elasticity. Reversal of the correlation between age and elasticity however, might suggest a small effect size, thus not significant with our small study

population. Nevertheless, the small effect size raises questions if normal lipofilling is 'just a filler' in aesthetic procedures in the aging face which involve only lipofilling. Improvement in outcome when lipofilling is combined with lifting procedures could be explained by the large wound surface created and ASC modulation during healing, downregulating fibrosis pathways. Recent publication on SVF boosted/ASC expanded lipofilling however, do show a significant clinical effect [29, 30] and seem the way forward.

In theory, adding PRP could affect overlying skin true several pathways and cell lines. Angiopoetin-1 and 2, abundantly present in platelets [31, 32]have shown to stimulate endothelial cell growth, migration and differentiation in cultured human dermal microvascular endothelial cells in vitro [22, 23]. Also, PRP-lysate is a strong proliferator for ASC [10, 33], essential for graft take [34] and a proven down regulator of fibrosis [26, 35].

Effects of lipofilling with or without PRP on nasolabial fold depth.

Grading of the nasolabial fold during follow-up showed no noticeable lasting effect of lipofilling nor lipofilling with PRP on the depth of the nasolabial fold. Even though the "Merz Scale' used in this study, has shown to successfully differentiate in small volume changes (e.g. filler injection) [36]. We could not determine these differences probably because the lipofilling increased overall facial volume, not altering relative differences between facial zones. In our opinion, only in combination with a facelift, lipofilling may additional demonstrate its effect on the nasolabial fold: lifting probably is definitely needed as such. Furthermore, changes in facial volume are minimal because of the limited amount of lipografts that is injected, with uncertainty about the clinical impact of these minor changes if not combined with a lifting procedure. To this date, only one study has been published that reported facial graft retention determined with external 3D photographic reconstruction [37] after aesthetic facial lipofilling. In this study, an overall retention of 32% was reported, however the range and variation of reported data questions its scientific merit. Moreover, the vast number of patients in this study also received some form of lifting procedure that most likely changed distribution of facial volume, and by this means influenced facial volume attributed to lipofilling. Again suggesting that lipofilling should be combined with a lifting procedure in aesthetic facial rejuvenation. Even though lipograft survival in the face has been documented with MRI imaging [38], the clinical relevancy of aesthetic facial lipofilling procedures without lifting procedures on facial fold depth remains to be determined.

With ongoing uncertainty about lipograft survival, several fundamental studies explored PRP addition [39-41] and found positive effects. Graft take might improve by PRP effects on ASC proliferation [39], blockage of apoptosis pathways [42] and differentiation into adipocytes [43]. Moreover, PRP lysate stimulates proliferation, migration and tube

formation of human umbilical vein endothelial cells both in vitro as well as in a nude mouse model [39]. PRP induces changes on endothelial cells that can contribute to (neo) angiogenesis of the fat graft and thereby enhance fat graft survival [44]. These findings however, fail to make a significant impact in the majority of available clinical PRP-lipofilling studies [45, 46], thus questioning clinical use of PRP addition to lipofilling for this reason.

PRP speeds up patient recovery

Patient reported recovery time was significantly reduced by the addition of PRP in this study. This finding is in line with previous data from our retrospective study [11] and current literature on aesthetic procedures like fractional carbon dioxide laser resurfacing treatment [47, 48].

Dermal- and wound closure effects observed after PRP injection might be explained by the effect from PRP on fibroblasts. In vitro study of Ramos-Torrecillas et al. [49]eases the growth of fibroblasts and induces their differentiation into myofibroblasts, thus playing a key part in wound contracture [22]. Collagen 1 and extracellular matrix remodeling by fibroblast is also affected by PRP. Fibroblast exposed to PRP lysate in vitro up regulates the expression of Matrix metalloproteinase (MMP)-1 [24], which in its turn plays a key role in collagen remodeling. Also, type 1 collagen expression is increased under these circumstances [50]. Increased fibroblast activity, along with changes in collagen production and a potentially stronger inflammation response [51]could also play a role in our observed reduced recovery time after surgery when the lipograft was combined with PRP.

The concentration paradox: Less is more?

A potential pitfall in evaluating the effect of PRP is the lack of uniform concentrations of created PRP. The studies of Yamaguchi et al. [52, 53] were the first publications that showed that a higher concentration of PRP (or more platelets) may produce counterproductive effects, possibly by unwanted cell differentiation. Most commercially available PRP kits capture a percentage of available platelets from whole blood, not a certain quantitative number of platelets. Considering the fact that normal human platelet counts are defined within a wide range and show large daily variations, the cumulative amount of growth factors in kit-isolated PRP is inconsistent [54]. This variation can inadvertently influence its effect in a way as is observed in vitro on different cell types [22]. Regarding cells present in the lipograft, PRP concentration alters ASC proliferation, function

and behavior. High PRP concentrations increase proliferation, but also changes ASC into a fibroblast like phenotype, with increased collagen RNA expression and altered paracrine signaling that negatively influences endothelial vessel formation [55, 56].

Although platelet counts were normal within our well-defined healthy patient cohort, combined with comparable fat-graft-PRP-or-placebo mixture ratios, our study is potentially biased and weakened by this concentration-depended effect. Moreover, this phenomenon could explain the failure of clinical studies.

Local growth factor conditions after lipofilling are also an issue that remains unclear; in a healthy patient, the release of platelets and pro-inflammatory factors due to damage caused by the lipofilling procedure itself could be of such an extent that the addition of PRP actually is insignificant and/or redundant or even too high.

CONCLUSION

This randomized double-blinded, placebo-controlled study clearly has shown that PRP significantly reduces post-operative recovery time but does not improve patient outcome when looking at skin elasticity, improvement of the nasolabial fold nor patient satisfaction. The reversal of the correlation between age and elasticity might indicate for some effect on skin, but requires more power of future studies.

Thus far, the use of PRP as an additive in lipofilling has shown great promises in vitro. These beneficiary effects, however, have only partially been reproduced in a clinical setting. A growing number of studies report a concentration depended effect of PRP in vitro, making optimal use in a clinical setting delicate and complex. Further studies of PRP interactions on both the lipograft as well as the receptor host site involved cells seems to be of paramount importance to determine the optimal use and concentrations of PRP in a clinical setting.

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The addition of tissue stromal vascular fraction to platelet-rich plasma supplemented lipofilling does not improve facial skin quality: a prospective randomized clinical trial

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ABSTRACT

Background

Lipofilling has become popular as a treatment to improve aging related skin characteristics *e.g.* wrinkles, pigmentation spots, pores or rosacea. Different additives such as platelet-rich plasma (PRP) or stromal vascular fraction (SVF) have been added to lipofilling to increase the therapeutic effect of adipose derived stromal cells (ASCs).

Objectives

In this study, we hypothesized that mechanical isolated SVF augments the therapeutic effect of PRP supplemented lipofilling to improve facial skin quality.

Methods

This prospective, double-blinded, placebo-controlled, randomized trial was conducted between 2016 and 2019. In total, 28 female subjects were enrolled with 25 completing the follow-up. All patients received PRP supplemented lipofilling with either mechanical isolated SVF or saline. SVF was isolated by means of the fractionation of adipose tissue (FAT) procedure (tissue-SVF). Results were evaluated by changes in skin elasticity and transepidermal water loss, changes in skin aging related features *i.e.* superficial spots, wrinkles, skin texture, pores, vascularity and pigmentation as well as patient satisfaction (FACE-Q), recovery and number of complications up to 1 year postoperative.

Results

The addition of tSVF to PRP supplemented lipofilling did not improve skin elasticity, transepidermal water loss nor skin aging related features. No improvement in patient satisfaction with overall facial appearance nor facial skin quality was seen when tSVF was added to PRP supplemented lipofilling.

Conclusions

PRP supplemented lipofilling with tSVF compared to PRP supplemented lipofilling alone does not improve facial skin quality nor patient satisfaction in a healthy population. PRP supplemented lipofilling with tSVF can be considered a safe procedure.

BACKGROUND

Lipofilling has rapidly become a popular treatment modality for facial rejuvenation to restore loss of volume and to decrease aging related skin characteristics *e.g.* wrinkles, pigmentation spots, pores or rosacea.[1] In literature, these effects are mainly ascribed to the presence of adipose tissue-derived stromal cells (ASCs) which reside in the stromal vascular fraction (SVF) of adipose tissue. The precursors of (cultured) ASCs are attached around vessels as peri-adventitial cells and pericytes.[2,3] ASCs secrete a plethora of growth factors, cytokines and proteins which could enhance tissue regeneration based on angiogenesis and matrix remodeling.[4,5] In this way, autologous lipofilling might reverse loss of facial skin elasticity.

To enhance the regenerative effects of lipofilling, different additives have been advocated to use such as platelet-rich plasma (PRP) or SVF to increase the number of ASCs. SVF can be isolated by means of enzymatic or mechanical isolation. Enzymatic dissociation yields a single cell suspension of SVF (cSVF) without cell-cell communications and extracellular matrix, while mechanical dissociation results mainly in a SVF with intact cell-cell interactions including extracellular matrix (tissue-SVF (tSVF)).[6,7] The use of tSVF might be advantageous over cSVF because intact cell-cell interactions warrant retention of ASCs after injection. Additionally, an intact native network of extracellular matrix binds and releases cells as well as trophic factors and thus preserves the regenerative function of tSVF. In contrast to mechanical isolation, enzymatic isolation is time-consuming, expensive and clinical use of enzymes is forbidden by law in an increasingly number of countries.[6]

PRP is defined as a portion of plasma of blood having a platelet concentration above baseline. Platelets serve as a source of regenerative growth factors and cytokines.[8] These regenerative factors have shown to influence ASCs in dose-dependent fashion in animal and *in vitro* studies.[9-11] A concentration of platelets above baseline results in increased cell proliferation and RNA expression of genes related to angiogenesis, matrix remodeling and wound healing.[9-11] To date, clinical studies using PRP as additive to autologous lipofilling have shown to reduce postoperative recovery time and showed preliminary evidence of increased dermal wound healing.[12-15] Plasma also contains fibrinogen, that will form fibrin fibers after activation with thrombin. Fibrin clots entrap platelet-released trophic factors and also serve as transient scaffolds for tissue repair.

We hypothesized that tSVF augments the therapeutic benefit of PRP-supplemented lipofilling in treatment of aged facial female skin.

MATERIAL & METHODS

Study overview

This study was conducted from July 2016 to November 2019 as a single-center, double-blind (patient as well as investigator), randomized, placebo-controlled clinical trial at Bergman Clinics The Hague, the Netherlands. The study protocol was carried out in compliance with the Declaration of Helsinki and was approved by the national medical ethics committee (CCMO) of The Netherlands (National legislator trial code: NL54409.000.15, Dutch trial register code: NTR5703). All subjects provided written informed consent prior to start of the study.

Patient population and randomization

A power calculation was performed to calculate the total number of subjects required for this study (n=64). Subjects were randomly divided into two groups: the experimental group received subcutaneous lipofilling with additional transcutaneous PRP and tSVF injections, while the control group received subcutaneous lipofilling with additional transcutaneous PRP as well as sterile saline injections, to serve as placebo. In- and exclusion criteria are listed in table 1. Patient enrollment started in June 2016 and ended prematurely in November 2019 because the required pace of inclusion was not met endanger completion of the study. After enrollment, subjects were prohibited to undergo any facial rejuvenation procedure as well as to start smoking. If subjects still did, subjects were excluded from the study.

Harvesting and injection of condensed lipoaspirate

Liposuction, processing and deep and superficial lipofilling was performed using Sorenson harvesting (2.4 x 22 cm) and smaller curved lipofilling cannulas (0.9 x 5 cm, Tulip, Medical Products, San Diego, CA).[16-18] In short, 100 ml of adipose tissue was harvested from the upper legs, centrifuged and 15-18 ml was injected immediately after processing against the inside of the skin to each side of the face. The part of the injection side that was used for assessment was outlined by the mandible bone, the nostril, lower eyelid, corner of the eyebrow till the earlobe. In this particular area 6 ml of condensed lipoaspirate was injected as superficial lipofilling keeping the round tip of the curved canula upwards against the inside of the skin. Remaining condensed lipoaspirate was used as deep lipofilling. All patients were treated under sedative anesthesia using Propofol and remifentanil intravenously.

Table 1. In- and exclusion criteria of subjects participating in this study.

Inclusion criteria	Exclusion criteria
Females	Facial surgical intervention 1 year prior start of the study
Age 35-60	Any oncological event in history
Stable BMI (20-15) at least 1 year prior start of the study	Known psychiatric condition
	Known systemic disease that will impair wound healing
	Smoking
	Pregnancy or active child wish Frequent exposure to known carcinogenic substances (e.g. work related)
	Active or previous use of hormone replacement therapy

BMI = body mass index

Platelet-rich plasma and tissue-stromal vascular fraction preparation

Prior to the surgery, 62 ml of whole blood was drawn from each subject in this study. 8 ml of anticoagulant citrate dextrose solution A (ACD-A) was added to 52 ml of whole blood and prepared following the Arthrex Angel system™ instructions. This resulted in 6 ml of non-activated PRP with a platelet concentration of 4 times the baseline. The other 10 ml of whole blood was collected in an ACD-A syringe and was analyzed for the number of platelets.

60 ml of the harvested 100 ml of adipose tissue was used to create three times 1 ml of tSVF using the previously described Fractionation of Adipose Tissue (FAT) procedure.[19] In short, all harvested adipose tissue was decanted and centrifuged at 3,000 rpm for 2.5 min. with a 9.5 cm radius fixed angle rotor (Medilite, Thermo Fisher Scientific, Waltham, MA) at room temperature (RT). After centrifugation, three times 10 ml of adipose tissue was mechanical dissociated with the use of a fractionator (a luer-to-luer transfer with three holes 1.4 mm inside, Tulip, Medical Products, San Diego, CA) by pushing lipoaspirate 30 times forward and backward. Then, the dissociated adipose tissue was again centrifuged at 3,000 rpm for 2.5 min. with a 9.5 cm radius fixed angle rotor (Medilite) at RT. 1 ml of tSVF was mixed with 2.5 ml of PRP and injected transcutaneously against the inside of the skin with the use of a 23G needle

(BD Microlance needle, blue) to each side of the face. As a control, 1 ml of sterile saline was mixed with 2.5 ml of PRP and used as a placebo. For all subjects in this study, 1 ml of tSVF was send to the lab for analysis. All procedures were performed by the senior author (HPS).

Immunohistochemical and immunocytochemical analysis of tissue-stromal vascular fraction

Samples of tSVF were formalin-fixed and embedded in paraffin. Then, 4 μm slides were cut, deparaffinized and stained following the previously published protocol by van Dongen et al.[19] Samples were stained for α-Smooth Muscle Actin (α -SMA, 1:200, Abcam, Cambridge, United Kingdom) to stain smooth muscle cells, von Willebrand Factor (vWF, 1:200, DAKO, Glostrup, Denmark) to stain endothelial cells and Perilipin A (1:200, Abcam) to stain adipocytes. As secondary antibodies, polyclonal rabbit anti-mouse for α-SMA, polyclonal swine anti-rabbit for vWF and polyclonal goat anti-rabbit for Perillipin A were used in 1:100 (DAKO). A third antibody was used for theα-SMA staining (polyclonal, swine anti-rabbit, 1:100, DAKO). Masson's trichrome staining was used to stain extracellular matrix deposition.

Measurement outcomes

All measurements of every patient were performed by the first author (JAD), who was blinded for treatment, at predetermined time-points: preoperative, 6 weeks postoperative, 3 months postoperative, 6 months postoperative and 12 months postoperative. Subjects did not apply any skin products on the day of surgery as well as on the day of the measurements. Only the right side of the subjects' face was measured.

Skin elasticity and transepidermal water loss measurements

Local skin elasticity was measured with the use of the non-invasive validated Cutometer MPA580 elasticity probe (Courage & Khazaka GmbH, Cologne, Germany).[20-23] Local transepidermal water loss (TEWL) was measured using the non-invasive validated Tewameter TM300 (Courage & Khazaka GmbH, Cologne, Germany).[24,25] Measurements were done at standardized evaluation zones (Fig. 1). True skin elasticity was defined as ratio of elastic recovery to total deformation (R7 parameter). Also, net elasticity (R5 parameter) and ratio of viscoelasticity to elastic extension (R6 parameter) were measured. TEWL is expressed as in grams per square meter per hour ($g/m^2/h$). Data was corrected for humidity, room temperature and age.

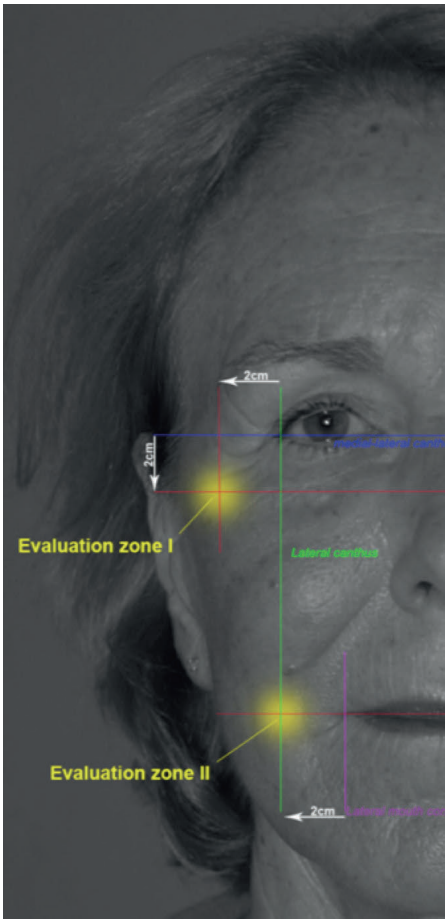

Figure 1. Locations of skin measurements used for the cutometer and tewameter. Evaluation zone 1: 2 cm lateral and 2 cm caudal from the lateral canthus. Evaluation zone 2: 2 cm lateral from the lateral commissure. Age of patient is 58 years old.

Skin quality measurements

Local skin quality was measured with the use of the non-invasive VISIA® camera (Canfield Imaging Systems, New Jersey, USA). The VISIA® camera containing the VISIA Complexion analysis software measures several skin parameters: superficial spots, wrinkles, skin texture, pores, vascularity and pigmentation. Results are given in absolute total number of occurrences of a particular parameter being evaluated and a relative score that combines size and intensity of a parameter. Prior to surgery, a predetermined evaluation mask was created with the use of fixed anatomic marks (Fig. 2).

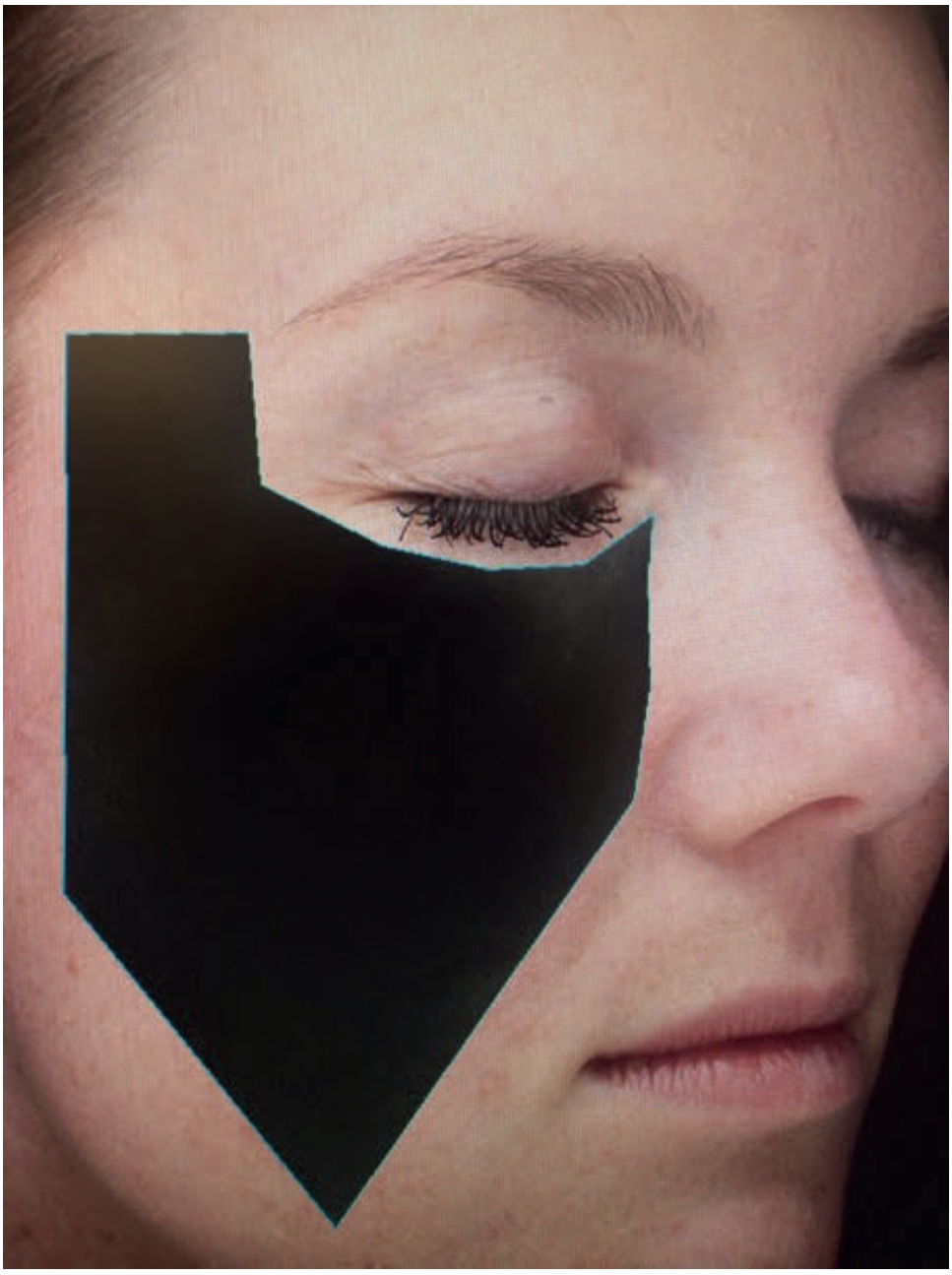

Figure 2. Mask used for the VISIA analysis. All masked were created according the same anatomical landmarks: medial canthus, around the eyelashes to the lateral canthus, lateral side of the eyebrow, 0.5 cm in front of the hairline to the height of the earlobe, perpendicular line to the mandible line ending 0.5 cm above the mandibula and nostril. Age of patient is 34 years old.

FACE-Q

The following FACE-Q were examined at the predetermined time-points except for 3 months postoperative: satisfaction with facial appearance overall, satisfaction with facial skin, appraisal of lines overall, appraisal of crow's feet lines, age appraisal with the use of visual analogue scale (VAS), aging appearance appraisal, psychological wellbeing, social function and satisfaction with outcome. Recovery of early symptoms and recovery early life impact were only examined one week postoperative. All questionnaires were validated and translated to Dutch using a linguistic validation. Raw FACE-Q scores were converted to RASCH scores according to the protocol of the FACE-Q editorial board.[26] VAS scores of age appraisal were not converted to RASH scores. FACE-Q scores of 155 Caucasian Dutch women between 18 and 80 years old who have never received any aesthetic facial procedure where asked to complete the baseline FACE-Q modules and served as control.

Statistical Analysis

Statistical analysis was performed under supervision of an independent statistician (KMV) that received blinded data from the first author (JAD) and the randomization from the senior author (HPS). All analysis was done with the use of SPSS 20 (IBM, Chicago, IL, USA). Prism 6 was used to design the graphs (GraphPad software, La Jolla, CA, USA). Visual inspection revealed normally distributed data for the cutometer and VISIA analysis, thus a paired samples *t*-test and regular *t*-test were used to analyze the outcomes of the cutometer and VISIA analysis. A Wilcoxon Signed Ranks test and Mann-Whitney test were used for the FACE-Q data. A two-sided $p<0.05$ was considered statistically significant.

RESULTS

Demographics and platelet counts

A total of twenty-eight subjects were enrolled in this study with twenty-five subjects completed the entire study (tSVF = 14, control = 11). Only twenty-eight of the calculated sixty-four subjects were included because the required pace of inclusion was not met endanger completion of the study. Four subjects failed to complete each follow-up moment of the study; one subject underwent a facial aesthetic procedure after six months of follow-up, two subjects did not show-up for the last follow-up moment and one subject missed only the six months follow-up moment due to personal circumstances. Fourteen subjects were included in both study arms (Table 2). Mean age

of subjects in the experimental group was 51.64 ± 4.24 [range 45 – 58] and 48.93 ± 7.08 [range 38 – 60] in the control group (p>0.05). No difference was seen in baseline platelet count between both groups (p>0.05).

Table 2. Age and platelet count at time of surgery of all included subjects in both groups.

	Group 1 Lipofilling + PRP + tSVF (n = 14)			Group 2 Lipofilling + PRP (n = 14)			
	Mean	SD	Range	Mean	SD	Range	p-value
Age at time of surgery	51.64	4.24	45 - 58	48.93	7.08	38 - 60	0.23
Platelet count at time of surgery	213.93	45.76	142 - 280	202.38	35.82	145 - 263	0.89

No significant difference between both groups. PRP = platelet rich plasma; tSVF = tissue stromal vascular fraction.

Immunohistochemical and immunocytochemical analysis of tissue-stromal vascular fraction

Fractionation of adipose tissue to obtain tSVF was effective in all patients because almost all adipocytes were disrupted. Reduction of adipocytes resulted in an enrichment of small vessels (determined as mean α-SMA positivity of 21.24% ± 5.02 and mean vWF positivity of 8.95% ± 3.26) and extracellular matrix per remaining 10% volume (Fig. 3, table 3). This study was not designed to focus on any correlation between clinical effect and histological composition of tSVF.

Platelet-rich plasma supplemented lipofilling with tissue-stromal vascular fraction does not improve skin elasticity nor reduces TEWL

The addition of tSVF showed no difference in total deformation (R7) between both groups at any moment for both evaluation zones except for evaluation zone one at twelve months postoperative (Fig. 4).

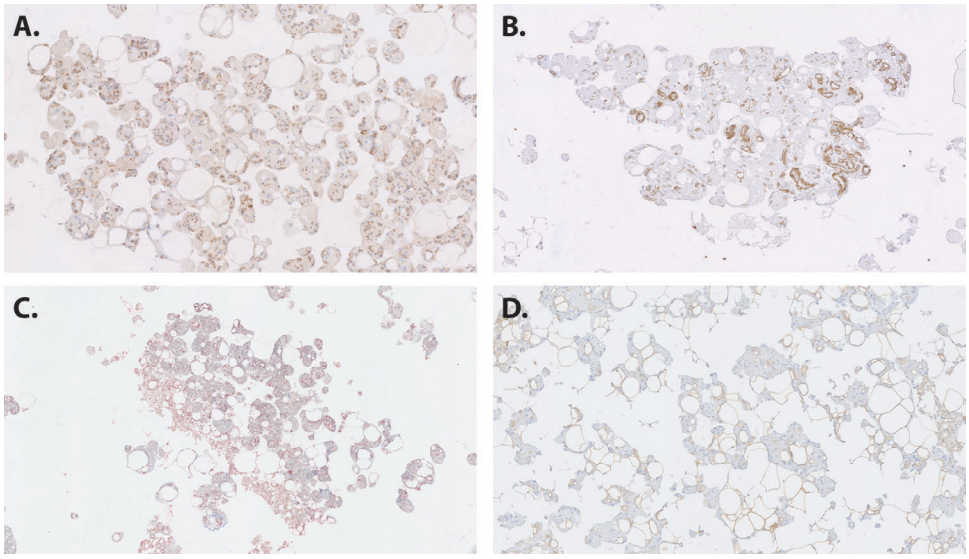

Figure 3. Light microscope images of **(A)** vWF staining of tSVF, **(B)** α-SMA staining of tSVF, **(C)** masson's trichrome staining of tSVF and **(D)** perilipin staining of tSVF. α-SMA = alpha-smooth muscle actin; vWF = von Willebrandfactor; tSVF = tissue stromal vascular fraction.

Table 3. Quantification of α-SMA and vWF staining of tSVF

	Staining results	
Patient	**vWF Mean % area (n= 3)**	**αSMA Mean % area (n= 3)**
1	14.40	18.26
2	8.13	11.51
3	10.10	22.82
4	7.79	25.33
5	13.71	16.23
6	9.12	23.56
7	8.07	16.16
8	7.58	28.79
9	5.86	20.99
10	12.48	23.91
11	3.40	16.50
12	4.23	24.76
13	11.00	28.23
14	9.46	20.34

α-SMA = alpha-smooth muscle actin; vWF = von Willebrandfactor; tSVF = tissue stromal vascular fraction.

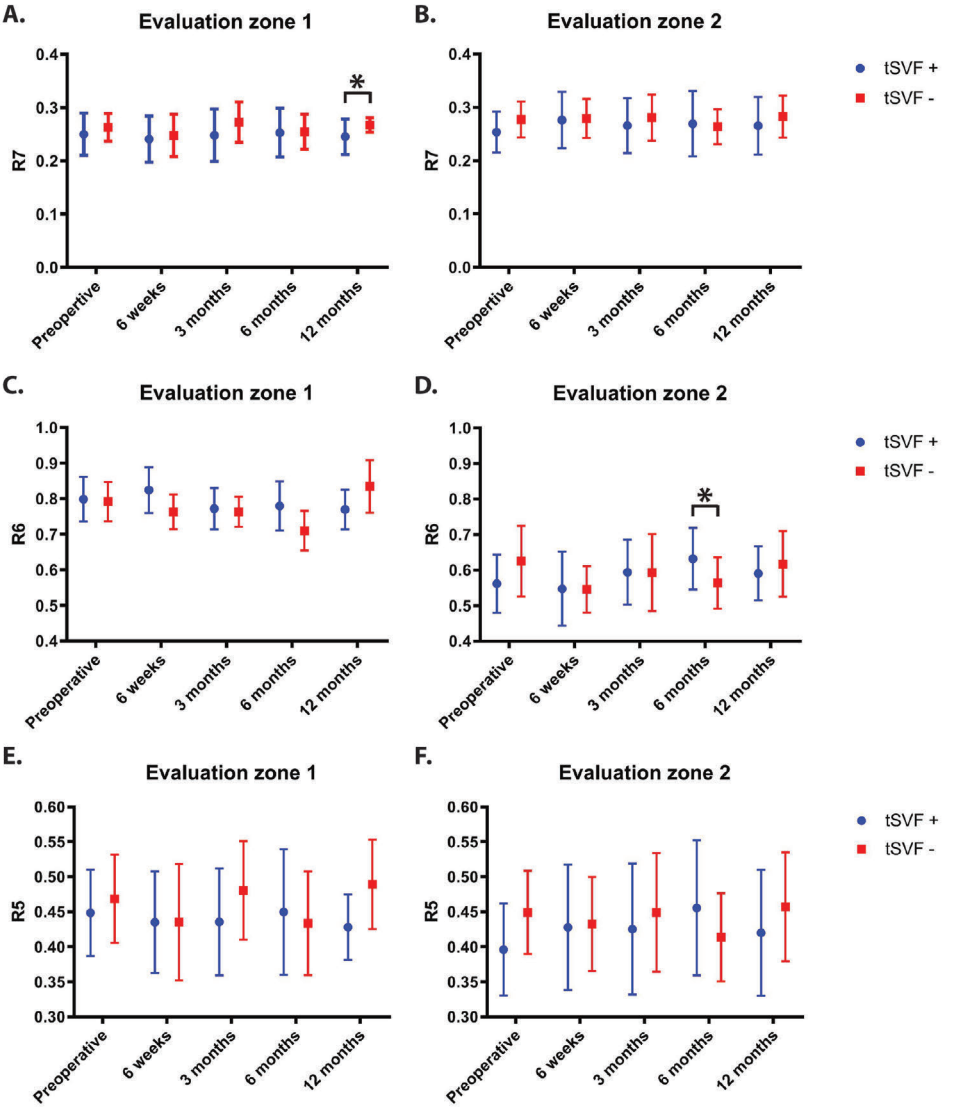

Figure 4. Changes in true skin elasticity as ratio of elastic recovery to total deformation (R7 parameter) for **(A)** evaluation zone 1 and **(B)** evaluation zone 2, ratio of viscoelasticity to elastic extension (R6 parameter) for **(C)** evaluation zone 1 and **(D)** evaluation zone 2 and net elasticity (R5 parameter) for **(E)** evaluation zone 1 and **(F)** evaluation zone 2 are presented between both the experimental and control group for all timepoints. * Higher R7 parameter was shown for evaluation zone one in the tSVF- group than in the tSVF+ group twelve months postoperative ($p<0.05$). * Higher R6 parameter was shown in the tSVF+ group in comparison with the tSVF- group six months postoperative ($p<0.05$). No difference was shown for the R5 parameter. tSVF = tissue stromal vascular fraction.

Evaluation zone one showed a higher skin elasticity in the control group with a mean of 2.7 ± 0.01 versus 2.4 ± 0.03 in the experimental group ($p<0.05$). On the other hand, the ratio of viscoelasticity to elastic extension (R6) was increased in the treatment group compared to controls but only for evaluation zone two at six months postoperative ($p<0.05$, fig. 4). Treatment did not influence net elasticity (R5). No difference in TEWL was seen between both groups at any follow-up moment for both evaluation zones (Fig. 5). Within each group, the effect of lipofilling and PRP with or without tSVF on R5, R6 and R7 was negligible compared to preoperative values.

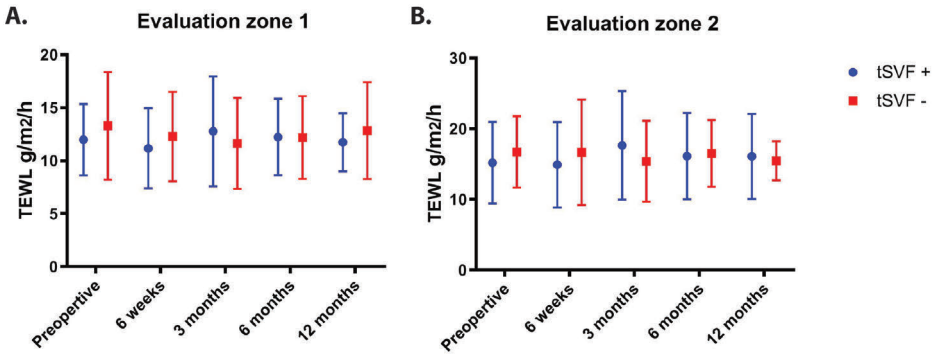

Figure 5. Changes in local TWEL for **(A)** evaluation zone 1 and **(B)** evaluation zone 2 are presented between both the experimental and control group for all timepoints. No difference was shown. TWEL = local transepidermal water loss; tSVF = tissue stromal vascular fraction.

Platelet-rich plasma supplemented lipofilling with tissue-stromal vascular fraction does not improve skin quality

The addition of tSVF showed no difference for the number of superficial spots, pores as well as skin texture, vascularity and pigmentation for both absolute and relative scores between both groups at any moment during follow-up (Fig. 6). The absolute number of wrinkles was reduced in the control group as compared to the experimental group at twelve months postoperative ($p<0.05$). In contrast, the relative score of wrinkles showed did not differ between groups. Within each group, PRP supplemented lipofilling with or without tSVF did not reduce the number of any parameter when preoperative and postoperative values were compared.

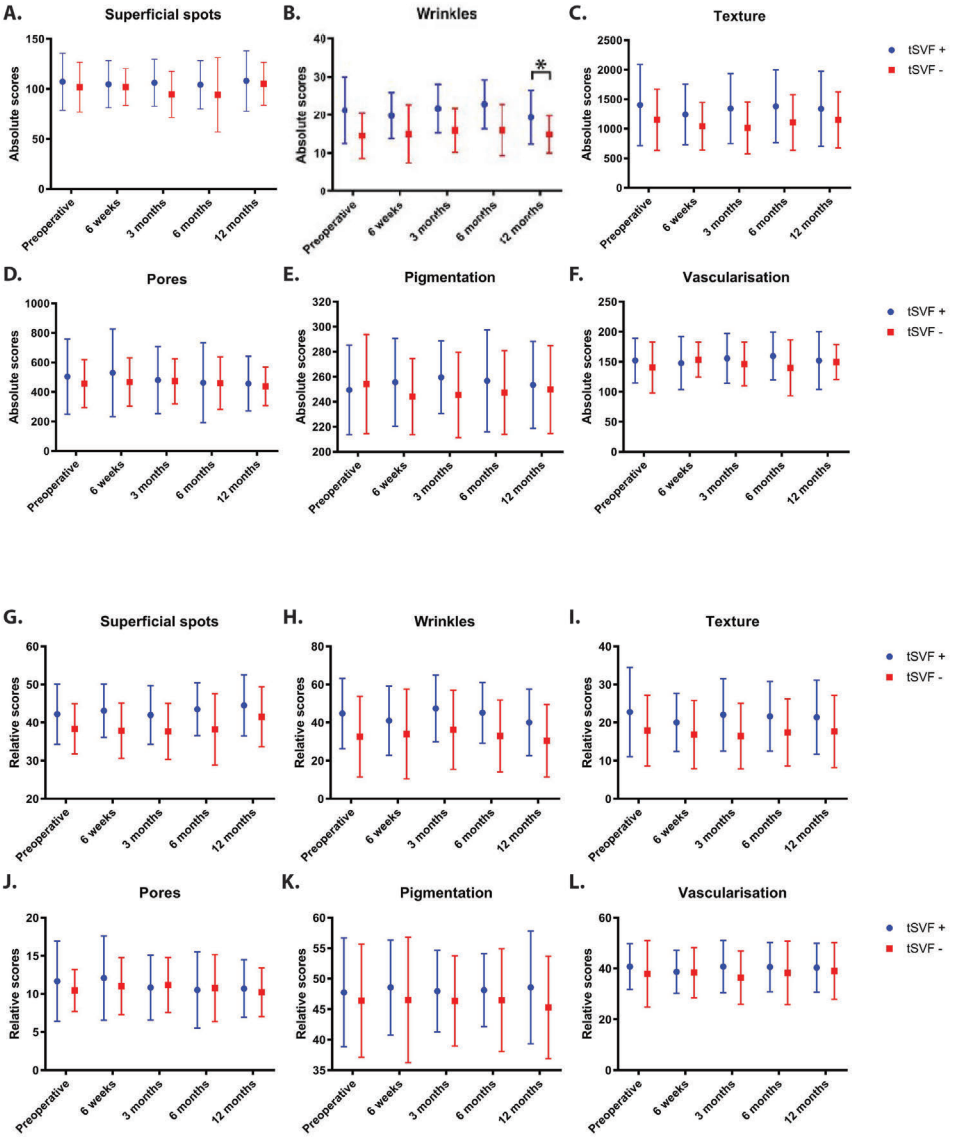

Figure 6. Changes in VISIA data *i.e.* absolute numbers of **(A)** superficial spots, **(B)** wrinkles, **(C)** texture, **(D)** pores, **(E)** pigmentation, **(F)** vascularization and relative scores of **(G)** superficial spots, **(H)** wrinkles, **(I)** texture, **(J)** pores, **(K)** pigmentation and **(L)** vascularization are presented between both the experimental and control group for all timepoints. * Higher absolute number of wrinkles was shown in the tSVF+ group than in the tSVF- group twelve months postoperative ($p<0.05$). tSVF = tissue stromal vascular fraction.

Platelet-rich plasma supplemented lipofilling with tissue-stromal vascular fraction does not improve patient satisfaction

No difference was seen in satisfaction with overall facial appearance nor facial skin quality between both groups at any follow-up moment (Fig. 7). In comparison with the control group of women who never seek aesthetic facial procedures, pre- and postoperative satisfaction rates were lower. All other FACE-Q modules regarding subject satisfaction of skin characteristics revealed no difference between both groups at any follow-up moment (Table 4). Psychological wellbeing, social function and recovery were similar for both groups pre- and postoperative. Subjects in both groups had comparable satisfaction rates regarding the final outcome of the treatment (Table 4).

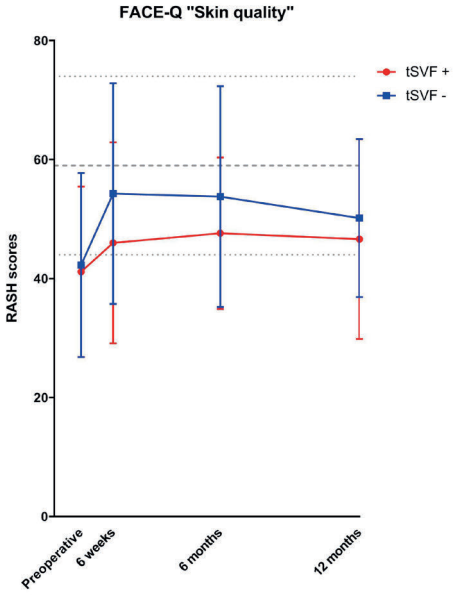

Figure 7. Changes in FACE-Q regarding facial skin quality are presented between both the experimental and control group for all timepoints. No difference was shown. tSVF = tissue stromal vascular fraction.

Lipofilling and PRP with or without tSVF did not improve any FACE-Q modules regarding subject satisfaction. However, only overall facial appearance in the control group was improved at twelve months postoperative ($p<0.05$, fig. 8). Psychological wellbeing and social function did not improve as well. No major complications occurred in both groups.

Table 4. Patient satisfaction and psychological wellbeing as well as social function measured by different FACE-Q modules at different timepoints.

Timepoint	N	Mean	Standard deviation	P-value
		Appraisal of lines overall		
		tSVF + / tSVF -		
Preoperative	14 / 14	29.00 / 37.64	16.72 / 20.57	0.27
6 weeks	14 / 14	44.86 / 46.14	21.58 / 26.61	0.89
6 months	13 / 13	36.54 / 44.85	17.88 / 25.02	0.27
12 months	14 / 11	42.64 / 39.45	25.83 / 19.77	0.74
		Appraisal of crow's feet lines		
		tSVF + / tSVF -		
Preoperative	14 / 14	42.43 / 48.29	16.08 / 26.93	0.85
6 weeks	14 / 14	56.64 / 56.57	25.06 / 33.06	0.98
6 months	13 / 13	46.54 / 55.23	18.99 / 23.22	0.43
12 months	14 / 11	47.79 / 40.73	22.29 / 20.00	0.36
		Age appraisal (VAS)		
		tSVF + / tSVF -		
Preoperative	14 / 14	3.21 / 2.14	6.48 / 6.33	0.62
6 weeks	14 / 14	1.71 / 1.21	4.85 / 5.95	0.72
6 months	13 / 13	2.62 / 1.38	5.71 / 5.58	0.47
12 months	14 / 11	2.21 / 1.55	4.85 / 4.54	0.48
		Age appearance appraisal		
		tSVF + / tSVF -		
Preoperative	14 / 14	34.50 / 51.14	21.62 / 25.83	0.06
6 weeks	14 / 14	46.43 / 52.36	23.94 / 25.23	0.52
6 months	13 / 13	42.46 / 60.54	21.59 / 28.80	0.16
12 months	14 / 11	46.36 / 46.64	23.09 / 26.90	0.72
		Psychological wellbeing		
		tSVF + / tSVF -		
Preoperative	14 / 14	59.43 / 65.5	15. 73 / 22.84	0.30
6 weeks	14 / 14	61.93 / 60.00	14.36 / 24.96	0.80
6 months	13 / 13	62.92 / 68.85	20.11 / 17.45	0.72
12 months	14 / 11	65.07 / 64.73	18.29 / 16.29	0.91
		Social function		
		tSVF + / tSVF -		
Preoperative	14 / 14	68.36 / 66.71	19.58 / 25.59	0.89
6 weeks	14 / 14	70.71 / 63.07	19.75 / 16.46	0.36
6 months	13 / 13	68.23 / 71.08	21.02 / 17.85	0.86
12 months	14 / 11	68.79 / 68.82	20.84 / 19.45	0.72
		Satisfaction with outcome		
		tSVF + / tSVF -		
6 weeks	14 / 14	36.14 / 37.79	26.68 / 30.09	0.98
6 months	13 / 13	32.08 / 36.85	24.66 / 35.39	0.88
12 months	14 / 11	33.57 / 34.90	28.80 / 25.27	0.81
		Recovery of early symptoms		
		tSVF + / tSVF -		
1 week	14 / 14	32.64 / 31.86	10.43 / 7.96	0.96
		Recovery early life impact		
		tSVF+ / tSVF-		
1 week	14 / 14	48.93 / 45.43	15.48 / 10.84	0.76

No significant differences between both groups. tSVF = tissue stromal vascular fraction; VAS = visual analogue scale.

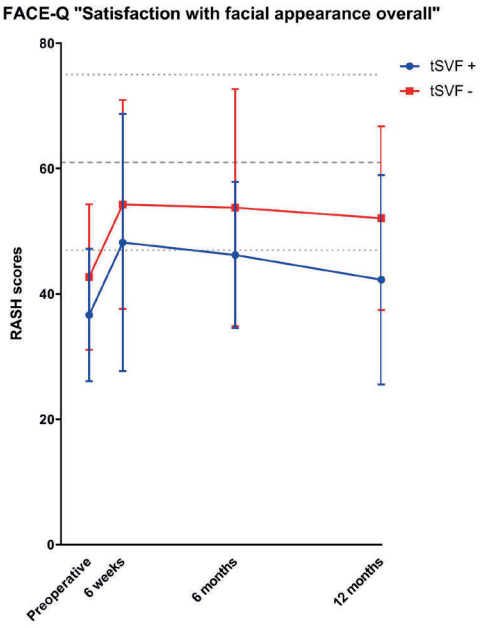

Figure 8. Changes in FACE-Q regarding satisfaction with facial appearance overall are presented between both the experimental and control group for all timepoints. * A higher satisfaction with facial appearance overall was shown in the control group as compared to the experimental group twelve months postoperative ($p<0.05$). tSVF = tissue stromal vascular fraction.

DISCUSSION

The addition of tSVF to autologous facial lipofilling with PRP did not show benefits for skin quality for aesthetic reasons, recovery or subject satisfaction in this study. Lipofilling with PRP alone did not improve skin quality compared to preoperative values, while patient satisfaction for overall facial appearance was improved. In literature, clinical reports studying autologous lipofilling or any component of adipose tissue *e.g.* cSVF or tSVF as a treatment of facial skin quality show different results compared to this prospective randomized controlled trial.[27-31] Those reports mentioned improvement in skin quality *e.g.* skin texture, wrinkles and pores size. However, the level of evidence in those clinical studies does not allow to draw strong conclusions.[1] Thus, those results should be interpreted with caution. To date, only one well-designed prospective double-blinded randomized controlled trial showed no increase of facial skin elasticity after treatment with autologous lipofilling with or without PRP.[12] However, a regression

analysis of true skin elasticity i.e. R7 parameter as a function of age showed a negative correlation preoperative. After treatment with lipofilling, this correlation reversed to a positive correlation, especially in the PRP group. The change from negative to positive correlation was not significant (p=0.056), probably due to the high number of dropouts. On the other hand, postoperative recovery in this study decreased by the addition of PRP to lipofilling.

Thus far, four studies used histological outcomes to evaluate the effect of autologous lipofilling on facial skin.[27,32-34] Three studies only subjectively described the histological outcomes and failed to correlate the histological outcomes to clinical outcomes.[32-34] It is well-known that changes in histology after application of a treatment does not always result in significant observable clinical effects. None of the histology studies used a control group with micro-needling alone. Several clinical and animal studies have shown that microneedling alone has substantial effects to the skin *e.g.* epidermal thickening and increased dermal density.[35-38] The described effects in the four studies using histological outcomes after autologous lipofilling for facial skin rejuvenation might largely be caused by needling.

The definition of 'skin rejuvenation' is broad and difficult to describe leading to noncomparable results between different clinical studies. In literature, a clear definition of skin rejuvenation is lacking which contributes to a large number of publications stating that autologous lipofilling improves skin quality *e.g.* disappearing of wrinkles or dark infraorbital circles.[39,40] These observed effects are volumetric effects rather than 'true' skin rejuvenation effects to our opinion. Lipofilling causes an increased subcutaneous volume on the site of injection. Increased subcutaneous volume stretches the overlaying skin thereby reducing wrinkle formation. Moreover, dark infraorbital circles are caused by increased transparency of the orbicularis oculi muscle due to aging related decrease in subcutaneous volume and thinning of the skin. Increasing subcutaneous volume by transplantation of adipose tissue results in a less visible orbicularis oculi muscle and thus decreased dark infraorbital circles. To our opinion, the definition of skin rejuvenation should only be used when ordinary aging related improvements of the skin without any volumetric component is mentioned *e.g.* improvements of thickness, skin texture, elasticity, pores size or pigmentation. There should also be a clear distinction between ordinary (physiological) aging related changes of the skins and changes due to pathological processes such as traumatic scars, fibrotic diseases or wound healing.

All the aforementioned clinical studies used lipofilling as a treatment to enhance skin rejuvenation in patients with ordinary aged skin. To our opinion, aging of the skin is a

physiological process where elastin gradually degrades due to ultraviolet exposure and wear-and-tear with no substantial changes in a short period of time. These changes over time cause no significant release of cytokines and thus transplanted ASCs are probably not sufficiently triggered by host environment to regenerate tissue. To induce a regeneration trigger, host environment might be damaged deliberately prior treatment using trichloroacetic (TCA) peeling. Aging related skin modifications cannot be considered damaged tissue and therefore lipofilling for skin rejuvenation is not a suitable indication and should only be used for a volumizing effect of the facial fat compartments in the aging face. In contrast, pathological processes are accompanied by a burst release of growth factors and cytokines initiating inflammation, a disturbance in extracellular matrix remodeling and reducing angiogenesis. These growth factors and cytokines trigger ASCs to release anti-inflammatory and pro-angiogenic cytokines as well as metalloproteinase to remodel extracellular matrix.[41] Thus far, several clinical studies have shown that autologous lipofilling might increase scar remodeling.[42]

Although, PRP contains a large number of pro-angiogenic growth factor and metalloproteinases, clinical data of skin rejuvenation remains insufficient.[8,43] A systematic review by Maisel-Campbell et al. evaluated studies using PRP as a monotherapy for skin rejuvenation.[43] In total, ten studies treating 180 patients with PRP showed improvement in satisfaction, skin texture, hydration and pigmentation. However, almost all studies used non-validated and subjective outcome measurements and most studies failed to report the concentration of platelets in PRP or baseline blood samples. It is well-known that platelet concentrations highly variable during the day and most PRP preparation devices use relatively increased number of platelets compared to the baseline at the moment of preparation. Studies have shown that high concentrations of PRP increases proliferation of ASCs but may also induces undesirable differentiation.[10,11] High concentrations of PRP may change ASCs towards a more fibrotic phenotype with a less angiogenic capacity as well.[9] Hence, the concentration of PRP was reduced from six to eight times above threshold in our previous study to four times above threshold in this study.[12] However, most suitable concentration of PRP to maximize tissue regeneration by ASCs remains unknown *in vivo* which is one of the limitations of this study as well. Yet, baseline concentration of platelets was comparable between both study groups.

Similarly, the ratio of SVF/lipofilling is still on debate. In this study, a ratio of 1:6 for respectively tSVF and superficial lipofilling was used based on pragmatic reasons. Ni et al. showed that a ratio of 1:3 for respectively cSVF and lipofilling was ideal in terms of volume maintenance in comparison with lower ratios in a rabbit model three months after injection.[44] Other studies confirmed the aforementioned results and showed that

a higher ratio than 1:1 for respectively cSVF and lipofilling did not increase the survival rate in animal models.[45,46] A higher survival rate of transplanted SVF enriched fat grafts might also result in a greater regenerative effect on the overlaying skin. Compared to our study, these animal models used a significant higher ratio of cSVF/lipofilling and therefore patients might undertreat in this study. However, all other studies only used animal models and did not investigate the effect of SVF enriched lipofilling on skin quality. Moreover, all other studies used enzymatic isolated SVF instead of mechanical isolated SVF. Hence, those results are difficult to compare to clinical outcomes of this study.

A limitation of this study is the use of the VISIA analysis to evaluate skin quality because this device is not validated; for that reason, the VISIA analysis was only used as secondary outcome, while the validated cutometer was used as primary outcome. There is also a lack of power in this study due to a low sample size with only fourteen subjects in each group and five subjects not completing the entire study. Besides, this study was terminated prematurely because the required pace of inclusion was not met endanger completion of the study. Designing a well-designed prospective randomized controlled trial with a strong power and completing it is challenging in regenerative aesthetic surgery, but definitely necessary to further develop the field.

In conclusion, lipofilling and PRP with tSVF did not improve skin elasticity, recovery nor subject satisfaction in a healthy population in this study. PRP supplemented lipofilling with tSVF can be considered a safe procedure. However, some controversy remains regarding tSVF/lipofilling ratios, concentration of PRP and optimal isolation method procedure to isolate cSVF or tSVF requiring further elaboration in the future.

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The effects of facial lipografting on skin quality: a systematic review

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ABSTRACT

Introduction

Autologous lipografting for improvement of facial skin quality was first described by Coleman in 2006. The current dogma dictates that adipose tissue-derived stromal cells (ASCs) which reside in the stromal vascular fraction (SVF) of lipograft contribute to skin rejuvenation *e.g.* increased skin elasticity, a more homogenous skin color and softening of skin texture. Nowadays, many studies have been reported on this 'skin rejuvenation' effect of autologous fat grafting. This systematic review was undertaken to assess the efficacy of autologous lipografting on skin quality.

Material & Methods

MEDLINE, Embase, Cochrane Central, Web of Science and Google Scholar databases were searched for studies evaluating the effect of autologous lipografting on facial skin quality (05-11-2018). Outcomes of interest were skin texture, color and elasticity as well as histological outcomes and number of complications.

Resul ts

Nine studies were included with 301 patients treated in total. No meta-analysis could be performed due to heterogeneity of the metrics and outcomes. Eight studies reported increased skin elasticity, improvement in skin texture as well as a more homogeneous skin color after treatment with lipografting, cellular SVF or Nanofat. One study reported no increased skin elasticity after lipografting. Histological improvement was seen after lipografting and ASCs injections. However, in general, the level of evidence of the included studies was low. No serious complications were reported.

Conclusion

Autologous facial lipografting as well as cSVF and ASCs injections hardly seem to improve facial skin quality but can be considered as a safe procedure.

INTRODUCTION

Aging of the face is characterized by many changes in a broad spectrum of facial skin features *e.g.* increased pigmented spots, increased wrinkle depth and rosacea formation. Aged facial skin can be categorized into two types of aging: intrinsic or programmed aging and extrinsic or photoaged aging. Intrinsic aging is caused by passage of time due to genetic influences, while extrinsic aging is mainly caused by ultraviolet radiation or cigarette smoke.[1,2] Major alterations of both intrinsic as well as extrinsic aging occur in the dermal extracellular matrix.[3,4] In aged skin, there is a reduced collagen syntheses and increased collagen fiber fragmentation resulting in collagen deficiency and therefore a thinner skin.[3,5] Photoaged skin is often histologically characterized by increased epidermal thickness, damaged dermal connective tissue as well as accumulation of disorganized elastin.[1,6] These extracellular matrix changes result in loss of elasticity and therefore formation of wrinkles over time.

In 2006, Coleman was the first to describe that lipografting or lipofilling, the transplantation of adipose tissue, reduced age-related skin changes such as wrinkle depth, pore size and pigmented spots.[7] These "skin rejuvenation" effects were ascribed to the regenerative potential of adipose tissue-derived stromal cells (ASCs). ASCs are attached around vessels as precursor cell types *e.g.* pericytes and supra-adventitial cells in the stroma *i.e.* stromal vascular fraction (SVF) of adipose tissue.[8,9] Adipose tissue comprises of parenchyma *i.e.* adipocytes and SVF that also consists fibroblasts, immune cells and vascular cells.[10,11] ASCs can be enzymatically isolated from large quantities of lipoaspirates and have the ability to remodel extracellular matrix.[12,13]

The enzymatic isolation of ASCs sparked an increase in clinical studies with respect to ASC-enriched lipofilling or cell-assisted lipofilling (CAL).[14-16] However, in many countries the use of enzymes and animal derived products to isolate cells (*e.g.* ASCs) from human tissue for clinical use is forbidden by legislation. Hypothetically, the animal-derived products increase the risk for zoonosis, while these multistep procedures are considered undesirable manipulations. Therefore, new intraoperative isolation procedures of ASC were developed without the use of enzymes and animal derived products, called mechanical isolation procedures.[11] Most of these mechanical isolation procedures isolate SVF that still contains cell-cell and cell-matrix connections (tSVF), in contrast to enzymatically isolated SVF that is a single cell suspension which obviously lack cell-cell connections and extracellular matrix (cSVF).[11,17]

Thus far, no studies have been published using tSVF or tSVF-enriched lipografting for skin rejuvenation purposes. Numerous other publications have described the clinical observation of improved skin quality after lipografting.[7,18-28] This systematic review now was undertaken to evaluate the effect of lipografting on skin quality.

METHODS

Protocol, information sources and search

This study was performed in accordance with the PRISMA statement.[29] The study was not registered. MEDLINE, Embase, Cochrane Central, Web of Science and Google Scholar databases were searched (08-12-2017). An update search was performed on 05-10-2018. The search was restricted to humans. Keywords used for the search on the effect of lipografting on human facial skin quality can be found in table 1, supplemental content.

Eligibility criteria

Studies were included if lipografting or a component of adipose tissue *i.e.* tSVF, cSVF or ASCs was used to improve human facial skin quality. Changes in skin quality were defined as clinical changes in skin texture, color and elasticity or any histological changes in skin epidermis or dermis (Table 1). Studies were included if adipose tissue was obtained by liposuction. Studies including patients with diseases and trauma that could affect skin quality or subcutaneous adipose tissue *e.g.* burn wounds, scars and disease-caused lipoatrophy were excluded. Studies only evaluating wrinkles or volumetric effect of lipografting or any components of adipose tissue without analyzing the skin elasticity were excluded as well. A decreased wrinkle depth after lipografting can be caused by either a volumetric effect of lipografting or increased skin elasticity. Improved skin elasticity is considered to be a positive effect on skin quality, while a volumetric effect of lipografting does not influence the skin quality. Furthermore, studies evaluating the effect of lipografting on infraorbital dark circles were excluded, because the therapeutic effect is based on reducing transparency of the orbicularis oculi muscle through the lower eyelid skin by increasing subcutaneous volume.[30,31] Additionally, studies analyzing the outcome of facial lipografting or any substance of adipose tissue in conjunction with other surgical procedures *e.g.* botulinum toxin injection or fillers were excluded. Case reports, conference abstracts and reviews were excluded as well *i.e.* only peer-reviewed original research papers were included (Table 1). The literature search was not restricted by date or publication status (Table 1, supplemental content).

Study selection

Two authors (JvD and ML) independently selected prospective and retrospective clinical studies that met the eligibility criteria (Fig. 1). Reference lists of the included studies were hand-searched for relevant studies that were not initially included using the aforementioned inclusion criteria. Inconsistences were discussed during a consensus meeting. In case of disagreement, the senior author (BvdL) gave a binding verdict.

Table 1. Inclusion and exclusion criteria.

Inclusion criteria	Exclusion criteria
Human skin	Diseases and trauma affecting skin quality *e.g.* burn wounds, scars, disease-caused
Adipose tissue obtained by liposuction	Studies evaluating volumetric effect of lipografting *e.g.* effect on wrinkle depth
Facial lipografting or any components of adipose tissue *i.e.* ASCs, cSVF, tSVF	Studies evaluating effect of lipografting on infraorbital dark circles
Clinical skin changes in texture, color and elasticity	Lipografting in conjunction with other treatments *e.g.* botulinum toxin or fillers
Changes in skin histology	Case reports, conference abstracts and reviews
Prospective and retrospective studies	

ASCs = adipose tissue-derived stromal cells; cSVF = cellular stromal vascular fraction; tSVF = tissue stromal vascular fraction

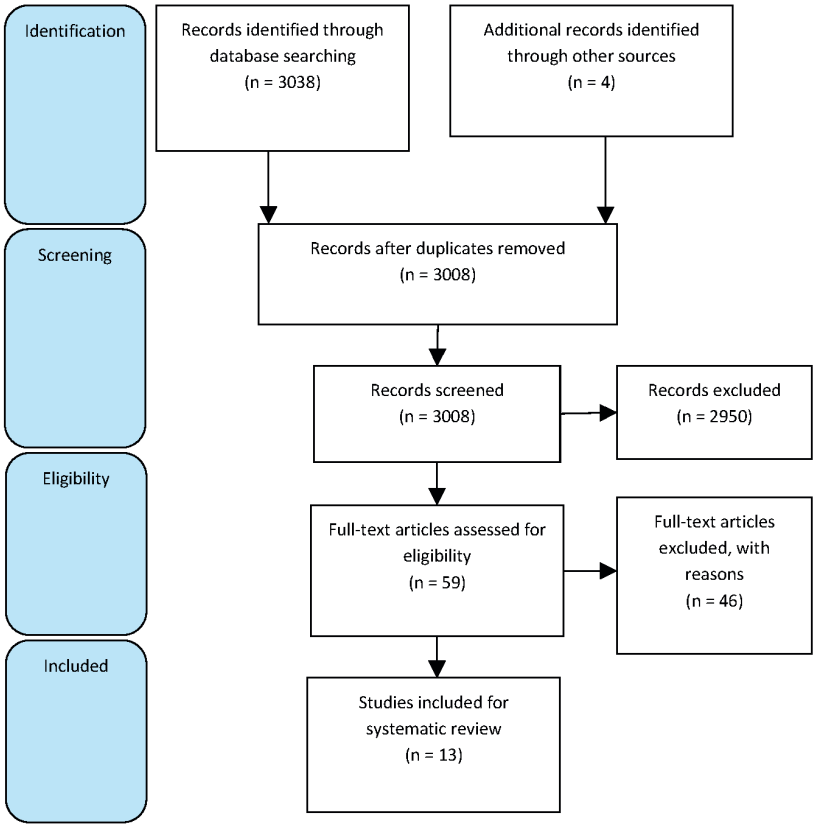

Figure 1. Flow diagram of study selection.

Assessment of quality of included studies

The included studies were graded on quality of evidence using the Oxford Center for Evidence-Based Medicine (OCEBM) criteria.[32] Disclosure agreements were reviewed for each study.

Outcomes

Outcomes of interest were clinical outcomes *i.e.* skin texture, skin color as well as skin elasticity, histological outcomes and number of complications. No meta-analysis could be performed due to the diversity of the metrics and outcomes.

Risk of bias in individual studies

The quality of lipografting and ASCs depends on age, comorbidity such as obesity and diabetes mellitus type 2, harvesting and processing techniques of adipose tissue.[33-38] For those reasons, detailed clinical information *e.g.* demographics, harvesting and processing techniques are included.

RESULTS

Included studies

The literature search yielded 4595 publications (Fig. 1). After abstract screening, 2267 articles were excluded. Seventy-three studies were read in full-text and assessed on eligibility criteria. Thirty-eight studies did not describe an outcome of interest and were therefore excluded.[7,26,30,31,39-72] Fourteen publications were reviews and therefore excluded.[7,73-84] Eight studies were excluded based on the use of lipografting or any substances of adipose tissue in combination with other treatments.[85-92] One study was excluded because it was an animal study.[15] Two studies were excluded based on the treatment of disease-caused lipoatrophy.[93,94] One study was excluded for being a letter to the editor.[95]

Quality assessment of included studies

Of the nine included studies; two studies scored a level of evidence of 2[21,25], two studies a level of evidence of 3 [19,96] and five studies a level of evidence of 4 (Table 2).[18,20,22-24] In one study, a disclosure agreement of support by a manufacturer was provided.[25]

Table 2. Quality assessment of included studies according to the OCEBM criteria.

Author, year	Level of evidence
Amirkhani et al. 2016	4
Botti et al. 2011	3
Charles-de-Sá et al. 2015	4
Covarrubias et al. 2013	2
Liang et al. 2018	3
Rigotti et al. 2016	4
Song et al. 2017	4
Trivisonno et al. 2017	4
Willemsen et al. 2017	2

OCEBM = Oxford Center for Evidence-Based Medicine

Study characteristics

Eight of the nine included studies were prospective clinical trials[19,21,22,96-100], six of these eight were controlled studies and of the latter mentioned six studies[19,21,22,96,98,100], two had been randomized.[21,100] In total, 301 subjects were treated. All studies included a 93% female study population. All studies reported the range of age while four studies reported the mean age too.[19,22,96,100] Willemsen et al. reported an overall Body Mass Index between 20-25, which was already stable for one year (Table 3).[100]

Adipose tissue harvesting and processing

All studies reported which donor site had been used. Eight studies used infiltration prior to liposuction[19,21-23,96,97,99,100] and one study by Charles-de-Sá et al. did not mention any infiltration step (Table 4).[20] Five out of the seven studies that used lipografting used decantation to process the adipose tissue prior to injection.[20-24] Two studies processed the adipose tissue by centrifugation of which one used the standard Coleman technique.[19,25] Three studies used enzymatic isolation to isolate SVF (cSVF).[18,20,22] One study used the so-called Nanofat which essentially is an emulsification procedure of lipoaspirates (Table 4).[96]

Table 3. Study characteristics and patient demographics.

Author, year	Study Type	Sex		Mean age (y) + sd	Range (y)	BMI
		Male	Female			
Amirkhani et al. 2016	Prospective, non-blinded, non-randomized, non-controlled clinical trial	1	15	-	38-56	-
Botti et al. 2011	Prospective, double-blinded, non-randomized, controlled clinical trial	4	21	46.3	21-72	-
Charles-de-Sá et al. 2015	Prospective, non-blinded, non-randomized, controlled clinical trial	1	5	-	45-65	-
Covarrubias et al. 2013	Prospective, single-blinded, randomized, controlled clinical trial		16	-	40-70	-
Liang et al. 2018*	Prospective, non-blinded, non-randomized, controlled clinical trial	12	91	28.5	24-55	-
Rigotti et al. 2016	Prospective, non-blinded, non-randomized, controlled clinical trial	2	11	56.2	45-65	-
Song et al. 2017	Retrospective, non-blinded, non-randomized, case-control study		76	-	26-53	-
Trivisonno et al. 2017	Prospective, non-blinded, non-randomized, non-controlled clinical trial	2	19	-	35-62	-
Willemsen et al. 2017	Prospective, double-blind, randomized, placebo-controlled clinical trial		25	52 ± 6.75	38-63	20-25 (1 year stable)

** Sex, mean age + sd and range are only given for the experimental group. No demographic data of the control group (n=128) could be extracted.*

Table 4. Adipose tissue harvesting and processing characteristics.

Author, year	Donor site	Infiltration solution	Harvesting cannula	Pressure	Processing method
Amirkhani et al. 2016	Abdominal	Saline solution + epinephrine 0.001%	3 mm	-	Enzymatic isolation of cSVF with collagenase type 1
Botti et al. 2011	Abdomen, knees, or thigh	Saline solution + mepivacaine 0.25% + epinephrine 1:500,000, ratio 1:1 solution:tissue	2 mm two-hole blunt	Manual negative pressure <2 cc	I1: filtering and washing with the use of strainer and 0.9% NaCl I2: centrifugation at 3000 rpm for 3 min.
Charles-de-Sá et al. 2015	Abdominal	-	3 mm	-	I1: non-enzymatic isolation of cSVF by centrifugation at 1286 *xg* for 3 min. cSVF was mixed with decanted lipografting I2: enzymatic isolation of cSVF and subsequent expansion of ASCs for 5 weeks
Covarrubias et al. 2013	Lower hemiabdominal	150 ml of saline solution + adrenaline 0.25g + 20 ml of lidocaine 2%	3 mm	-	Decantation for 15 min.
Liang et al. 2018	Medial and lateral thigh	Tumescent technique	3.5 mm polyporous	Low negative pressure	Centrifugation at 1000 rpm for 2 min., washing with NaCl and subsequently the Nanofat procedure. Nanofat was mixed with PRF.
Rigotti et al. 2016	Infraabdominal	Lidocaine 0.5% + epinephrine 1:500,000	3 mm three-hole	Manual vacuum	I1: non-enzymatic isolation of cSVF by centrifugation at 1286 *xg* for 3 min. cSVF was mixed with decanted lipografting I 2: enzymatic isolation of cSVF and subsequent expansion of ASCs for 4-5 weeks I 3: decanted lipografting was mixed with PRP
Song et al. 2017	Abdominal, thighs and buttocks	Tumescent technique (not specified)	-	Low pressure	Decantation and wicking

Table 4. Continued.

Author, year	Donor site	Infiltration solution	Harvesting cannula	Pressure	Processing method
Trivisonno et al. 2017	Thighs and hip	Tumescent technique (250 ml saline solution + adrenaline 0.5 mg + lidocaine 20 mg)	2.1 mm multiperforated, rounded-tip	Manual negative pressure	Decantation for 20-30 min.
Willemsen et al. 2017	Upper legs	Standard Coleman procedure	2.4 mm x 22 cm	Manual low negative pressure	Standard Coleman procedure

I= intervention; cSVF = cellular stromal vascular fraction; NaCl = natriumchloride; rpm = rounds per minute; ASCs = adipose derived stromal cells; PRF = platelet rich fibrin; PRP = platelet rich plasma; wicking = inserting a cottonoid strip into the syringe touching the fat graft to wick off any remaining oil.

Intervention and injection methods

Seven studies used lipografting[19,21-23,98-100], while one study used cSVF[97] and one study used Nanofat as a treatment to improve facial skin quality.[96] Botti et al. compared different adipose tissue processing techniques *i.e,* filtering and washing versus centrifugation.[19] Two studies used cSVF enriched lipografting and compared this with cultured ASCs injection of which Rigotti et al. included a third group using platelet rich plasma (PRP) enriched lipografting.[20,22] Willemsen et al. compared PRP enriched lipografting to saline 0.9% enriched lipografting.[25] Covarrubias et al. compared lipografting with no treatment.[21] Liang et al. compared platelet rich fibrin (PRF) enriched Nanofat with hyaluronic acid injection (Table 5).[96]

The injected fat volumes differed highly among all studies, ranging from 0.05 ml – 43 ml per region of the face (Table 4). In five of the nine studies the same subject was used for both the intervention group as well as the control group.[19,21-23,98] Song et al. compared different adipose tissue injection techniques *i.e.* conventional hand push injection versus an electric injection device (YSZTQ-01, Lanzhou Wenhe Medical Instrument R&D Co., Ltd, Lanzhou).[23] Seven out of the nine studies mentioned the injection plane (Table 5).[21,22,96-100]

CLINICAL OUTCOME

Skin texture

Skin texture improvement after lipografting was reported in three studies (n=149).[19,24,96] Trivisonno et al. reported 25% improvement in skin texture measured with a non-validated skin surface profilometry analyzer (Antera 3D multispectral analyzer, Miravex Limited, Dublin, Ireland) ninety days postoperative ($p<0.01$).[24] A dermatologist also reported improvement of skin texture with the use of a non-validated 3-grade scale.[24] The skin texture homogeneity score decreased from 2.43 ± 0.68 to 1.19 ± 0.4 and the skin roughness score decreased from 2.33 ± 0.73 to 1.19 ± 0.4 ($P<0.05$). Trivisonno et al. did not use a control group.[24] Liang et al. reported improvement in skin texture after PRF enriched Nanofat measured with a non-validated VISIA skin imaging analyzer (VISIA Canfield Imaging Systems, Fairfield, NJ, USA) one, twelve and twenty-four months postoperative.[96] Botti et al. reported that 68% and 72% of the included subjects scored 'high' on improvement in skin texture for filtered and washed adipose tissue and centrifuged adipose tissue, respectively.[19] The level of improvement in skin texture was measured with a non-validated self-evaluated questionnaire. No statistical analysis was mentioned in this study (Table 6).

Table 5. Intervention types and injection characteristics.

Author, year	Intervention type	Injection technique	Injection cannula/ needle	Injected volume/ number of cells	Injection site	Injection plane	Number of lipografting sessions
Amirkhani et al. 2016	I1: cSVF	-	18-gauge blunt needle	2×10^7 of cells for each side of the face	Nasolabial fold	Subcutaneous	1
Botti et al. 2011	I1: filtered and washed lipografting I2: centrifuged lipografting	Retrograde and fanning	1-, 1.5-, or 2-mm blunt cannula with a lateral opening	Temporal 2-4 ml, eyelids 1-3 ml, tear-through 0.5-1 ml, malar 3-4 ml, cheek 5-7 ml, nasolabial fold 2-3 ml, mandible 4-6 ml, marionette fold 3-5 ml, chin 2-4 ml, lips 3-6 ml	I1: left side of the face I2: right side of the face	-	1
Charles-de-Sá et al. 2015	I1: cSVF-enriched lipografting I2: cultured ASCs	I1: retrograde and fanning I2: -	I1: 1.5 mm blunt cannula I 2: 30-gauge needle	I1: cSVF + 1 ml of lipografting I2: 2×10^6 of cells in 0.4 ml saline 0.9%	I1: right preauricular area I2: left preauricular area	Subdermal	1
Covarrubias et al. 2013	I: lipografting C: no treatment	Fanning	1 mm blunt cannula	5 ml of lipografting	I: preauricular region on one side of the face C: preauricular region on the other side of the face	Intramuscular and subcutaneous	1
Liang et al. 2018	I: Nanofat + PRF C: hyaluronic acid	Needle hydro lifting	-	4-5 ml	Forehead, cheeks, chin	Intradermal	1
Rigotti et al. 2016	I1: cSVF-enriched lipografting I2: cultured ASCs I3: lipografting + PRP	I1: retrograde and fanning I2: - I3: -	I1: 1.5 mm blunt cannula I2: 30-gauge needle I3: 1.5 mm blunt cannula	I1: - I2: 2×10^6 of cells in 0.4 ml of saline 0.9% I3: 1 ml of lipografting + 1 ml of PRP	I1: right preauricular area I2: left preauricular area 2 cm distal from the tragus I3: left preauricular area 2 cm forward from the lobe	Subdermal	1
Song et al. 2017	I1: lipografting with an electric injection device (YSZTQ-01) I2: lipografting with a conventional hand push injection	I1: retrograde I2: -	-	0.05–26.43 mL of lipografting per region	I1: left side of the face I2: right side of the face	-	1-2

Table 5. Continued.

Author, year	Intervention type	Injection technique	Injection cannula/ needle	Injected volume/ number of cells	Injection site	Injection plane	Number of lipografting sessions
Trivisonno et al. 2017	I: lipografting	-	23-gauge needle	12-18 ml of lipografting	-	Intradermal	1
Willemsen et al. 2017	I: lipografting + PRP C: lipografting + saline 0.9%	Standard Coleman procedure	0.9 mm x 5 cm injectors	*Temporal 2 ml, nasojugal groove 1 ml, central midface 2 ml, nasolabial fold 2 ml, marionette-line/ prejowling/chin 3 ml **Temporal/midface 4 ml, lower midface cheek 2 ml, while rolls 2 ml	See column injected volume	Subcutaneous and subdermal	1

*I= intervention; C= control; cSVF = cellular stromal vascular fraction; ASCs= adipose derived stromal cells; PRF = platelet rich fibrin; PRP = platelet rich plasma; YSZTQ-01 = code of an electric lipografting injection device. *Subcutaneous **Subdermal.*

Table 6. Results of lipografting or any substance of adipose tissue on facial skin quality**.**

Author, year	Method of measurement	Follow-up Period
Amirkhani et al. 2016	Skin thickness, elasticity and pigmentation assessed with a cutometer, and a skin scanner using ultrasound (DUB-TPA).	Preoperative, 15, 30, 60, and 180 days postoperative
Botti et al. 2011	Subjective patient self-evaluation of skin texture improvement using a non-validated questionnaire.	6 months postoperative
Charles-de-Sá et al. 2015	Histological and histomorphometric analysis by optical and electron microscopy of skin biopsies.	Preoperative and 3 months postoperative
Covarrubias et al. 2013	Histological evaluation of skin biopsies.	60-90 days postoperative
Liang et al. 2018	Skin texture assessed with the VISIA skin imaging analyser. Skin elasticity assessed with a skin scanner (SOFT5.5).	Preoperative, 1, 6, 12 and 24 months postoperative
Rigotti et al. 2016	Histological and histomorphometric analysis by optical and electron microscopy of skin biopsies.	Preoperative and 3 months postoperative
Song et al. 2017	Visual evaluation of photographs by a group including the patient, a plastic surgeon and a third party unrelated to the study.	Preoperative, directly postoperative and at 6-24 months postoperative (mean follow-up 10.7 months)
Trivisonno et al. 2017	Skin surface profilometry (Antera 3D® multispectral analyser), clinical assessment by a dermatologist using a 3-grade scale.	Preoperative, 30 and 90 days postoperative
Willemsen et al. 2017	Skin elasticity was measured with a cutometer.	Preoperative, 1 week, 3 months and 12 months postoperative

*PRP = platelet rich plasma. * $p<0.05$, ** $p<0.01$, *** $p<0.001$*

Results	Complications
An increase in skin elasticity***, dermis thickness**, dermis density*** and epidermis thickness** after 6 months. No difference was seen in epidermis density. There was no difference in pigmentation and melanin production 6 months post-operative.	None
No difference in skin texture between the two groups. Improvement in skin texture was rated 'high' in 68% of the patients of the filtered and washed adipose tissue group vs72% of the patients of the centrifuged adipose tissue group.	None
After both treatments analysis showed a decrease in elastic fiber network and collagen fibers in the reticular dermis. The elastic fibers were more dissociated and reduced in diameter with a smoother surface. An increase of oxytalan elastic fibers was visible in the papillary dermis. No quantitative data was shown and no differences were noticed between both groups.	Not mentioned
An increase in dermis thickness, immature collagen and presence of arteries was found in the intervention group compared to the control group.*** No difference in presence of mature collagen was found between the two groups.	Not mentioned
Improvement in skin texture and elasticity scores 1, 12 and 24 months postoperative for intervention group**. No comparisons made between intervention group and control group.	5 transient infections, 7 temporarily paraesthesia, 2 pigmentation changes N
Lipografting + PRP resulted in an increased number of small oxytalan elastic fibers present in an irregular network in the papillary dermis. Reticular dermis showed a decrease of elastic fibers with a reduced diameter and smoother surface. Moreover, an induced inflammatory infiltrates and increased vasculature was visible. No quantitative data was shown.	Not mentioned
Improvement in skin pigmentation was rated 'high' in 80% vs 72.2% for respectively the use of lipografting with an electric injection device and conventional hand push injection. No difference between the groups.	None
Clinical assessment: improved skin texture, homogeneity and skin colour after treatment.* Skin surface profilometry: 25% improvement in skin texture**, and declined facial haemoglobin and melanin concentrations* after 90 days.	None
Lipografting with PRP did not improve skin elasticity as compared to lipografting without PRP or compared to the baseline at any follow-up moment.	None

Skin color

A more homogeneous skin color was noticed after lipografting in two studies (n=97).[23,24] Trivisonno et al. reported declined concentrations of hemoglobin and melanin measured with a validated skin surface profilometry analyzer (Antera 3D multispectral analyzer, Miravex Limited, Dublin, Ireland), ninety days postoperative ($p<0.05$).[24,101] A dermatologist also reported decreased pigmentation and redness of the skin with the use of a non-validated 3-grade scale.[24] The skin redness score decreased from 2.29 ± 0.64 to 1.14 ± 0.36 and the skin melanin pigmentation score decreased from 2.33 ± 0.58 to 1.24 ± 0.44 ($p<0.05$). Song et al. reported that 80% and 72.2% of the included subjects scored 'high' on improvement in skin pigmentation measured by visual evaluation of photographs for respectively two different injection techniques: 1) lipografting with an electric injection device and 2) conventional hand push injection ($p>0.05$).[23] However, no statistical analysis was used to analyze this improvement. Amirkhani et al. assessed the effect of cSVF on the skin color (n=16). No difference in pigmentation and melanin production was measured with a validated Mexameter six months postoperative ($p>0.05$) (Table 6).[18,101]

Skin elasticity

Increased skin elasticity was reported in two studies, while another study failed to show increased skin elasticity after cSVF and Nanofat injections as well as lipografting, respectively (n=144).[18,25,96] In a non-controlled study, Amirkhani et al. showed increased skin elasticity after injection of cSVF measured with a validated cutometer (C&K Electronic, Cologne, Germany), six months postoperative compared to preoperative $P<0.001$).[18] Liang et al. reported increased skin elasticity using the non-validated SOFT5.5 in a controlled study following PRF enriched Nanofat one, twelve and twenty-four months postoperative. Although, no comparisons between the intervention and the control group have been made.[96] In a double-blinded, randomized placebo-controlled trial, Willemsen et al. demonstrated no increase in skin elasticity after lipografting with or without the addition of PRP measured with a validated cutometer, twelve months postoperative ($p>0.05$).[25] Yet, reversal of the correlation between true skin elasticity and age (from negative to positive) might suggest a small effect of lipografting. The reversal of this correlation was stronger when using lipografting with PRP in comparison with lipografting alone. However, these results were not significant, most likely due to the small sample size caused by too many dropouts ($p=0.055$) (Table 6).

Histological outcome

Three studies showed histological and/or histomorphometric improvement of skin biopsies after treatment with lipografting, PRP-enriched lipografting, cSVF-

enriched lipografting and cultured ASCs.[20-22] All studies used the same patients for the intervention group as well as the control group. In an observer-blinded, randomized clinical trial, Covarrubias et al. compared lipografting with no treatment and demonstrated an increase in dermis thickness, presence of immature collagen and arteries sixty-nine days postoperative (n=16, $p<0.001$).[21] No increase or decrease was seen in the presence of mature collagen.

In two non-blinded, non-randomized studies conducted by the same research group, three different types of treatments were performed: 1) PRP-enriched lipografting; 2) cSVF-enriched lipografting; and 3) injection of cultured ASCs (n=13).[20,22] Three months postoperative skin biopsies were compared with preoperative skin biopsies. The different types of treatment were not compared with each other. After all three types of treatment, the reticular dermis showed a decrease in elastic fiber network with more dissociated elastic fibers, a smoother surface and a smaller diameter.[20,22] Additionally, after cSVF-enriched lipografting and cultured ASCs the reticular dermis showed a decrease in collagen fibers.[20] Additionally, the number of oxytalan elastic fibers in the papillary dermis was increased.[20,22] Moreover, after injection of PRP-enriched lipografting increased inflammatory infiltrates and vasculature was seen (Table 6).

Complications

Six out of the nine studies reported on the occurrence of no significant complications after lipografting, cSVF or Nanofat treatment (n=196, Table 6).[18,19,23-25,96] Liang et al. reported fourteen small complications: five patients had a transient infection, seven patients suffered from temporarily paresthesia and two patients noticed pigmentation changes which lasted for more than twelve months.[96]

DISCUSSION

This systematic review demonstrates that substantial evidence is lacking that the use of lipografting or a component of adipose tissue *i.e.* cSVF or ASCs rejuvenates healthy human facial skin, as was advocated by Coleman.[7] This review also demonstrates that the use of facial lipografting or a substance of adipose tissue can be considered to be a safe procedure.

In general, most included studies of this review reported positive results. However, the level of evidence in five out of nine included studies was low with an OCEBM evidence level of just 4.[18,20,22-24] Therefore, the reported outcomes of these studies should be interpreted with caution. Low levels of evidence were caused by poor study designs:

two studies lacked a control group[18,24], four studies used non-validated methods of measurements[19,23,24,96], two studies did not report any quantitative data[20,22] and two studies did not define the outcome of interest *i.e.* skin texture.[19,96] Trivisonno et al. defined improved skin texture as decreased wrinkle depth, folds and fine lines when analysed with the profilometry analyser. The observed improved skin texture might therefore be caused by a volumetric effect of lipografting.[24] The volumetric effect of lipografting can decrease wrinkle depth and give the patients' face a rejuvenated appearance. Decrease in wrinkle depth is not necessarily related to an improved skin quality. Furthermore, each skin parameter *e.g.* skin texture, wrinkles, pigmentation, pores, is affected by one another. For example, skin texture is defined as softening of the skin which is affected by wrinkles, pores and birthmarks. Hence, the improvement in skin texture is partly caused by a volumetric effect of lipografting due to decreased wrinkle depth. This shows that the definition of "skin rejuvenation" or "skin quality" is rather broad and difficult to confine.

The heterogeneity between studies is high caused by the absence of standardization of fat harvesting and processing techniques of lipoaspirates as well as variation in patient demographics. Cell yield and viability of both lipoaspirate and cSVF depend on age, comorbidity *e.g.* obesity, diabetes mellitus type 2 and probably harvest location and processing techniques such as centrifugation and decantation.[33-38,102] The majority of the included studies did not mention these donor characteristics, which makes comparison of the included study populations difficult.

Animal experiments corroborate the clinical findings that administration of adipose tissue or its components is of little influence on skin quality. Two mice experiments demonstrated increased dermal thickening due to increased numbers of type 1 collagen fibers and angiogenesis after lipografting.[103,104] Another study investigating the effect of lipografting, ASC transplantation and ASC-enriched lipografting on the upper eyelid of pigs showed no increase in epidermal thickening.[105] However, the use of ASC-enriched lipografting resulted in an increased dermal thickening as well as increased epidermal cell proliferation, collagen content and number of arterioles in comparison with placebo.[105]

In aesthetic plastic surgery, it is challenging to design well-defined prospective randomized clinical trials with the use of validated equipment and questionnaires to assess skin quality. A study on the number of publications in three major plastic surgery journals found only 1.83% of the publications to be randomized clinical trials.[106] Plastic surgery literature consists mainly of relatively small sample sized studies, as compared to the general medical literature. Moreover, a small sample size makes it more difficult

to establish significant differences between the experimental and the placebo group.[107] Also, to treat a patient with placebo and subject the patient to all the potential risk of surgery is generally seen as ethically undesirable. However, treating a patient with a non-scientifically proven treatment is ethically undesirable as well. Hence, most clinical trials compare two different treatments, which makes it challenging to establish any significant difference in outcome and real effect of the treatment. To date, the readouts to quantify skin rejuvenation are mainly based on pre- and postoperative photographic comparisons, which often are not or poorly standardized. These pre- and postoperative photographs are often analysed in a descriptive manner instead of being blinded analysed by an objective and independent third observer or are the result from objective, validated, computer analysis.

To improve future clinical trials using facial lipofilling in aesthetic plastic surgery, we propose a statement for designing a proper randomized clinical trial with validated and objective readouts (Table 7). In this way, comparing outcomes of future clinical trials can draw a definite conclusion on the effect of facial lipofilling on skin quality. The study population should consist of a standardized group with ASA1 classification and a minimum age of 35, because skin elasticity is higher in younger patients.[108] Patients with obesity, systemic diseases, smokers and hormonal fluctuations should be excluded because all of these factors influence adipose tissue quality as well as skin quality.[38,109-111] Moreover, patients with preceding facial interventions within the last twelve months should be excluded because of late effects of priory interventions. Patients with a known history of psychiatric disorder should also be excluded because this could influence patient satisfaction outcomes. To date, there is no consensus on the quality of lipoaspirate harvested from different locations *e.g.* abdomen, upper legs as well as injection volumes and lipoaspirate processing techniques *e.g.* centrifugation, decantation.[33,35,37] Thus, we propose a standard harvesting location in all trial patients and to centrifuge lipoaspirate as first described by Coleman to ensure the right amount of lipoaspirate by losing infiltration fluid.[112] Injection volumes should be standardized during the entire study and there should be a maximum time of 30 min. between harvesting and injection to prevent ischemic cell death. Finally, we propose the use of the validated cutometer as primary outcome since loss of skin elasticity is strongly correlated with ageing.[113] Secondary outcomes should include: patient reported outcome measures *i.e.* satisfaction as measured with the validated FACE-Q questionnaires, clinical photographs analyses (by blinded and independent observers) and complications (number and type).

Nowadays, the indication for lipografting has already proven its efficacy for other clinical applications *e.g.* as a treatment to increase volume for cosmetic and/or

oncological breast augmentation as well as anti-scarring treatment for (posttraumatic) scars and burn wounds.[114-117] In a systematic review on lipografting in cosmetic breast augmentation, Groen et al. showed high retention volumes after lipografting after long term follow up (average volume retention 62.4% [range 44.7%-82.6%], mean time follow-up 16.6 months) with high satisfaction rates among patients (92%) and surgeons (89%).[115] Two systematic reviews on lipografting and ASCs in burn wounds showed that autologous lipografting significantly restores volume, improves scar appearance and scar related pain and itchiness.[116,117] Apparently It seems to be that the severity of the skin trauma/damage plays a key role whether lipografting or ASCs could be effective in skin reparative/ wound reparative effects. In case of low amounts of damage *e.g.* aging of the extracellular matrix in physiological processes such as ageing of the skin, lipografting or ASCs are not or hardly able to remodel the extracellular matrix. However, in case of severe skin damage, as in pathological processes *i.e.* burn wounds or scarring, lipografting or ASCs seem to be highly effective in remodelling the damaged skin.[116,117]

CONCLUSION

This systematic review demonstrates a lack of scientific evidence that autologous facial lipografting or any other substance of adipose tissue *i.e.* cSVF and ASCs improves normal aged facial skin quality but also demonstrates that the procedure can be considered to be safe.

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Table 7. Statement for designing a proper and well-designed clinical trial for facial lipofilling.

Study design	Patient demographics	Treatment	Outcomes
• Placebo vs. treatment or • Treatment vs. treatment • Lipofilling without other treatments* • Randomized • Double-blinded** • Follow-up with a minimum of 1 year	**Inclusion criteria** • ASA1 with a BMI <30 for at least 1 year • Non-smokers: >1-year non-smoking • Uniform population of females or males • Age: minimum of 35 **Exclusion criteria** • Diabetes mellitus type 1 or type 2 • Human immunodeficiency virus • Immunological diseases • Collagen diseases • Interventions of the face 1 year prior to the date of surgery • Active child wish • Active use of hormone replacement therapy∞ • History of a psychiatric disorder	• Standardized harvesting location • Centrifuged adipose tissue as • described by Coleman • Well documented injection volumes per deep, superficial and intradermal anatomical location^{o} • Number of sessions need to be standardized • Maximum time of 30 min. between lipoharvesting and injection	**Primary outcome** • Skin elasticity measured with the cutometer **Secondary outcome** • Patient satisfaction measured with the FACE-Q • Clinical photographs analysed by a blinded and independent observer • Number of complications

**Lipofiling can only be combined with cSVF, tSVF or PRP-like treatments. **Patient as well as investigator performing measurements preoperative and during follow-up. ∞Hormonal birth control treatments are excluded. ºTemporal, nasojugal groove, central midface, nasolabial fold, marionette-line/prejowling/chin. ASA = American Society of Anaesthesiology*

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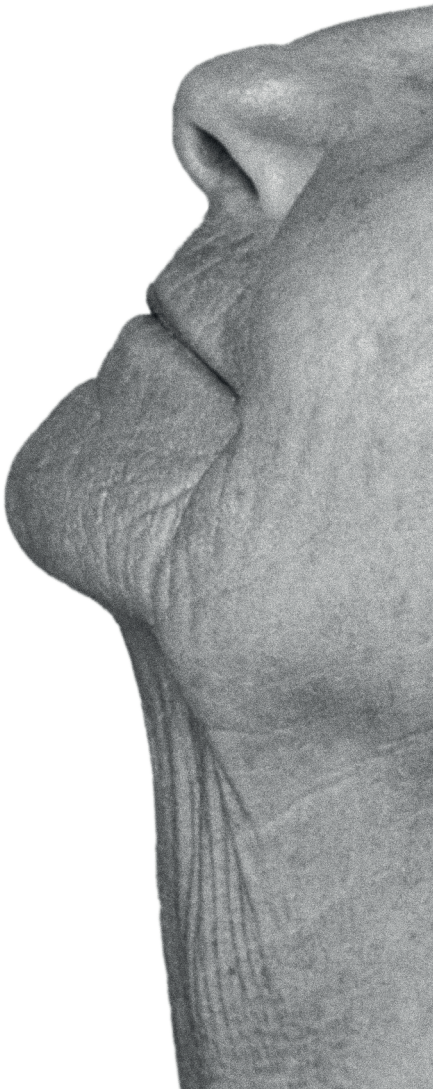
SUPPLEMENTAL CONTENT

Table S1. Specific search terms of databases.

Databases	Search terms
Embase	((('mesenchymal stem cell transplantation'/de OR transplantation/de OR 'tissue transplantation'/de OR 'tissue graft'/de OR injection/de OR 'cell therapy'/de OR 'stem cell transplantation'/de OR 'stem cell'/de) AND ('adipose tissue'/exp OR 'adipose tissue cell'/exp OR fat/de OR 'lipectomy'/de OR liposuction/de)) OR 'lipofilling'/de OR 'fat transplantation'/de OR 'fat injection'/de OR 'adipose tissue transplantation'/de OR 'autologous fat transfer'/de OR 'adipose derived stem cell'/de OR 'fat transfer'/de OR 'fat grafting'/de OR 'stromal vascular fraction'/de OR lipografting/de OR (lipofill* OR Lipograft* OR lipoinject* OR microlipofill* OR Microlipograft* OR microlipoinject* OR ((fat OR dermofat OR microfat OR Nanofat OR adipose OR facelift* OR face-lift* OR lipectom* OR liposuct*) NEAR/6 (transplant* OR graft* OR autotransplant* OR autograft* OR inject* OR stem-cell* OR Progenitor-Cell* OR transfer* OR autolog* OR implant* OR redistribut* OR filler OR filling)) OR (stroma* NEAR/3 vascul* NEAR/3 fraction*)):ab,ti) AND ('rhytidoplasty'/exp OR 'facial rejuvenation'/de OR 'face skin'/de OR 'face'/exp OR 'wrinkle'/de OR 'face surgery'/de OR 'facies'/de OR (Rhytidoplast* OR facelift* OR face-lift* OR midface* OR midfacial* OR face OR facial OR cheek* OR chin OR forehead* OR fore-head* OR periocul* OR periorbit* OR peri-ocul* OR orbit* OR nasolabial* OR wrinkle* OR bucca* OR (Crow* NEAR/3 feet*) OR ((frontal OR frontopariet* OR fronto-orbital*) NEAR/3 area*) OR facies):ab,ti) NOT ([animals]/lim NOT [humans]/lim) NOT ([Conference Abstract]/lim OR [Letter]/lim OR [Note]/lim OR [Editorial]/lim) AND [english]/lim
Medline Ovid	(((Mesenchymal Stem Cell Transplantation/ OR Transplantation/ OR Tissue Transplantation/ OR Injections/ OR Cell- and Tissue-Based Therapy/ OR Stem Cell Transplantation/ OR Cell Transplantation/ OR Stem Cells/) AND (exp Adipose Tissue/ OR Lipectomy/)) OR exp Adipose Tissue/tr OR (lipofill* OR Lipograft* OR lipoinject* OR microlipofill* OR Microlipograft* OR microlipoinject* OR ((fat OR dermofat OR microfat OR Nanofat OR adipose OR facelift* OR face-lift* OR lipectom* OR liposuct*) ADJ6 (transplant* OR graft* OR autotransplant* OR autograft* OR inject* OR stem-cell* OR Progenitor-Cell* OR transfer* OR autolog* OR implant* OR redistribut* OR filler OR filling)) OR (stroma* ADJ3 vascul* ADJ3 fraction*)).ab,ti.) AND (Rhytidoplasty/ OR exp Face/ OR Skin Aging/ OR Facies/ OR (Rhytidoplast* OR facelift* OR face-lift* OR midface* OR midfacial* OR face OR facial OR cheek* OR chin OR forehead* OR fore-head* OR periocul* OR periorbit* OR peri-ocul* OR orbit* OR nasolabial* OR wrinkle* OR bucca* OR (Crow* ADJ3 feet*) OR ((frontal OR frontopariet* OR fronto-orbital*) ADJ3 area*) OR facies).ab,ti.) NOT (exp animals/ NOT humans/) NOT (letter OR news OR comment OR editorial OR congresses OR abstracts).pt. AND english.la.
Cochrane CENTRAL	((lipofill* OR Lipograft* OR lipoinject* OR microlipofill* OR Microlipograft* OR microlipoinject* OR ((fat OR dermofat OR microfat OR Nanofat OR adipose OR facelift* OR face-lift* OR lipectom* OR liposuct*) NEAR/6 (transplant* OR graft* OR autotransplant* OR autograft* OR inject* OR stem-cell* OR Progenitor-Cell* OR transfer* OR autolog* OR implant* OR redistribut* OR filler OR filling)) OR (stroma* NEAR/3 vascul* NEAR/3 fraction*)):ab,ti) AND ((Rhytidoplast* OR facelift* OR face-lift* OR midface* OR midfacial* OR face OR facial OR cheek* OR chin OR forehead* OR fore-head* OR periocul* OR periorbit* OR peri-ocul* OR orbit* OR nasolabial* OR wrinkle* OR bucca* OR (Crow* NEAR/3 feet*) OR ((frontal OR frontopariet* OR fronto-orbital*) NEAR/3 area*) OR facies):ab,ti)
Web of Science	TS=(((lipofill* OR Lipograft* OR lipoinject* OR microlipofill* OR Microlipograft* OR microlipoinject* OR ((fat OR dermofat OR microfat OR Nanofat OR adipose OR facelift* OR face-lift* OR lipectom* OR liposuct*) NEAR/5 (transplant* OR graft* OR

PART III

Regenerative components of stromal vascular fraction



12

Adipose tissue-derived extracellular matrix hydrogels as a release platform for secreted paracrine factors

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ABSTRACT

Introduction

Fat grafting is an established clinical intervention to promote tissue repair. The role of the fat's extracellular matrix (ECM) in regeneration is largely neglected. We investigated *in vitro* the use of human adipose tissue-derived ECM hydrogels as release platform for factors secreted by adipose derived stromal cells (ASC).

Material & methods

Lipoaspirates from non-diabetic and diabetic donors were decellularized. Finely powdered acellular ECM was evaluated for cell remainders and DNA content. Acellular ECM was digested and hydrogels were formed at 37 °C and their viscoelastic relaxation properties investigated. Release of ASC-released factors from hydrogels was immune-assessed and bio-activity was determined by fibroblast proliferation and migration and endothelial angiogenesis.

Results

Acellular ECM contained no detectable cell remainders and negligible DNA contents. Viscoelastic relaxation measurements yielded no data for diabetic-derived hydrogels due to gel instability. Hydrogels released several ASC-released factors concurrently in a sustained fashion. Functionally, released factors stimulated fibroblast proliferation and migration as well as angiogenesis. No difference between non-diabetic and diabetic hydrogels in release of factors was measured.

Conclusion

Adipose ECM hydrogels incubated with released factors by ASC are a promising new therapeutic modality to promote several important wound healing related processes by releasing factors in a controlled way.

INTRODUCTION

Autologous fat grafting has been widely investigated and used for soft-tissue defects and regenerative purposes such as the treatment of open wounds as well as anti-scarring treatments [1-4]. Diabetes is associated with dysfunctional wound healing and occurrence of non-healing ulcers. A significant proportion of diabetics do not respond to revascularization therapies which regularly results in amputation of the affected limb. Few non-controlled clinical trials and case reports have shown that diabetic wound healing is augmented by lipografting [1,2,5,6]. One of the key components of this regenerative potential of adipose tissue that dictate tissue regeneration, is the stromal vascular fraction (SVF), which is rich in adipose tissue-derived stromal cells (ASC). In the SVF, ASC are present as pericytes or periadventital vascular cells [7,8]. The clinical efficacy of SVF is often ascribed to these resident ASC or other precursor cells, but definitive proof still lacks. Another important but neglected contributor to regeneration is the extracellular matrix (ECM) which provides the tissue architecture and structural support as well as key biochemical signaling cues. The role of ECM in tissue regeneration is but poorly studied, if at all. ASC secrete a plethora of growth factors, cytokines, lipid-based mediators and matrix molecules such as structural components and matrix metalloproteases (MMPs) that remodel ECM. For instance, MMPs can degrade the excessively deposited ECM by myofibroblasts in scars, while vascular endothelial growth factors (VEGF) and fibroblast growth factors (FGF) promote wound healing [9,10]. The ECM in SVF supports the proliferation and survival as well as differentiation of cells by binding of their surface-expressed integrins to ECM molecules. Moreover, paracrine factors bind and are retained by proteoglycans and glycoaminoglycans of ECM. In this way, ECM stores paracrine factors and functions as a physiological controlled 'on-demand' release system [11,12].

Concentration of the SVF of adipose tissue *i.e.* to get rid it of the large amounts of adipocytes and thereby reducing volume, might augment the clinical observed regenerative potential compared to standard autologous fat grafting. To concentrate SVF, the SVF can be isolated enzymatically or mechanically [13]. Enzymatic isolation destructs the ECM and its interaction with cells and thus yields a suspension of single SVF cells (cellular SVF or cSVF). Via centrifugation, adipocytes are readily removed with enzymatic isolation [13]. When a non-enzymatic isolation procedure is used, adipocytes are mechanically destructed and the ECM is still intact as are its interactions with bound cells while it also acts for sustained release of bound factors (tissue-like SVF or tSVF) [12,13]. Therefore, we anticipate a non-enzymatically obtained SVF to have a higher therapeutic benefit than enzymatic SVF or unprocessed lipografts.

Systemic, often chronic, diseases such as diabetes mellitus (DM) impact the clinical efficacy of autologous SVF injections. For DM, long term hyperglycaemic exposure of ASC and ECM influences their biological properties [14-16]. High levels of glucose impair the regenerative function of ASC by intracellular reactive oxygen species (ROS) accumulation [17]. ROS inhibits the proliferation and proangiogenic capacities of ASC, which are important processes in wound healing [17]. Furthermore, high levels of glucose and their glycolytic products such as methylglyoxal result in glycation of proteins. These so-called advanced glycation end products (AGEs), bind to a receptor of AGE (RAGE) that is expressed by virtually all tissue cells, and causes pro-inflammatory activation. Inflammation is relevant for wound repair, but increased chronic inflammation acts adverse and results in decreased vascularization and tissue necrosis as observed in diabetic ulcers. Glycation affects ECM molecules rather than circulating proteins due to their slow turnover i.e. long half-life [18]. Elevated AGEs damage ECM potentially in three different critical sites: compromised cellular binding site in the ECM which inhibits cell adhesion, survival and proliferation. Secondly, molecular sites in the ECM that are involved in turnover (both proteolytic degradation and cross-linking) may be affected which may result in adverse ECM remodelling, accumulation, changes in mechanical features and associated changes of biological responses of bound tissue cells [14,19,20]. Affected subunits of ECM molecules lead to more crosslinks between proteins and thus an unwanted higher stability and stiffness of ECM proteins [14]. In soft tissues, increased stiffness promotes fibrotic processes in a feed-forward looped fashion.

For those reasons, non-diabetic SVF is likely to be more suitable than diabetic SVF as treatment for *e.g.* wound healing of diabetic ulcers in diabetic patients. This would, however, implicate an allogeneic tissue transplantation, which will result in an immunological rejection of the administrated SVF. We surmised that the SVF's biological activity is primarily contained within the ECM and its bound paracrine factors in which parenchymal and stromal cells no more than serve to continuously replenish this biological entity. In particular, for short(er) term applications such as wound healing, ECM loaded with paracrine factors might show therapeutic efficacy. ECM can be obtained by decellularization of (adipose) tissue. Literature is littered with a host of decellullarization protocols that often dictate the use of detergents such sodium dodecyl sulphate (SDS) as well as acids and bases to remove cellular constituents. This procedure not only removes all cell types but also the paracrine factors [21]. A decellularized extracellular matrix that is devoid of cells and paracrine factors is of reduced clinical value compared to mechanically derived SVF in which both ECM and paracrine factors are retained. Therefore, re-charging of ECM with factors *e.g.* released from cultured ASC might restore a comparable clinical effect as mechanically derived SVF while preserving the option for inter-patient administration.

In this study, we interrogated uptake and release of ASC-released paracrine factors by human adipose tissue-derived ECM hydrogels *in vitro*. The prepared adipose ECM hydrogels were first incubated with factors released by ASC *i.e.* conditioned culture medium and subsequently studied for wound healing purposes. Additionally, structural differences between diabetic and non-diabetic ECM regarding binding of factors was investigated.

MATERIAL & METHODS

Processing and decellularization of adipose tissue

Adipose tissue was harvested from the abdomen of non-diabetic (n=5) and diabetic (n=5) patients during regular liposuction procedures and processed anonymously using the FAT procedure, as previously described by van Dongen et al. [22]. Informed consent was obtained according to the local ethical committee of the University Medical Center of Groningen. Briefly, 50 ml tubes containing lipoaspirate were centrifuged at 960*xg* at room temperature (RT) for 2.5 min. Next, 10 ml of centrifuged adipose tissue was pushed forward and back thirty times through a Luer-to-Luer connector with three holes of 1.4 mm. The fragmented adipose tissue was again centrifuged at 960*xg* at RT for 2.5 min. This procedure resulted in 1 ml of tSVF, which was then decellularized, as previously described by Roehm et al. [23]. Briefly, 10 ml of tSVF was mixed with 30 ml of a 50% ethanol/water mixture and frozen at -80 °C and thawed at RT in 30 min. for four cycles. Then, tSVF was incubated in 0.05% trypsin/ 0.05 mM ethylenediaminetetraacetic acid (EDTA) (1:1 v/v) for 1.5h at 37 °C. Afterwards, samples were sonicated (70W) in 0.5% SDS at 46 °C for 20 min. Samples were then lyophilized and immersed in xylene (1:10 w/v) for 17 min. Next, samples were washed with 96% ethanol and incubated in DNAse solution (LS002007, Worthington) (containing a final concentration of 30 μg/ml DNAse in 1.3 mM $MgSO_4$ and 2 mM $CaCl_2$) for 24h. Finally, samples (acellular matrix) were lyophilized again and homogenized with the use of an UltraTurrax fragmer (PM Tamson Instruments) and stored at -80°C before used in experiments.

Histological characterization of acellular adipose matrix

One-time centrifuged adipose tissue and tSVF (controls) as well as acellular matrix of non-diabetic (NAM) and diabetic patients (DAM) (n=3) were formalin-fixed and embedded in paraffin. Four μm thickness sections were cut, deparaffinized, then incubated overnight with 0.1 M Tris/HCL buffer (pH 9.0) at 80 °C and stained with antibody against Perilipin A (1 : 200, Abcam) to visualize adipocytes as previously described.[22] Samples were visualized under a light microscope Leica Microsystems,

DM IL). A Masson's Trichrome staining and Hematoxylin & Eosin (H&E) staining were performed on deparaffinized four µm of slides. Then, samples were mounted and visualized under a light microscope (Leica Microsystems, DM IL).

DNA content measurement

NAM and DAM samples (n=3) were used to assess presence of DNA. A solution containing 5 µL proteinase K (2U/mg) (Sigma Aldrich, 3115828001), 50 µL 10% SDS and 500 µL SE-buffer (75 mM NaCl, 25 mM EDTA and pH 8.0) were added to 10 mg of acellular matrix. Solutions were mixed gently and incubated overnight at 55 °C. Then, 6M NaCl and chloroform were added, and samples were thoroughly mixed with the use of a top-over-top rotator for 30 min. Samples were centrifuged at 650*xg* at 20 °C for 10 min. Next, supernatant was taken and mixed with ice-cold isopropanol. Samples were centrifuged at 18,000*xg* at 4 °C for 5 min. Then, supernatant was discarded, and pellet was washed with 70% ethanol. Afterwards, pellet was dissolved in TE buffer (containing 10 mM Tris and 0.1 mM EDTA, pH 8.0). DNA was quantified with the use of a Nanodrop meter. Additionally, a DAPI/phosphate buffered saline (PBS) staining was used to stain nuclei. Samples were visualized under a fluorescence microscope (Leica Microsystems, DM IL).

Sulphated Glycosaminoglycans measurement

The concentration of sulphated glycosaminoglycans (sGAG) was measured with the use of a 1,9-dimethylmethylene blue (DMMB) assay according to the protocol of Farndale et al. [24]. NAM and DAM samples (n=3) were digested in 1% proteinase K/SE solution at 55 °C for 24h. As a control, a standard solution of 10 µgl/ml chondroitin sulphate C (#C4384-250 mg, Sigma-Aldrich, St Louis MO) was used. After the addition of the DMMB staining solution, extinction was measured at 525 and 595 nm. Additionally, an Alcian blue staining was used to visualize the total amount of glycosaminoglycans in non-diabetic and diabetic adipose tissue as well as tSVF and NAM and DAM samples. Samples were visualized under a light microscope (Leica Microsystems, DM IL).

Cell isolation, characterization and collection of conditioned medium of ASCs

Adipose tissue was harvested by normal liposuction procedures from healthy donors (n=3) used for cell isolation. Briefly, samples were washed with PBS three times. Then, 0.1% collagenase A/1% bovine serum albumin (BSA) in PBS was added as dissociation medium. The samples with 0.1% collagenase A/BSA in PBS were then stirred in a water bath at 37 °C for 1.5h. Next, cells were placed in lysis buffer on ice for 5 min. to disrupt all erythrocytes. Cells were then centrifuged and cultured at 37°C at 5% CO_2 in

humidified incubator in Dulbecco's Modified Eagle's Medium (DMEM) (BioWhittaker Walkersville, MD: 10% fetal bovine serum (FBS), 1% L-Glutamine (L-Glut), 1% Penicillin/Streptomycin (P/S)). Medium was refreshed twice a week. After culture, cells were characterized for ASC characteristics. Cells were characterized based on CD marker surface expression using flow cytometry (CD29, CD31, CD44, CD45, CD90 and CD105), adipogenic, osteogenic and smooth muscle cell differentiation capacity as well as colony formation capacity. Pooled non-diabetic ASC (n=3) of passage 4 – 6 were used to prepare ASC conditioned medium (ASC-CMe) (containing: DMEM, 1% L-Glut, 1% P/S) or RPMI 1% L-Glut, 1% P/S). Medium was collected and filtered through a 0.22 μm filter after 24h of culture. ASC characterization was confirmed in accordance with the International Federation of Adipose Therapeutics and Science (IFATS)/International Society of Cellular Therapy (ISCT) criteria [25].

Generation of hydrogels incubated with released factors by ASCs

NAM and DAM samples (n=3) were digested with 2 mg/ml of porcine pepsin (3,200 I.U. Sigma-Aldrich) in 0.01 M hydrochloric acid. 1 mg of porcine pepsin was used to digest 10 mg of lyophilized acellular matrix. Acellular matrix was digested under constant stirring for 6h at RT. Afterwards, the porcine pepsin was inactivated by pH neutralization (pH 7.4) with 0.1 M sodium hydroxide to reach 0.01M final concentration (Fig. 1). Then, ASC-CMe was mixed in order to allow released factors to bind to acellular matrix and finally, salt was added in order to allow for self-assembly gelation. To maintain the appropriate concentration of acellular matrix in hydrochloric acid to facilitate gelation, ASC-CMe and PBS were added in a concentrated form. ASC-CMe was concentrated with the use of 3 kDa cut-off Amicon® Ultra-Centrifugal filters (Sigma-Aldrich), respectively twenty-, forty- and eighty-times. Twenty-times, forty-times or eighty-times concentrated ASC-CMe was added and carefully mixed to reach final dilutions of respectively one-time (ASC-CMe1), two-times (ASC-CMe2) and four-times (ASC-CMe4) concentrated ASC-CMe (Fig. 1). The released factors bind to the acellular matrix pre-gel solutions at 4 °C for 24h. Next, the pre-gel solutions were brought to physiological conditions *i.e.* 1x PBS by adding twenty times concentrated PBS. Finally, the pre-gel solution was placed in an incubator to allow for self-assembly gelation for 1h at 37 °C (Fig. 1). Released factors from the incubated hydrogels by ASC-CMe1, ASC-CMe2, ASC-CMe4 were harvested in serum free medium after 24h and stored at -80 °C for immunoassaying. The respective concentrated ASC-CMe served as input controls (2.8). Released factors from the incubated hydrogel by ASC-CMe1 were harvested in serum free medium after respectively 24h, 48h and 96h and stored at -80 °C for biological assays. For fibroblast migration and endothelial angiogenesis, the respective concentrated ASC-CMe1 as well as DMEM serum free incubated hydrogels

harvested after 24h served as controls (2.9 and 2.11). For fibroblast proliferation, the mean cell proliferation of fibroblast after using the respective concentrated ASC-CMe was set at 100% (2.10). Moreover, hydrogels were not cytotoxic (data not shown).

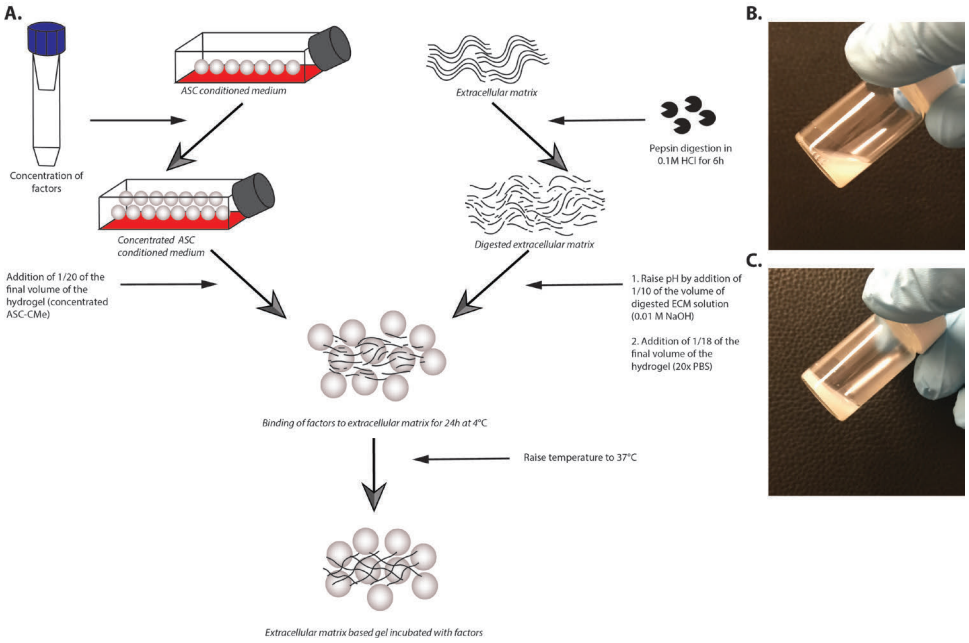

Figure 1. (A) Overview of the generation of ECM-derived hydrogels incubated with concentrated ASC-CMe. **(B)** Pre-gel solution prior to warming to 37°C. **(C)** Hydrogel after after gelation at 37°C. HCl = hydrogen chloride, NaOH = sodium hydroxide, ASC-CMe = adipose stromal cell conditioned medium, ECM = extracellular matrix, ASC = adipose stromal cell, PBS = phosphate buffered saline.

Characterization of viscoelastic relaxation properties of the hydrogels

NAM and DAM hydrogels (n=3) were formed in 0.8 mm polydimethylsiloxane rings and placed on a glass slide derived from three different pre-gel solutions per donor in duplicates (three technical replicates per donor) (Table 1). However, due to technical errors single data was obtained of pre-gel solution C of donor one and two. Then stress relaxation tests were performed on the NAM and DAM hydrogels with the help of a low-load compression tester in a non-hydrated environment at RT [26]. A stainless steel plunger with a diameter of 0.25 cm was lowered towards the hydrogels at 5 µm/s till the plunger came in contact with the gel (touch load defined as 10 mg). This position was recorded and similarly the position of the top of the glass slide was determined. Hydrogel

thickness was calculated with the difference of the two positions. Subsequently, the hydrogels were deformed by 20% and the deformation was held constant for 100s while the force response was monitored over time [27,28]. Force was converted in stress by dividing with the area of cross-section of the plunger, whereas the deformation was converted in strain (deformation/100). The slope of the straight-line plot between stress and strain during the deformation was taken as stiffness (*E*). Then the strain was held constant at 0.2 for 100 s and the stress was monitored. Since hydrogels are viscoelastic in nature the stress did not remain constant but decreased (relaxed) with time. Total relaxation in 100 seconds was also recorded. Relaxing stiffness (*E(t)*) was calculated by dividing relaxing stress with constant strain. The relaxation was understood in terms of a generalized Maxwell model by fitting the experimental data with the equation using the optimization routine in the solver Add-in of Microsoft Excel 2007. Fitting started with one Maxwell element and gradually more elements were added till the decrease in chi-squared (error function) value became insignificant. Each Maxwell element was characterized by the stiffness (E_i) and relaxation time constant (t_i) for which it remained active (Fig. 5 B, C, D). The stiffness values were converted into relative importance using the equation $RI_1=100*E_1/(E_1+E_2..... E_n)$.

Table 1. Nomenclature of NAM and DAM hydrogels derived from different pre-gel solutions.

	Donor 1	Donor 2	Donor 3
Pre-gel solution A	NAM1A/DAM1A	NAM2A/DAM2A	NAM3A/DAM3A
Pre-gel solution B	NAM1B/DAM1B	NAM2B/DAM2B	NAM3B/DAM3B
Pre-gel solution C	NAM1C/DAM1C	NAM2C/DAM2C	NAM3C/DAM3C

NAM = non-diabetic acellular matrix; DAM = diabetic acellular matrix

Immunoassay of ECM hydrogels

NAM and DAM hydrogels (n=3) were produced as previously described for the immunoassay (two technical replicates) (2.6). Multiplex immunoassaying (Luminex, R&D systems) was used to measure release of eleven representative ASC-released proteinaceous factors according to the manufacturer's protocol: vascular endothelial growth factor A (VEGF-A), angiopoetin-1 (Ang-1) and angiopoetin-2 (Ang-2), matrix metalloproteinase 1 (MMP-1), tissue inhibitors of metalloproteinase 1 (TIMP-1), interleukin-1β (IL-1β), IL-6, IL-8/CXCL8, fibroblast growth factor 1 (FGF-1), hepatocyte growth factor (HGF) and monocyte chemotactic protein 1 (MCP-1/CCL2)).

Fibroblast scratch assay

NAM and DAM hydrogels (n=3) were produced as previously described (three technical replicates) (2.6). PK84 fibroblasts were cultured in DMEM (containing 10% FBS, 1% L-Glut, 1% P/S). After confluency, PK84 fibroblasts were seeded into a 24-wells plate with a cell density of 17,500 cells/cm^2. Then, cells were serum-starved overnight. The next day, a scratch was made with the use of a 1000 µl pipette tip. Subsequently, cells were washed with PBS three times and all the different types of medium were applied. Scratches were analysed after 9h.

Fibroblast proliferation assay

NAM and DAM hydrogels (n=3) were produced as previously described (three technical replicates) (2.6). PK84 fibroblasts were cultured in DMEM (containing 10% FBS, 1% L-Glut, 1% P/S). After confluency, fibroblasts were seeded into a 96-wells plate with a cell density of 17,500 cells/cm^2. After 24h, the different types of medium were applied for 24h (2.6). After 24h, 20µl of MTT (5 mg/ml in PBS) was added and incubated for 3h at 37 °C. Next, medium was removed and 100 µl of dimethyl sulfoxide was added to dissolve the formazan crystals. Plates were read at 570 nm. The fibroblast proliferation assay was repeated twice. Results were expressed as percentage of cell proliferation compared to normal culture medium (DMEM containing 10% FBS, 1% L-Glut, 1% P/S).

Human umbilical vein endothelial cell sprouting assay

NAM and DAM hydrogels (n=3) were produced as previously described (three technical replicates) (2.6). Instead of DMEM serum free, RPMI serum free incubated hydrogels were used as a control (2.6). Human umbilical cord vein endothelial cells (HUVEC) were isolated and cultured in RPMI (containing 10% FBS, 1% L-Glut, 1% P/S). µ-Slide Angiogenesis plates (Ibidi GmbH, Germany) were coated with 10 µl of Matrigel™ (BD Bioscience, CA) and incubated at 37 °C for 2h. HUVEC were resuspended in all different types of medium. 60 µl of medium containing 10,000 cells were added per well. The slides were incubated at 37 °C for 6h. The sprouting networks that had formed were photographed with the use of an inverted light microscope (Leica Microsystems, DM IL) and the number of loops was counted manually.

Statistics

Scratch assay images were analysed using ImageJ, version 1.4.3.67 (NIH). Descriptive statistics were used to evaluate the amount of DNA, sGAG, stiffness and factor release profile of the hydrogels, scratch surface area, fibroblast proliferation as well as the number of loops. Data was expressed as mean with standard error of the mean, except

for the controls (serum free culture medium and ASC-CMe1) and stiffness as well as relaxation data of the hydrogels. Data of the controls as well as stiffness and relaxation of the hydrogels was expressed as mean with standard deviation. A one-tailed *t*-test was used to determine the statistical difference between the hydrogels and hydrogels *vs* controls with the use of Graphpad, version 7.0c (Graph Pad Software Inc., Los Angeles, CA).

RESULTS

Fractionation and decellularization of adipose tissue results in an acellular matrix

The fractionation of adipose tissue and its subsequent decellularization process resulted in a completely acellular adipose matrix. Macroscopically, acellular matrix was white of color, while one-time centrifuged adipose tissue and tSVF were yellow colored (Fig. 1, left panels, supplemental content). Microscopically, the H&E staining together with the absence of perilipin staining showed that the acellular matrix contained neither adipocytes nor other remaining cells. In contrast, the cell number in tSVF was higher as compared to one-time centrifuged adipose tissue as observed by dense clusters of stromal cells (Fig. 1, second series of panels, supplemental content). In accordance with our previous experience, fractionation of adipose tissue disrupted adipocytes while other cell types and extracellular matrix were preserved [22]. The absence of perilipin staining confirmed the disruption of adipocytes (Fig. 1, third series of panels, supplemental content). Furthermore, Masson's Trichrome staining demonstrated visibly higher amounts of collagen (blue) per unit area in acellular matrix as compared to one-time centrifuged adipose tissue and tSVF (Fig. 1, right panels, supplemental content).

Low amount of DNA left in acellular adipose matrix

NAM and DAM samples contained negligible amounts of DNA with 12.83 +/-2.62 ng/mg per dry weight tissue for NAM and 60 +/53.01 ng/mg per dry weight tissue for DAM ($p>0.05$) (Fig. 2A, supplemental content). The amount of DNA in DAM is, however, higher than the amount of DNA in NAM. All samples, except for one DAM sample, contained a lower amount of DNA than 50 ng/mg per dry weight tissue, which is the standard for successful decellularization. DAPI staining could not reveal nuclei in both NAM as well as DAM as compared to the high number of nuclei after the fractionation of adipose tissue procedure (Fig. 2B, supplemental content).

No difference in sulphated glycosaminoglycans

There was no difference in amount of sGAGs in NAM and DAM samples with 0.93 +/- 0.31 and 0.69 +/-0.31 µg sGAG per mg dry weight ECM respectively ($p>0.05$) (Fig. 3A, supplemental content). This was confirmed by Alcian blue staining of control adipose tissue, tSVF as well as ECM samples: here similar levels of staining were detected (Fig. 3B, supplemental content).

Viscoelastic relaxation properties of NAM hydrogels

Proteolytic treatment at room temperature of NAM and DAM liquified these matrices, which formed stable hydrogels upon warming to 37°C, albeit of relatively low mechanical strength. Viscoelastic relaxation properties were determined for all NAM and DAM hydrogels, however, all DAM hydrogels and one NAM gel collapsed during the measurements and yielded no data. This indicates that structural differences are present in ECM derived from diabetic donors in comparison with ECM derived from non-diabetic donors. Average stiffness for all measured NAM hydrogels was relatively low with 1.81 +/- 0.02 kPa (Fig. 2A). For each donor, three independent pre-gel solutions derived from the same donor and ECM isolation were produced by pepsin digestion (Table 1). Different pre-gel solutions showed a large intra-donor variation of stiffness (Fig. 2A). The large intra-donor variation of stiffness warrants standardization of the gelation procedure. The intra-donor variation was also present in the relaxation properties of the measured NAM hydrogels. Relaxation of stiffness showed a fast decrease with the stiffness reaching zero within about 20 seconds (Fig. 2B, C, D). This was typical for most of the replicates *i.e.* NAM1, -2 and -3 with each requiring 2 to 3 (Table 1, Fig. 2E). The fast decrease shows that adipose tissue-derived ECM hydrogels are much more viscous than elastic. A more viscous gel is less resistant to mechanical stress *i.e.* more prone to collapse and therefore less suitable for clinical applications which relate to movement and friction such as wound healing. Stress relaxation as a function of time was measured with the use of a Maxwell model with each element in this model having a spring constant related to the elastic part of the gel and a relaxation time constant representing a viscous part of the gel. Two or three elements were sufficient and the addition of more elements did not result in an improved quality of the fit. The first element having a high relative importance and a relaxation time constant of less than 1 sec. was most likely liquid that was pushed out of the gel (Fig. 2). The second element with a relaxation time constant between 1 and 10 sec, which was most likely ECM. Interestingly, in some hydrogels a third element was shown with a relaxation time constant between 10 and 100 sec. Hydrogels that present a third element were also the hydrogels with the highest stiffness and lowest relaxation.

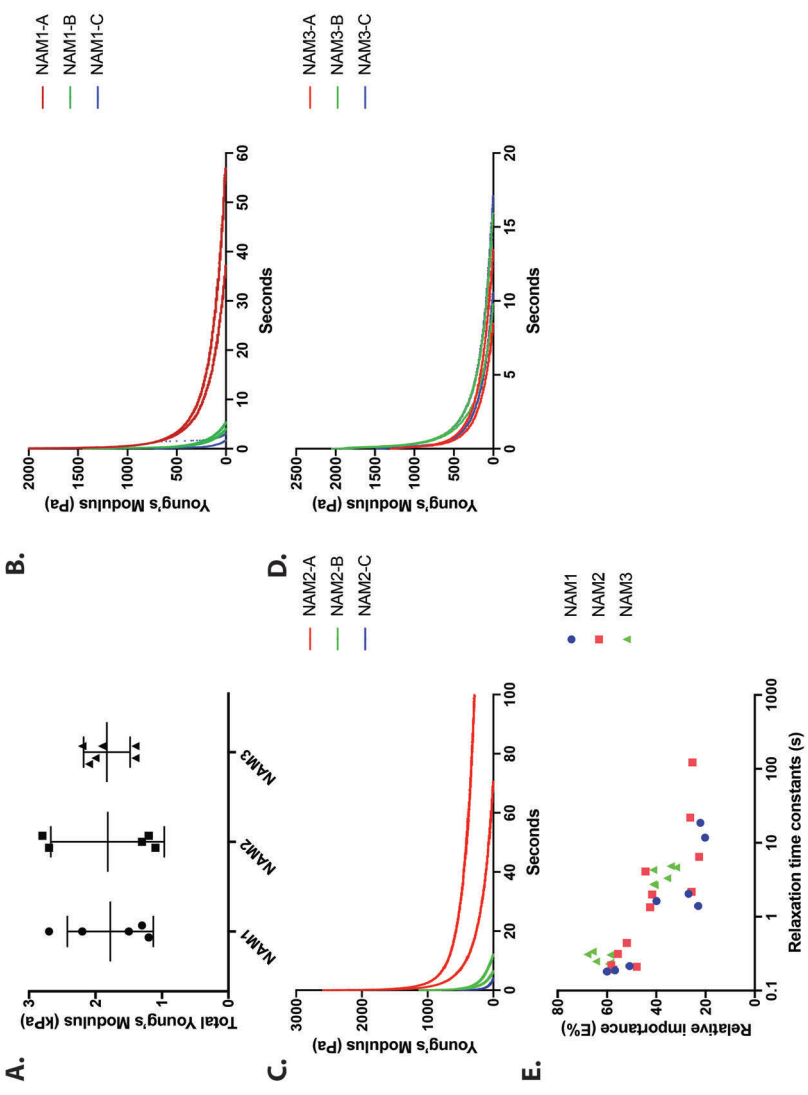

Figure 2. (A) The stiffness (kPa) for only NAM hydrogels (n=3). DAM hydrogels collapsed and could not be evaluated. **(B), (C), (D)** Decrease in stiffness of NAM hydrogels displayed as a function of time. All replicates of each donor (n=3) are presented. **(E)** Relative importance of individual Maxwell elements as a function of relative time constants in seconds for each replicate of all donors (n=3). Each point represents one Maxwell element. NAM = non-diabetic acellular matrix, DAM = diabetic acellular matrix, kPa = kiloPascal.

Hydrogels bind and release ASC-secreted factors

Hydrogels derived from NAM and DAM samples mixed with concentrated CMe from cultured ASC bind released factors from ASC and had released part of the measured factors after 24h. The concentrations of factors were lower in the conditioned medium derived from NAM and DAM samples in comparison with the three baseline concentrations of ASC-CMe for MMP-1, CXCL8 and IL-6. The difference in concentration of factors indicates that the release of bound factors extends well beyond 24h. For Ang-1, Ang-2 and FGF-1 a higher concentration in conditioned medium derived from hydrogels as compared to the baseline concentrations was found. This implies that the hydrogels release more factors than initially were mixed in with ASC-CMe. This indicates that there were still factors present in ECM after decellularization that were detectable by the immunoassay, albeit that their bioactivity might not be retained after the harsh decellularization procedures. There was no significant difference in release pattern of any of the measured factors between non-diabetic and diabetic-derived hydrogels ($p>0.05$) (Fig. 3). The concentrations of IL-1β and HGF were below the detection limit. In contrast, TIMP-1 concentrations exceeded the maximal detection limit and were therefore not plotted. These high concentrations of TIMP-1 indicate that most of the MMP-1 released by ASC and, therefore, released by the hydrogels is likely rendered inactive by binding of TIMP-1. Moreover, large interdonor variations in releasing patterns were observed, especially in diabetic derived hydrogels and mainly when two and four times concentrated ASC-CMe was mixed with the gels. This indicates a donor dependent binding capacity limitation of specific factors such as VEGF-A, Ang-1, Ang-2, MMP-1 and CCL2.

Similar PK84 fibroblast migration when treated with released factors by CMe-loaded NAM and DAM hydrogels

In comparison to serum free controls, conditioned medium from ASC (ASC-CMe1) promoted closure of damaged fibroblast monolayers (Fig. 4). Similarly, factors released from either NAM or DAM gels, promoted closure even after 96h of release (Fig. 4). A similar migration speed was detected between fibroblasts treated with factors from CMe-loaded NAM and DAM hydrogels released after 24h, 48h as well as 96h ($p>0.05$) (Fig. 4). However, in comparison to ASC-CMe1, factors from CMe-loaded DAM hydrogels released after 24h and 96h showed a smaller decrease in scratch surface area ($p<0.05$). For the NAM groups, all groups showed a larger decrease in scratch surface area in comparison to controls *i.e.* serum free culture medium ($p<0.05$). For the DAM group, released factors from CMe-loaded hydrogels after 48h and 96h in comparison with serum free controls showed a larger decrease in scratch surface area ($p<0.05$). This suggests that acellular adipose tissue matrix hydrogels released factors for at least 96h as observed by stimulated migration of fibroblasts.

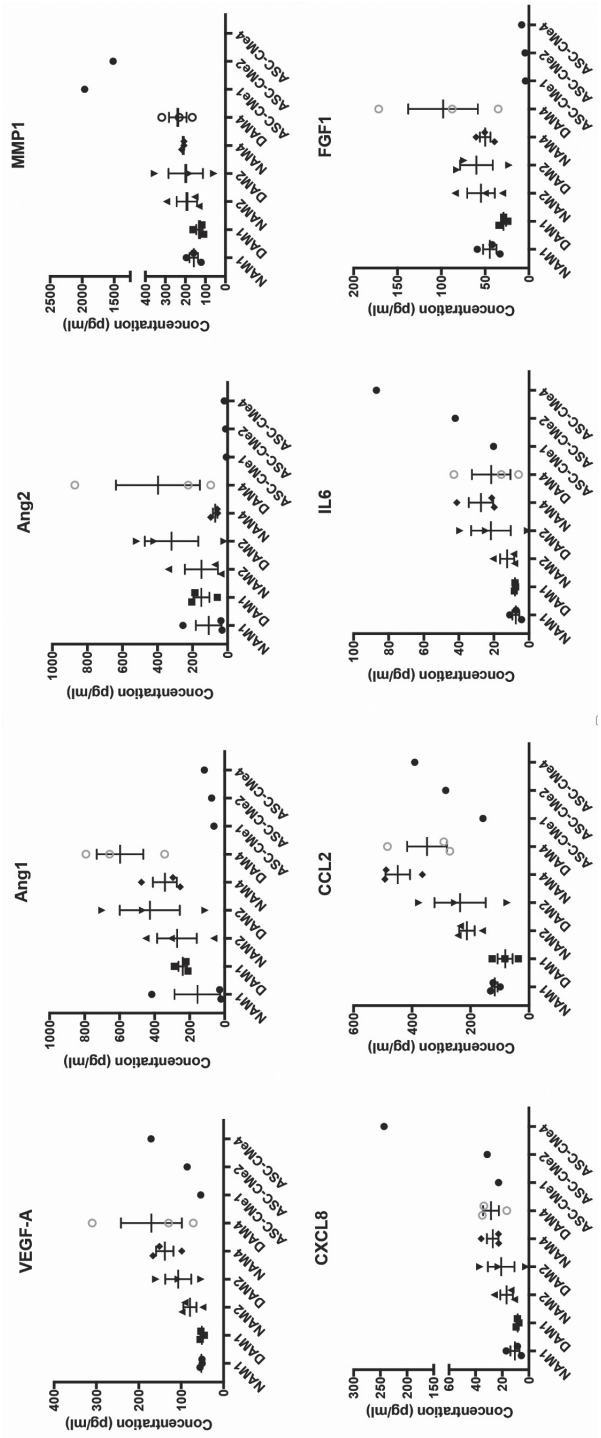

Figure 3. Statistical analyses of the concentration of released factors in baseline ASC-CMe and conditioned medium derived from NAM and DAM samples (n=3). ASC-CMe = adipose derived stromal cell conditioned medium. NAM = non-diabetic acellular matrix, DAM = diabetic acellular matrix. One, two and four displays the concentration factor of the factors in ASC-CMe as well as the concentration of the hydrogel released factors injected in NAM and DAM samples. The concentration of MMP1 exceeded the upper limit of the assay in ASC-CMe4 and was excluded. Results are expressed as mean with standard error of the mean.

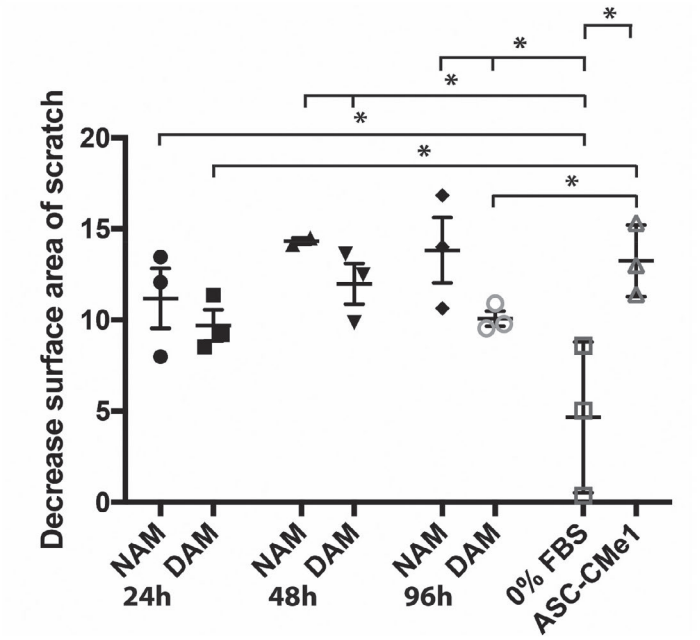

Figure 4. Statistical analyses of changes of the scratch surface area (9h) compared to controls (serum free culture medium and ASC-CMe1) for conditioned medium derived from NAM and DAM hydrogels (n=3) with released factors after 24h, 48h and 96h. NAM = non-diabetic acellular matrix, DAM = diabetic acellular matrix, ASC-CMe1 = one time concentrated (undiluted) adipose derived stromal cell conditioned medium, FBS = fetal bovine serum. *Significant smaller scratch surface area visible after the use of NAM24h, NAM48h, NAM96h as well as DAM48h and DAM96h in comparison with serum free culture medium ($p<0.05$). Moreover, the use of ASC-CMe1 reduced the scratch area in comparison to serum free culture medium ($p<0.05$). Significant smaller scratch area visible after the use of ASC-CMe1 in comparison with DAM24h and DAM96h ($p<0.05$). Results are expressed as mean with standard error of the mean for NAM and DAM hydrogels. Results are expressed as mean with standard deviation for serum free culture medium and ASC-CMe1.

Proliferation of PK84 fibroblasts is increased with released factors by CMe-loaded DAM hydrogels

Factors released from both NAM and DAM gels promoted proliferation for 96h (Fig. 5). Proliferation was higher in the DAM group as compared to the NAM group with factors being released for 24h and 48h (respectively $p<0.01$, $p<0.05$) (Fig. 5). Furthermore, the proliferation was increased in the NAM group with released factors after 24h *vs* 48h ($p<0.001$) as well as after 24h *vs* 96h ($p<0.05$) and was higher in the DAM group after 24h *vs* 96h ($p<0.01$).

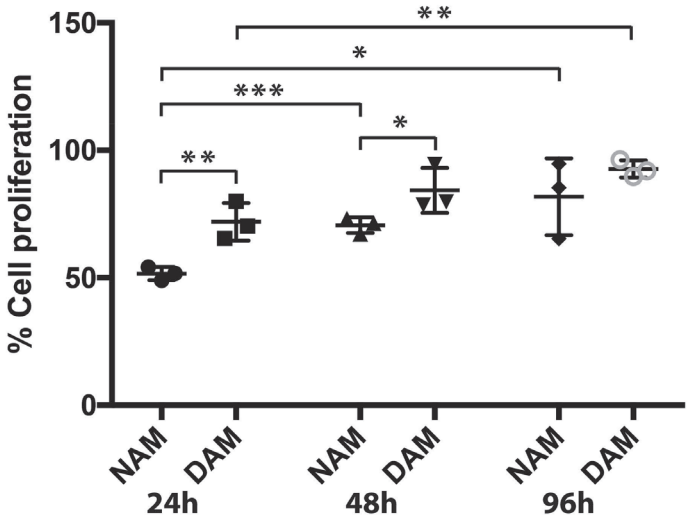

Figure 5. Statistical analyses of fraction of proliferating cells compared to ASC-CMe for conditioned medium derived from NAM and DAM hydrogels (n=3) with released factors after 24h, 48h and 96h. NAM = non-diabetic acellular matrix, DAM = diabetic acellular matrix, ASC-CMe1 = one time concentrated (undiluted) adipose derived stromal cell conditioned medium. *Significantly more proliferation occurred between NAM24h *vs* NAM96h and NAM48h *vs* DAM48h ($p<0.05$). **Significantly more proliferation occurred between NAM24h *vs* DAM24h and DAM24h *vs* DAM96h ($p<0.01$). ***Significantly more proliferation occurred between NAM24h *vs* NAM48h ($p<0.001$). Results are expressed as mean with standard error of the mean for NAM and DAM hydrogels.

Similar sprouting ability of HUVEC when treated with released factors by CMe-loaded NAM and DAM hydrogels

Sprouting of HUVEC was similar after treatment with factors released from CMe loaded NAM hydrogels compared to DAM hydrogels ($p>0.05$) (Fig. 6). Moreover, a comparison of the total number of loops between different times *i.e.* 24h, 48h and 96h of released factors was similar for both types of hydrogels ($p>0.05$) (Fig. 6). For the NAM groups, released factors from CMe-loaded hydrogels after 24h as well as 96h in comparison with serum free controls showed more loops ($p<0.05$). Unexpectedly, no significant difference in the group of released factors from CMe loaded NAM hydrogels after 48h compared with serum free medium was found. This is probably caused by large interdonor variation, because the mean number of loops is higher in the group of released factors from CMe loaded NAM hydrogels after 48h. Moreover, these results indicate that CMe loaded hydrogels function as a controlled slow release scaffold of factors that stimulate sprouting even after 96h. For the DAM group,

released factors from CMe-loaded hydrogels after 24h as well as 48h in comparison with serum free culture medium showed more loops ($p<0.05$). The mean number of loops for ASC-CMe1 was comparable with the mean number of loops for the NAM and DAM hydrogels ($p>0.05$).

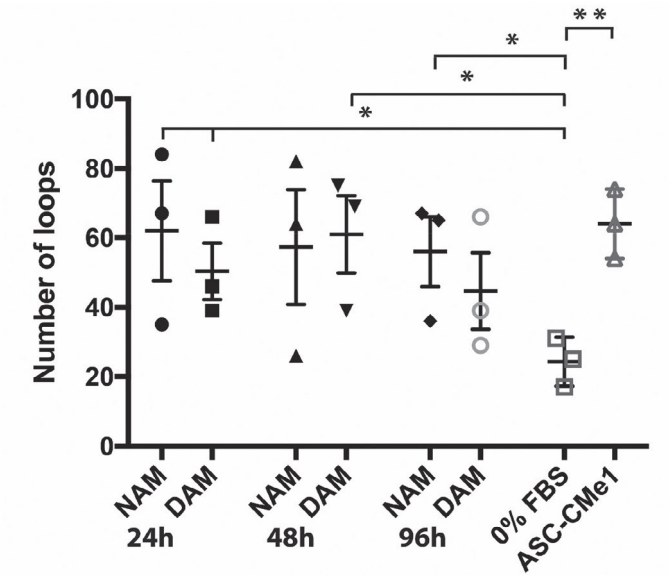

Figure 6. Statistical analyses of the number of loops of the 6h sprouting assay compared to controls (serum free culture medium and ASC-CMe1) for conditioned medium derived from NAM and DAM hydrogels (=3) with released factors after 24h, 48h and 96h. NAM = non-diabetic acellular matrix, DAM = diabetic acellular matrix, ASC-CMe1 = one time concentrated (undiluted) adipose derived stromal cell conditioned medium, FBS = fetal bovine serum. *Significantly more loops visible after the use of NAM24h, NAM96h, DAM24h as well as DAM48h in comparison with serum free culture medium ($p<0.05$). **Significantly more loops visible after the use of ASC-CMe1 in comparison with serum free culture medium ($p<0.01$). Results are expressed as mean with standard error of the mean for NAM and DAM hydrogels. Results are expressed as mean with standard deviation for serum free culture medium and ASC-CMe1.

DISCUSSION

In this study, we demonstrated that acellular ECM hydrogels from decellularized adipose tissue incubated with factors released by ASC functioned as a controlled slow release scaffold. These ECM hydrogels bound a series of different factors which were released in an incremental fashion for at least 96h and maintained their biological activity. These ECM hydrogels showed to be non-cytotoxic as well. Diabetic origin of

the hydrogels did not substantially affect the biological activity nor concentrations of the released factors; small differences in kinetics of biological assays were seen for unknown reasons. Yet, diabetic ECM derived hydrogels had a too low mechanical strength and are therefore less suitable for clinical applications. Thus, CMe incubated non-diabetic derived hydrogels seems to be a promising new treatment modality to augment wound healing.

The released factors by ASC-CMe-loaded ECM hydrogels stimulated several important wound healing related processes including an increased fibroblast proliferation and migration as well as sprouting by HUVECs *i.e.* surrogate angiogenesis. Diabetic origin did not affect either of these processes, except that cell proliferation of fibroblasts was reduced 24h and 48h after release of factors by non-diabetic hydrogels in comparison with diabetic hydrogels. Yet, the immunoassay showed similar concentrations of factors released by both types of hydrogels. This suggests that the releasing pattern of some factors might be different when released by hydrogels of diabetic origin *e.g.* a faster release. A faster release of factors results, however, in a faster depletion of factors in ASC-CMe-loaded hydrogels and thus a shorter time to promote wound healing. Moreover, factors will be actively subtracted from the hydrogel *in vivo* instead of accumulation of factors until a concentration balance is reached between inside and outside the hydrogel. Additionally, fibroblast cell proliferation increased over time after using released factors in an incremental fashion up to 96h.

Wound healing comprises different processes that show spatiotemporal overlapping phases with different processes of which some *e.g.* angiogenesis, fibroblast proliferation and migration are investigated in this study. Our results suggest that released factors from ASC-CMe loaded hydrogels influence both the course and kinetics of these phases. These human ECM-derived hydrogels allow the allogenic administration because both factors released by ASC as well as ECM will not induce an adverse immune reaction. In fact, ECM components are evolutionary highly conserved which warrants the use of animal-derived ECM hydrogels *e.g.* pig dermal ECM hydrogels. The potential therapeutic value of this 'one-donor-for-all' treatment modality for wound healing purposes is based on biological properties as well as physical properties. Biologically, a plethora of factors can be bound and released by ECM. In this way, wound healing can be enhanced by influencing processes like angiogenesis, apoptosis, chemoattraction of immune cells and support of local mesenchymal or progenitor cells [29]. Angiogenesis is enhanced by VEGF and FGF1, especially under hypoxia [30]. ASC-CMe, which contains VEGF and FGF1, increases endothelial proliferation and suppresses cell apoptosis under hypoxia. Both proliferation of endothelial cells and survival are important mechanisms in wound healing [31]. Additionally, VEGF also functions as a chemoattractant for local progenitor cells as well as macrophages [32]. Macrophages, attracted by chemokines

such as ASC-released CXCL8, play an important role in the different phases of wound healing [33]. During the inflammation phase, macrophages phagocytose pathogens and cellular debris. During the proliferation phase, epithelial proliferation is enhanced by activated macrophages [33]. Furthermore, re-epithelialization by keratinocyte proliferation and migration is also stimulated by CXCL8, which was bound and released by our ECM hydrogels [29]. Besides increased re-epithelialization, CXCL8 is also responsible for the attraction of leukocytes which results in an increased phagocytosis during the inflammation phase [34]. The presence of leukocytes and macrophages are prolonged by the presence of MCP1, as MCP1 is a chemoattractant for macrophages, mast cells and T-cells [29]. A prolonged presence of leukocytes and macrophages result in a sustained pro-inflammatory state of the wound [35]. After the inflammation phase, a decrease in inflammatory signaling is needed to induce formation of granulation tissue and subsequently re-epithelialization. *In vitro* and *in vivo* experiments have shown that GAG-binding sites are able to modulate chemokine gradients of MCP1 and IL8 by subduction in excisional wounds. [36] In this way, ECM hydrogels incubated with ASC-CMe can modulate inflammation in wounds by both releasing and attracting growth factors and chemokines.

Besides the biological function of our studied hydrogel, its physical properties are also of crucial importance. Physical properties of the hydrogel such as stiffness affect ASC and other cells present in the surrounding tissue of the wound. Low stiffness hydrogels promote adipogenic differentiation of native ASC, while stiff hydrogels promote their osteogenic differentiation [37]. The soft non-diabetic hydrogels support adipogenic differentiation of host tissues ASC, however, the bound factors together with the stiffness of the hydrogel dictate final cell fate [37]. A soft hydrogel is also easier infiltrated by host cells like ASC and endothelial cells than stiff hydrogels. In this way, both ASC and endothelial cells can stimulate angiogenesis inside the hydrogel which might result in increased wound healing rates [38]. It remains to be determined whether the stability and longevity of these hydrogels supports cells' maintenance, migration and differentiation. Also, it is unknown if the hydrogels with a low stability can resist all mechanical stress during application of the hydrogel *in vivo*. The enzymatic degradation to convert complex ECM structures into gels has inspired physical chemists to compile prediction models [39,40]. However, from a pragmatic point of view, production of (pre)gels from freeze-dried, fine-powdered ECMs, requires careful standardization of enzyme treatment. These should consider *e.g.* the disease background of the ECM, because our results show differences between diabetic and non-diabetic ECM hydrogels *i.e.* gels of diabetic origin collapse. Regarding future application, diabetic hydrogels will not suffice due to their limited physical strength. As a matter of fact, viscoelasticity measurements proved impossible because diabetic hydrogels collapsed readily. This indicates the presence of structural differences in matrix between non-diabetic and diabetic donors. This difference could be caused by accumulation of AGEs in ECM

which results in more crosslinks between proteins and therefore a high stability of the ECM proteins [14]. The major component of ECM is collagen type I and glucose-based AGEs may cause a high level of intramolecular Lys-Arg and Lys-Lys crosslinks that increase the molecule's stiffness [41]. The glycation and crosslinking masks potential pepsin digestion sites and thus yields different, likely larger, fragments upon pepsin treatment. As it appeared these fragments did not yield hydrogels from diabetic ECM. Instead of adipose tissue, other sources of matrices with a higher stiffness might also suit to enhance wound healing. Our recent data show that ECM hydrogels from heart or aorta tissue have a higher viscoelasticity (to be published elsewhere).

To date, most studies that evaluate decellularized adipose tissue focus on tissue engineering of a new subcutaneous fat layer rather than its use to augment wound healing [42-46]. Moreover, all these studies used adipose matrix derived hydrogels without incubation of paracrine factors. Without the use of additional paracrine factors and cytokines, the soft adipose matrix will most likely only regenerate a soft subcutaneous fat layer. Yet, to regenerate a dermal layer for wound healing purposes, the addition of factors released by ASC is warranted. A combination of factors released by ASC and a soft adipose matrix derived hydrogel might, therefore, be an ideal treatment modality for full thickness wounds.

Both types of gel released functional factors over a longer time. However, a large inter donor variation remains regardless the origin of the ECM. The inter donor variation might be caused by the fact that ECM derived from different donors with different 'pack years' of diabetes. In this way, the amount of AGEs accumulation is different between donors. Moreover, liposuction procedures are performed on lean patients and patients with obesity. However, obese patients frequently acquire diabetes mellitus type 2 with high levels of glucose prior to diagnosis. It is feasible that ECM derived from non-diabetic donors was exposed to high levels of glucose already. Finally, the medical history of the anonymous donations was unknown. We cannot exclude the influence of age, gender, BMI and other confounding factors on the donor variation [47-50]. This warrant to standardize the generation of factor-loaded ECM hydrogels with regard to both producing ASC as well as to source of the ECM. The low yield of adipose-derived ECM (w/v base) hampers clinical application of adipose tissue-derived hydrogels.

In summary, this study shows that several important wound healing related processes *i.e.* angiogenesis, fibroblast migration and proliferation are stimulated by a sustained release of growth factors from ECM-derived hydrogels. Therefore, the use adipose tissue ECM-derived hydrogels incubated with released factors from ASCs become a promising new treatment modality to augment disturbed dermal wound healing *e.g.* diabetic ulcers. Animal studies are warranted as an essential prelude to clinical trials.

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SUPPLEMENTAL CONTENT

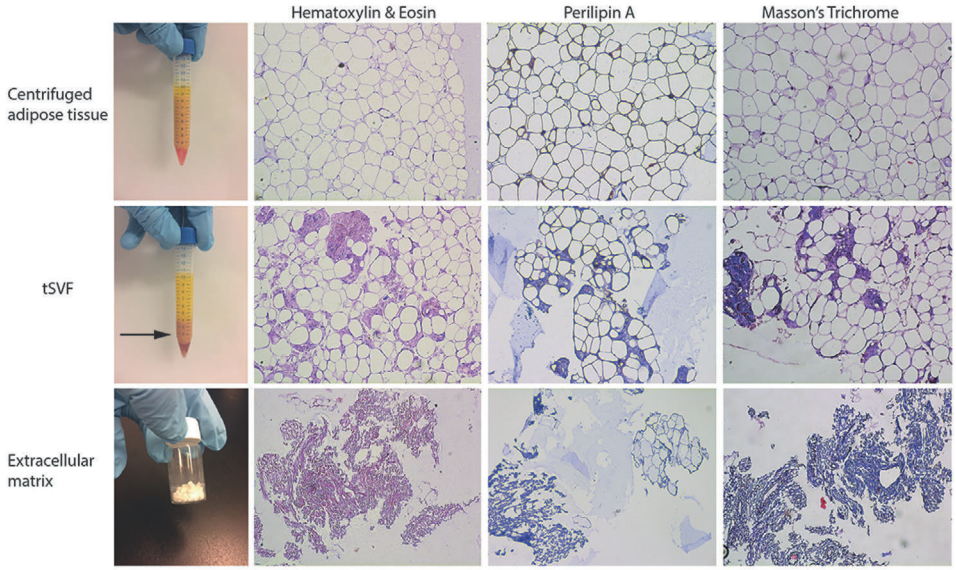

Figure 1. Light micrographs of hematoxylin & eosin staining, perilipin A staining and Masson's trichrome staining of respectively centrifuged adipose tissue, tSVF and extracellular matrix. tSVF = tissue stromal vascular fraction.

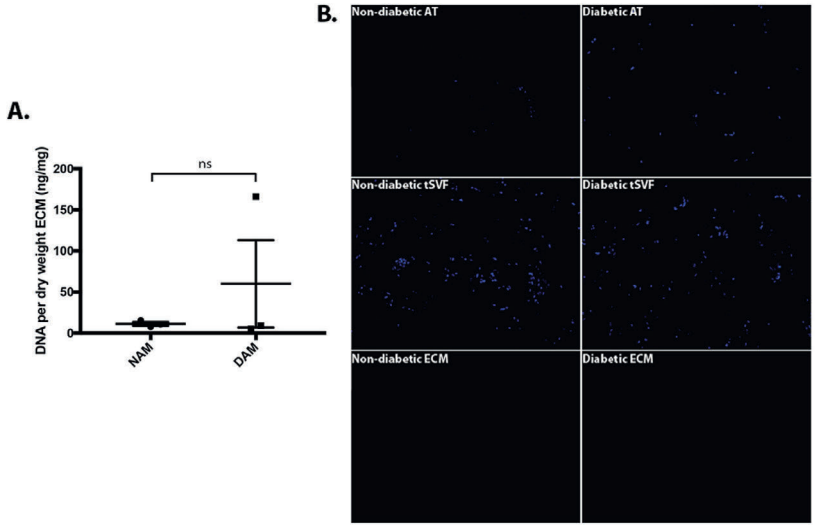

Figure 2. (A) Statistical analyses of DNA contents per dry weight ECM (ng/mg) of NAM and DAM samples (n=3). **(B)** Immunofluorescent microscope photographs of DAPI staining. ECM = extracellular matrix, NAM = non-diabetic acellular matrix, DAM = diabetic acellular matrix, AT = adipose tissue, tSVF = tissue stromal vascular fraction, NS = non-significant. Results are expressed as mean with standard error of the mean.

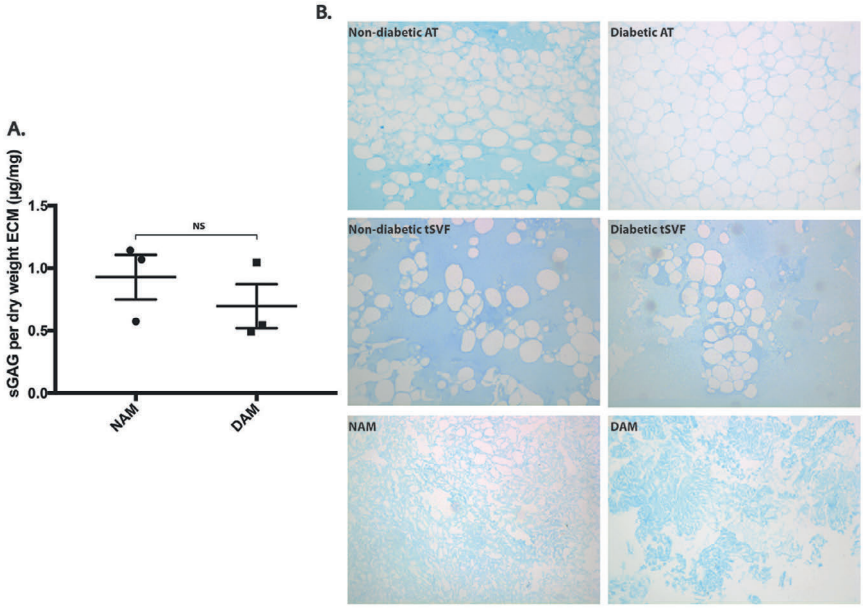

Figure 3. (A) Statistical analyses of sGAG contents per dry weight ECM (µg/mg) of NAM and DAM samples (n=3). **(B)** Light microscope photographs of alcian blue staining. sGAG = sulphated glycosaminoglycan, ECM = extracellular matrix, NAM = non-diabetic acellular matrix, DAM = diabetic acellular matrix, AT = adipose tissue, tSVF = tissue stromal vascular fraction, NS = non-significant. Results are expressed as mean with standard error of the mean.

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Adipose tissue-derived ECM hydrogels and their use as 3D culture scaffold

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ABSTRACT

Adipose tissue has therapeutic capacity in the form of a fat graft *e.g.* for treatment of irradiation-induced scars and difficult to heal dermal wounds. For large-scale clinical application, an off-the-shelf product is warranted. In recent years, ECM-derived hydrogels are postulated to harbour therapeutic capacity and might even replicate the beneficial effects of adipose tissue. In normal homeostasis, the natural ECM acts as a deposit of growth factors, that releases them over time. In the healing of lesions, this might promote cell accumulation and proliferation which in turns stimulate angiogenesis and repair. The decellularization of tissue and generation of hydrogels may leave cytotoxic traces. Therefore, our research assessed the cytotoxic effect of human adipose tissue-derived ECM hydrogels on connective tissue cells i.e. fibroblasts. The results showed no cytotoxicity, meaning the hydrogels caused no cell death. Cell migration and survival were observed when cultured in ECM hydrogels and followed for 7 days. Cell survival in the hydrogel was confirmed with CFDA staining and also cells showed the ability to penetrate and migrate throughout the gel. We conclude that ECM hydrogels are promising to use as innovative therapy for wound healing.

INTRODUCTION

Adipose tissue has a beneficial role in wound healing.[1,2] Yet the underlying pathways are not completely understood. This renders adipose tissue a novel, intriguing and exciting territory in science, especially in the field of wound healing.

Lipografting is used in reconstructive- and plastic surgery to restore loss of volume after breast surgery or to treat burn wounds and augment wound healing.[3,4] Short term results of lipografting are good, however, on the long-term; graft take survival is variable ranging from 20-90% loss of volume after injection.[1] In this way, the clinical efficacy of lipografting is only temporary. To improve tissue grafting, scientists implemented a technique where they combined adipose tissue-derived stromal cells (ASC) with fat tissue.[5]

The adipose tissue comprises parenchyma (adipocytes) and stroma. Stroma comprises vasculature (endothelial and smooth muscle cells as well as pericytes, adipose tissue-derived stromal/stem cells (ASCs) and fibroblasts embedded and held together by the extracellular matrix (ECM). The stroma is also known as the stromal vascular fraction (SVF). In general, most studies focus on ASCs as optimal candidates for repair of tissue damage or to facilitate angiogenesis, for example in orthopaedics and cardiology.[6-9] The secretome of ASCs stimulates angiogenesis which augments local tissue perfusion. This paracrine action of ASCs comprises secretion of pro-regenerative factors such as VEGF, FGF, and HGF. In addition, ASCs contribute to repair via differentiation into parenchymal or stromal cells, such as smooth muscle cells, pericytes, fibroblasts, adipocytes or osteoblasts, depending on the damaged tissue.[8] We have shown that ASC-derived pericytes both promote and stabilize vascularization in vitro and in vivo in a NOTCH2-dependent fashion.[10,11] In these studies we also cultured ASCs in 3D matrices of commercial extracellular matrix i.e. Matrigel®.

In the last 5 years, there is a new, promising player arising in this field: the stromal vascular fraction.[12-14] Mechanical isolation of SVF is faster and easier than the isolation and culture of ASCs.[15] SVF is shown to improve wound healing rates faster compared to the injection of ASCs alone because it contains multiple cell types including vasculature, fibroblasts and ECM, which are important in the wound healing process. Moreover, ECM serves as a slow growth factor reservoir to which cells are able to attach to and can be supported by in growth and proliferation.

In the past years, ECM has been studied increasingly and different regenerative therapies, with the use of ECM, are developing in various medical fields.[16] ECM

comprises the non-cellular component of the tissue, occupies the space between cells and functions as a scaffold to give structural support to cells. ECM is composed of extracellular macromolecules secreted by professional connective tissue cells such as fibroblasts. The two main classes of macromolecules in ECM are 1) strongly negatively charged polysaccharides such as glycosaminoglycans (GAGs) and proteoglycans (PGs) that embed the 2) proteinaceous fraction of ECM such as collagens. Collagen is the most abundant protein; and comprises about 30% of the tissue mass (percentage various within different tissues types) and there are 28 different types of collagens. PGs can either stimulate or inhibit angiogenesis in a context-dependent manner. By virtue of their charge, PGs and GAGs are very hydrophilic molecules that regulate tissue volume by water retention which makes ECM in fact a natural hydrogel.[17] Moreover, ECM serves as a slow release reservoir of growth factors that regulate behaviour of proximal cells. This ranges from suppression of apoptosis by joint action ECM molecules and growth factors to proliferation, differenation and migration. Growth factor binding the GAGs in the ECM depends on the presence of the so-called aminoterminal heparin-binding domain but also plain electrostatic or Van der Waals interactions may keep soluble factors bound to ECM.

A manner to implement the extracellular matrix as a treatment or bioengineering tool is to produce ECM hydrogels. Various human or porcine tissues qualify to produce organ-specific ECM hydrogels including heart[18,19], lung[20], pancreas[21], skin[22], and adipose tissue.[23] We surmise that these ECM hydrogels may act to retains growth factors with the benefit that these are released with distinct kinetics over time. The released growth factors are able to induce angiogenesis by stimulation of endothelial cells to form new blood vessels which in turn stimulates tissue regeneration resulting in wound healing.[24] Wound healing is especially impaired in diabetic patients because of decreased peripheral blood flow leading to diabetic ulcers formation. Diabetes affects ASCs and SVF, therefore, clinical use of an ECM-derived hydrogel from healthy donors is more suitable for patients suffering from diabetes mellitus.

ECM hydrogels may also be applicable for 3D culturing, 3D bioprinting and in vivo tissue regeneration.[25] These can be in a donor-independent fashion and over a species barrier while SVF which can be used only as an allogeneic transplant otherwise immunological rejection would occur. More preliminary studies are needed before ECM hydrogel can enter clinical trials. The decellularization of adipose tissue precedes the generation of ECM hydrogels, the latter is done by mild digestion under low pH with pepsin. In particular, decellularization involves multiple steps with harsh chemicals such as detergents that are incompatible with survival of cells. Therefore,

the aim of this research was to assess the influence of human adipose tissue-derived ECM hydrogels on viability, proliferation and migration of therapeutic cells *i.e.* ASCs and fibroblasts.

MATERIALS AND METHODS

Fractionation of adipose tissue procedure

The fractionation of adipose tissue (FAT) procedure was performed as previously described.[15] Briefly, adipose tissue was harvested during normal liposuction procedures and transferred cooled from the operation room. Informed consent was obtained according to the local ethical committee of the University Medical Center of Groningen. For the FAT procedure, lipoaspirates were warmed to room temperature (RT) and divided into 50 ml tubes. Then, lipoaspirate was centrifuged at 956xg for 3 min. at RT to separate adipose tissue into three layers: oil, adipose tissue and infiltration fluid. The oily fractions as well as the infiltration fluid were discarded. Next, the centrifuged adipose tissue was placed in a 10 ml syringe and connected to the fractionator (a luer to luer connector with three holes of 1.4 mm inside). An empty 10 ml syringe was connected on the other side of the fractionator. Adipose tissue was pushed 30 times forwards and back and the adipocytes were mechanically disrupted. Finally, adipose tissue was centrifuged again at 956xg at RT for 3 min. The second round of centrifugation yielded four fractions: oily fraction, SVF and infiltration fluid containing a small pellet. SVF was collected and washed with phosphate-buffered saline (PBS) and stored at -20ºC until further use (Fig. 1).

Decellularization of stromal vascular fraction

SVF was frozen with 50% ethanol/water in a -80ºC fridge for 2h and thawed for 30-60 min. for four cycles.[26] After thawing, the 50% ethanol/water mixture was replaced every time. Next, the SVF was incubated with 0.05% trypsin/ 0.05 mM ethylenediaminetetraacetic acid (EDTA) (1:1 v/v) under constant stirring (Bambino machine) at 37ºC for 90 min. Then, samples were washed with phosphate buffered saline (PBS) and sonicated (70W) with 0.5% sodium dodecyl sulphate (SDS) at 46ºC for 20 min. Samples were centrifuged and washed with PBS to completely remove SDS. Finally, samples were lyophilized and subsequently immersed in xylene and placed on a rolling bench for 17 min. Afterwards, samples were washed with PBS and subsequently washed with 100% ethanol until the solution became clear. Samples were incubated with DNAse solution (1:1 v/v) (LS002007, Worthington, final concentration of 30 µg/ml DNAse in 1.3 mM MgSO4 and 2 mM CaCl2) overnight at 37ºC. Next day, samples

were washed with PBS and again lyophilized. Finally, samples were ground to a fine powder with an UltraTurrax device (PM Tamson Instruments) and stored at -80°C until further use (Fig. 1).

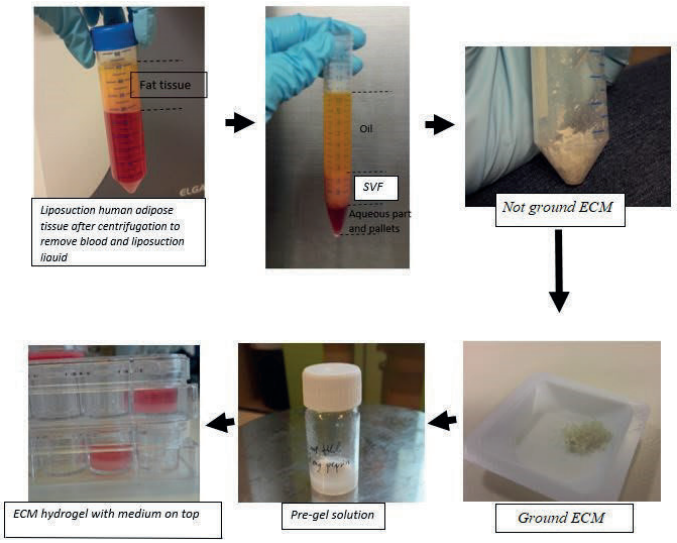

Figure 1. Overview of preparation method of an ECM hydrogel from human adipose tissue. SVF = stromal vascular fraction; ECM = extracellular matrix.

Gelation of decellularized adipose derived extracellular matrix

ECM (20 mg) was mixed with porcine pepsin powder (2 mg, 3,200 I.U. Sigma-Aldrich) in 1 ml 0.01 M hydrochloric acid (HCl). ECM was digested under constant stirring on a magnetic stirring device at 500 rpm at RT for 6h. Afterwards, pH was raised to 7.4 to neutralize the pepsin with 100 µl of 0.1 M sodium hydroxide (NaOH) to reach a final concentration of 0.01 M NaOH. The pre-gel solution was buffered with 110 µl of 10x PBS to reach a final concentration of 1x PBS. Then, the pre-gel solution was mixed well and incubated for one hour at 37˚C to allow for gelation (Fig.1).

Immortalization and lentiviral tagging of ASCs

Cultured human ASCs (pool of five donors, 1 million at passage 4) were transfected with 1µg pMC1neo-polyA (Stratagene) which is a plasmid encoding the large T antigen of SV40 with neomycin as selectable marker.[27,28] At 48h post-transfection, transfected cells were selected by adding 250 µg/ml geneticin (G418) to the medium. After approximately three weeks, colonies that remained were picked, subcultured and

propagated in medium with 250 μg/ml G418 for a second round of selection. Stable cell lines were propagated and stored in liquid nitrogen. For the current study, clone iADSC13 was used and characterized. Tagging with CMV promoter-driven reporter genes respectively EGFP (green fluorescence) or dTomato (red fluorescence) was with third generation VSV-pseudotyped replication-deficient lentiviruses. Up to three rounds of transduction were done to increase the fraction of reporter-expressing ASCs. These were named iADSC13EGFP and iADSC13dTomato respectively. Additionally, transfected cells were FACS-sorted, propagated and cryopreserved in liquid nitrogen. Between 80 and 95% of the sorted ASCs showed reporter expression that was detectable with a fluorescence inversion microscope, which sufficed for the experiments.

Characterization and differentiation potential of immortalized ASC

Flow cytometry

Immortalized ASC, lentivirally tagged with either EGFP or dTomato, were analysed using low cytometry (FACS) for CD surface marker expression. Cells were stained with the following anti-human monoclonal antibodies: CD31- phycoerythrine/cyanine7 (Pe/Cy7; eBioscience, Vienna, Austria), CD45-fluorescein isothiocyanate (FITC; IQ Products) and CD90-allophycocyanin (APC; BD Bioscience, San Jose, CA). CD29-APC (eBiosience), CD44-FITC (BD Bioscience) and CD105-Pe/Cy7 (eBiosience). For controls we used the following monoclonal antibodies: Mouse IgG1 kappa-Pe/Cy7, Mouse IgG1 kappa-APC (both eBioscience), IgG2b FITC (BD Bioscience) and IgG1 FITC (IQ products). Cells were mixed with the antibodies and incubated for 30 mins.

Adipogenic, osteogenic and smooth muscle cell differentiation assay

Basal medium DMEM (BioWhittaker Walkersville, MD) containing 10% foetal bovine serum (FBS), 1% penicillin/streptomycin and 1% glutamine was used. Immortalized ASC, tagged with EGFP or dTomato, were cultured to confluency and medium was changed to promote differentiation. For adipogenic differentiation this was basal DMEM plus 0.1 μM dexamethasone, 1 nM insulin, 0.5 mM isobutymethylxanthine. For osteogenic differentiation this was basal DMEM plus 0.1 μM dexamethasone, 10 mM β-glycerophosphate and 0.05 mM ascorbic acid. Finally, smooth muscle cell differentiation was in basal DMEM plus with 10ng/ml TGF-β1. After 14 days culture in the differentiation medium, cells were fixed with 2% PFA and stained for Oil Red O (Sigma-Aldrich, St. Louis, MO) for adipogenic differentiation, Alizarin Red (Sigma Aldrich) for osteogenic differentiation and Phalloidin-FITC (Invitrogen, Thermo Fisher Scientific) in DAPI for 30 minutes for smooth muscle cell differentiation.

Colony formation assay

Tagged ASCs were seeded at respectively hundred and thousand cells per well of a six-well culture plate in duplicate and were cultured for 14 days. Cell were washed with PBS and fixed with 2%PFA in PBS for 15 mins. Cells were washed well with PBS and stained with Crystal Violet (Sigma-Aldrich). Plated were scanned and the ability to form colonies was assessed by determining the area of the colonies.

Histological characterization of acellular matrix by haematoxylin & eosin

Cryo-sections of 12 μm were made from snap-frozen adipose tissue, SVF and ECM derived from the same donor (n =17). Samples were stained with haematoxylin solution for 5 min. After staining, samples were washed with tap water for 5 min. Afterwards, samples were stained with eosin solution for 10 min. and subsequently washed with tap water for 5 min. Finally, samples were mounted with Aquatex and visualized under light microscope (Leica Microsystems, DM IL).

MTT conversion assay for cell viability

Hydrogels (n=3) in triplicate were prepared in a 24 well plate with a volume of 0.5 ml. One ml of culture medium (basal DMEM) was placed on top of each hydrogel and collected every 24h for four days. Conditioned medium from each hydrogel for each time point was used in triplicate. A serial twofold dilution series of the hydrogel-derived medium was used. Human dermal fibroblasts (PK84) were cultured in 96 well plates in culture medium (as previously described) until confluency. Culture medium was replaced with the dilution series of hydrogel-derived medium for 48h. As a positive control for cytotoxicity, a twofold serial dilution series of puromycin (Gibco, 10mg/ml) in medium was used, starting at 10 ul per well. Normal culture medium served as negative control. After 48h, 5 mg/ml MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) in PBS was added to each well. Plates were incubated at 37ºC for 3h. Afterwards, the medium was removed and the purple formazan crystal dissolved in 200 μl of dimethyl sulfoxide (DMSO) by careful mixing. Optical density was measured at 650 nm and 585 nm. The difference in optical density between 650 nm and 585 nm was plotted against the log dilution to generate cytotoxicity graphs. Results were analysed with GraphPad Prism 7 using a nonlinear regression for a dose-response inhibition. The half maximal inhibitory concentration (IC_{50}) was used as an indicator of cytotoxicity. The IC_{50} value indicates how much of the substance is needed to induce 50% of cell death.

Live/dead staining of ASCs on hydrogels

Hydrogels (n=3) in triplicate were prepared in a 24 well plate with a volume of 0.5 ml Hydrogels were incubated with one ml of basal medium (as previously described in 2.5) for eight days and replaced every day. A suspension of 300 µl of culture medium containing 200,000 immortalized ASCs was added on top of each hydrogel. Immortalization was performed with the large T antigen of SV40 and lentivirally tagged (Harmsen lab, described in 2.4) with enhanced GFP (green, iASC13EGFP) or with Dtomato (red, iADSC13Dtomato). Cells were evaluated for seven days. Culture medium was changed every day and micrographs were taken every day. After 7 days a live/dead staining was performed with CFDA-SE (1:2000), PI (1:500) and DAPI (1:5000) for iASC13GFP. For iADSC13dTOMATO only CFDA-SE and DAPI were used, PI staining was not used as it has the same wavelength as already pre-stained cells, therefore they are both the same colour and it cannot be differentiated. As a control, 1,300 µl of culture medium containing 200,000 iASC13EGFP in a 24 well plate was used. Samples were visualised with an immunofluorescence microscope (EVOS® FL Cell Imaging System). After evaluation, samples were fixed with 2% paraformaldehyde in PBS for 30 min. Finally, samples were embedded in paraffin for immunohistology staining.

Immunohistology staining of ASCs inside hydrogels

From the above-mentioned paraffin embedded hydrogels (2.6), 4 µm sections on adhesive slides were made with Leica Reichert-Jung 2055 microtome. Samples were deparaffinised and incubated overnight with 0.1 M Tris/HCl solution (pH 9.0) at 80°C. Next day, samples were stained with haematoxylin for 4 min. and mounted with Aquatex. Results were evaluated with a light microscope (Leica Microsystems, DM IL).

RESULTS

Characterization of immortalized ASC

Immortalized ASC retain ASC characteristics

A mean of 98.9% , 99.0%, 95.9% and 23% of the immortalized ASCs showed expression of CD29, CD44, CD90 and CD105. Endothelial marker CD31 and common leukocyte marker CD45 was not detected. These results show that the lentivirally tagged immortalized ASC have a similar surface marker expression as ASC (Fig.2).[15]

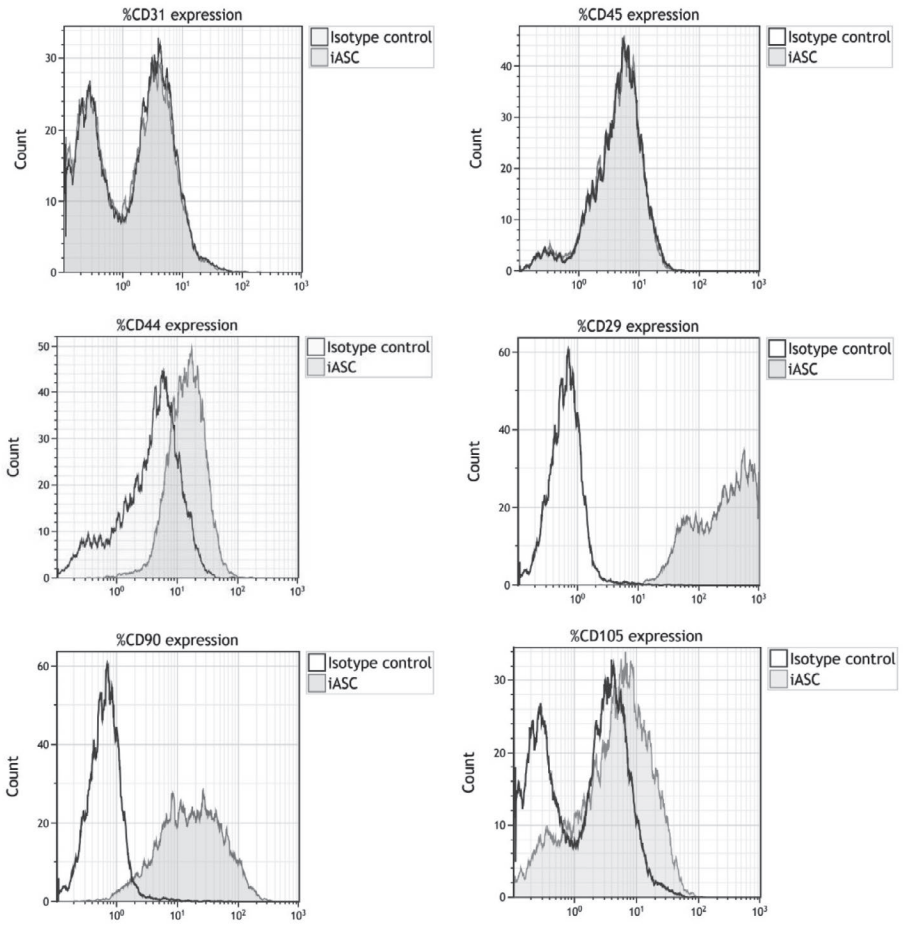

Figure 2. Representative data of CD-surface marker expression in immortalized ASCs.

Immortalized ASC retain cell differentiation capacity to adipocytes, osteoblasts and smooth muscle cells

Immortalized ASC successfully differentiated to adipocytes, osteoblasts and smooth muscle cells. This is coherent with the differentiation ability of normal, human isolated ASC (Fig.3).[15]

Immortalized ASC retain colony formation capacity

The number of colonies in each well consisting of more than 50 cells varied between 3-7%. This is coherent with results reported from colony experiments with normal, human ASC, were the number of colonies should be between 0.1-5%.[15]

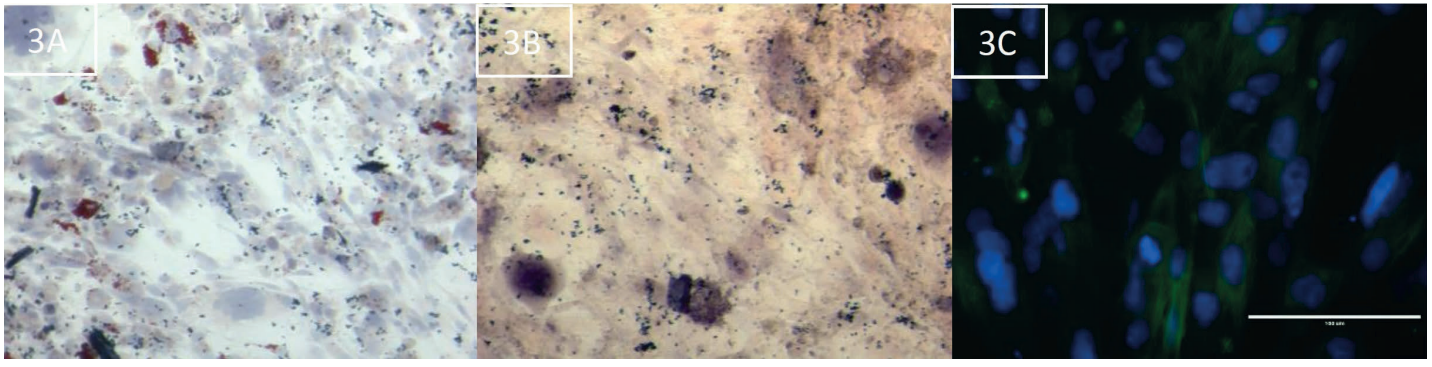

Figure 3. Immortalized ASC retain cell differentiation capacity to adipocytes, osteoblast, smooth muscle cells. 3A=Immortalized ASC differentiated to adipocytes. 3B=differentiated to osteoblasts. 3C=differentiated to smooth muscle cells.

Extracellular matrix derived hydrogels are non-cytotoxic

The serial sampling at 24h intervals of possible cytotoxic elutes from hydrogels showed no difference in MTT conversion compared to medium controls. Therefore, no IC_{50} could be calculated for all time points of each hydrogel indicating that the hydrogels did not release any cytotoxic compounds over time (Fig. 4). Results showed that the positive control (puromycin) induced a strong cytotoxic response ($LogIC_{50}$ 3.1 – 3.6 mM).

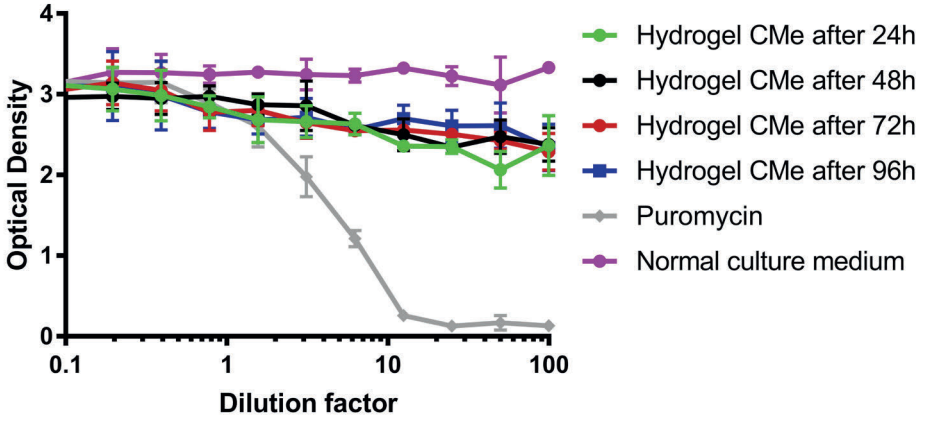

Figure 4. Optical density plotted against the serially diluted CMe from human adipose tissue-derived hydrogels after four different time points (log scale). PK84 fibroblasts were treated with hydrogel CMe or controls for 48h. Puromycin was used as positive control. Normal culture medium was used as negative control. Results are presented as mean with standard error of the mean of triplicates of three independent donors. CMe = conditioned medium.

No dead cells present in extracellular matrix derived hydrogels

Live/dead staining showed no dead cells (PI-stained nuclei) in hydrogels nor control (Fig. 5) ASCs appeared to adhere to the hydrogels and acquire the typical spindle-shape of mesenchymal cells; similar to the controls seeded on flat tissue culture plastic (Fig. 5B, D, F). It appeared that ASCs were more stretched when cultured in hydrogels than on tissue culture plastic (Fig. 5). Cell density did not appear to differ between ASC seeded on hydrogels as compared to tissue culture plastic controls, although quantification was not possible. The visualization even of fluorescently labelled ASCs (Fig. 5A, C, E) on and in hydrogels is challenging due to the limited depth of field with the microscope.

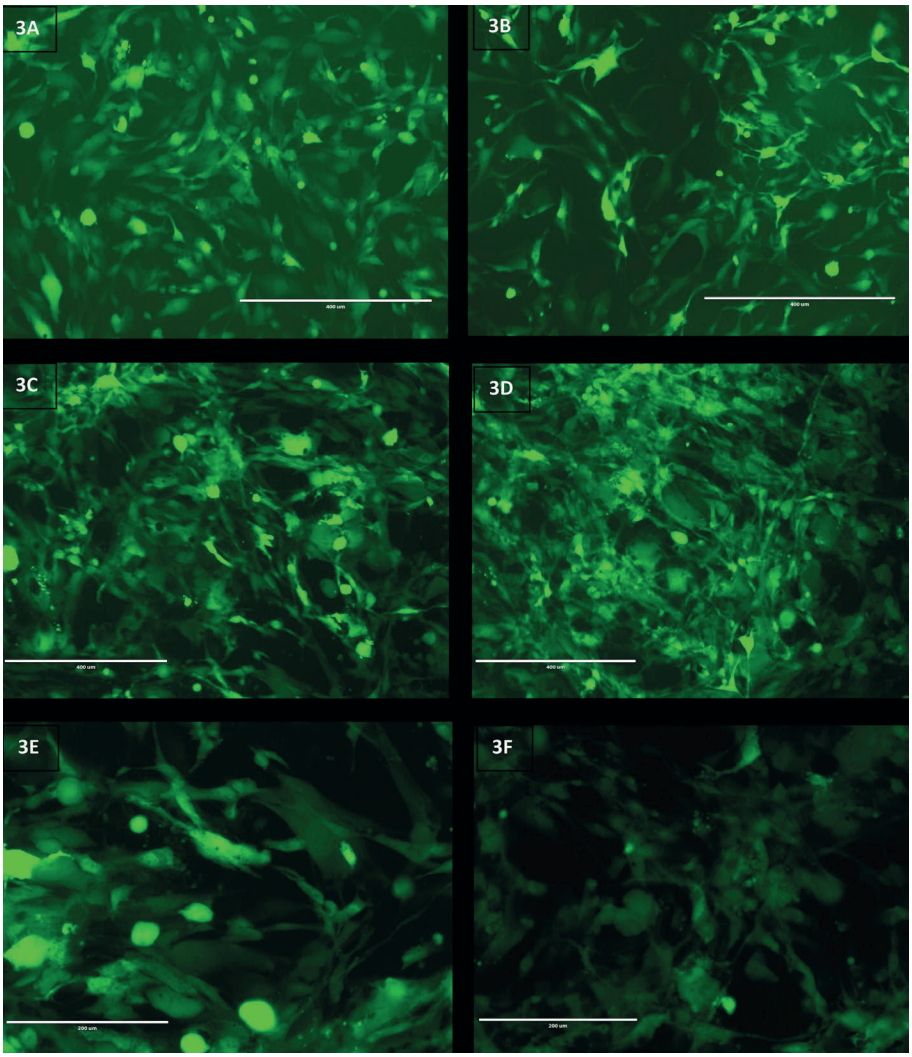

Figure 5. (**A,C,E)** = Representative fluoromicrographs of iASC13EGFP cultured on top of human adipose tissue-derived ECM hydrogels after respectively one, six and six days. (**B,D,F)** = Representative fluoromicrographs of iASC13EGFP cultured on tissue culture plastic (control) for respectively one, six and six days. Scale bar: A-D: 400 μm, E-F: 200 μm. iASC13EGFP = immortalized EGFP-tagged adipose derived stromal cells; ECM = extracellular matrix.

Histological confirmation of ASCs present inside the hydrogels

Haematoxylin-stained thin section of paraffin-embedded hydrogels confirmed the presence of ASCs inside the hydrogels (Fig. 6A) and at the periphery below the gel surface (Fig. 6B).

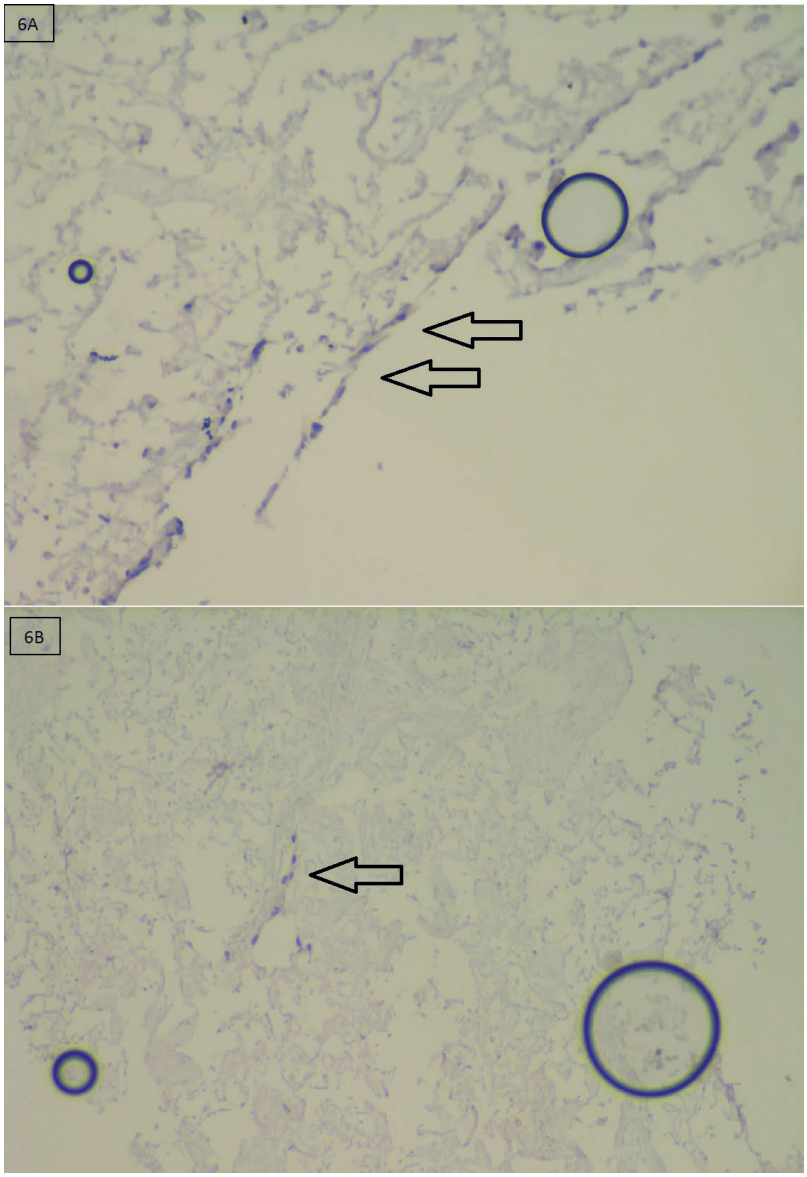

Figure 6. Hematoxylin-stained sections of iASC13EGFP cultured on top of human adipose tissue-derived ECM hydrogels after six days. Note that cells (visible by their dark blue nuclei) have migrated into the gel (arrows) at the periphery **(A)** and to the centre **(B)**. iASC13EGFP = immortalized EGFP-tagged adipose derived stromal cells; ECM = extracellular matrix.

DISCUSSION

The results of this research show that human adipose tissue-derived ECM hydrogel are non-cytotoxic and support adhesion, survival as well as migration and proliferation of immortalized ASCs. These are promising results that warrant further implementation e.g. as a delivery vehicle of stem cells, in animal and later also clinical trials. Cells cultured in the 3D hydrogel environment showed different morphology with respect to extending and elongating structures compared to cell morphology in plain cell culture plastic 2D environment culture. We also showed that immortalized ASC show the same characteristics as normal ASC (CD markers, colony forming ability and differentiation ability). ECM derived hydrogels can have various implementations.. ECM hydrogels are currently being used as a 3D culturing model and as bioengineering printing bio-ink. As shown by this study, culturing of cells differs in a 2D environment as compared to a 3D environment, especially in morphology. A study by Sung et al. showed that fibroblast in 3D culture released more signalling molecules which resembles more precisely their behaviour in the human organism.[29] Therefore, a 3D ECM hydrogel culture system is a better representative of how processes in the human body function and, therefore, an ideal culture system to study the effect of treatments on different cellular processes. The ECM hydrogels will likely impact bioengineering, as bio-ink for printing organoids together with vascular and connective tissue cells. Currently, ECM hydrogels from different origins have been tested in a variety of medical fields.[18-22] A study by Ghuman et al. induced a stroke in rats and subsequently injected an ECM hydrogel derived from adult porcine urinary bladder tissue into the necrotic stroke area.[30] This resulted in increased tissue regeneration in the brain after injection of an ECM hydrogel. The injected ECM hydrogel caused more cell infiltration as compared to the control group. Although different origins of extracellular matrix are available, the use of human adipose tissue-derived ECM provides several advantages as compared to decellularized tissue from other origins. First, human adipose tissue is more easily accessible without adverse side-effects compared to other organs of the human body such as skin. Second, the human adipose tissue is widely available since patients frequently donate liposuction adipose tissue. Third, the obtained adipose tissue-derived ECM can be used as both autologous as well as allogeneic treatment modality, while autologous transplantation is not possible for most of the other decellularized organs of the human body (*e.g.* heart, lung). Allogeneic transplantation is possible due to the lack of a cellular component causing an immune response after transplantation. This study served to setup a platform technology and focused on potential adverse influence of ECM hydrogels on two stromal cell types ASC and fibroblasts. Current research comprises of co-culture systems in ECM hydrogels of different organs and investigating processes relevant to tissue regeneration such as vascularisation. Thus, co-cultures of two or more

different cell types are under investigation. Besides, physical features of hydrogels such as matrix stiffness and viscoelasticity are under investigation now. In conclusion, the results of this research showed that human adipose tissue-derived ECM hydrogels are not cytotoxic and support adhesion and (in)growth of human immortalized ASCs. These results are promising and warranted further research about implementation of adipose tissue-derived hydrogels as *e.g.* a delivery vehicle of cell types or bioengineering printing bio-ink.

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14

General discussion and future perspectives

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Transplantation of adipose tissue *i.e.* lipofilling has been used to restore volume defects due to ageing as well as due to congenital or traumatic deviations for decades.[1] Adipose tissue, similar to almost all tissues, comprises of parenchyme (adipocytes) and supporting tissue. Obviously, the filler feature of adipose tissue is to be attributed to the 90% volume effect of adipocytes. In the past decades it has become clear that supporting tissue holds potency to support tissue repair and regeneration. Supporting tissue comprises connective tissue cells and vasculature that is embedding in an extracellular matrix. This is called the stromal vascular fraction of tissue (SVF). The current dogma is that the regenerative engine of adipose tissue comprises of adipose tissue-derived stromal cells (ASCs). ASCs reside in the SVF attached around vessels as precursor cell types *e.g.* pericytes and supra-adventitial cells.[2,3] Zuk et al. was the first to culture ASCs from enzymatically isolated adipose tissue and showed the multilineages differentiation capacity of ASCs *in vitro e.g.* ectodermal, endodermal and mesodermal.[4] During culture, ASCs secrete a plethora of growth factors, cytokines and proteins which are in potential able to support regeneration of damaged tissue.[5]

Therefore, in order to make full use of the regenerative capacity of SVF, isolation procedures are warranted that yield all components. However, for reasons of simplicity, intra-operative enzymatic isolation procedures have been developed to isolate a single cell suspension of SVF cells containing ASCs by degrading all intercellular connections including extracellular matrix.[6] Nowadays, this single cell suspension of SVF cells is used for a variety of different clinical indications *e.g.* skin rejuvenation, scar remodeling, osteoarthritis or wound healing to mention a few.[7-9] However, clinical use of intra-operative enzymatic isolation procedures is expensive and time-consuming. Therefore, mechanical isolation procedures were developed to be faster and less expensive, although mechanical isolation procedures are not capable of disrupting all adipocytes in contrast to enzymatic isolation procedures that separate all adipocytes from SVF cells. For instance, the mechanical Lipogems procedure is able to process up to 130 ml of lipoaspirate to isolate about 60-100 ml of SVF, while 90% of the volume of adipose tissue is contributed by adipocytes.[10] Hence, a large number of adipocytes remain intact after performing the Lipogems procedure. Therefore, we have developed a mechanical isolation procedure to isolate SVF cells by effectively disrupting adipocytes.[11,12] This mechanical isolation procedure is called the Fractionation of Adipose Tissue (FAT) procedure yields SVF that is devoid of almost all adipocytes while preserving vasculature and SVF cells as well as cell-cell communications, extracellular matrix and cell- matrix interactions. Moreover, a large fraction of ASCs was present, which were phenotypically as well as functionally intact. The volume of the obtained SVF was

reduced tenfold in comparison to the starting volume of lipoaspirate.[11,12] A reduction of volume is an indirect indication of the effectiveness of a mechanical isolation procedure because 90% of the volume of adipose tissue can be ascribed to adipocytes.

The main difference between the FAT procedure and other mechanical isolation procedures is the centrifugation step prior to fractionation. Centrifugation is necessary to remove all infiltration fluid in harvested lipoaspirate. Infiltration fluid is used for local anesthetics and the fluid component has a protective effect on mature adipocytes during fractionation. The principle of fractionation is based on shearing of tissue and large cells; powerfully pushing adipose tissue through a small hole in a luer-to-luer hub. Adipocytes are flexible by changing their shape while being pushed through a small hole. Once adipocytes pass the small hole, reformation occurs into their natural shape. However, without a protective fluid around the adipocytes giving counter pressure, adipocytes will disintegrate. A comparable technique used in literature is the Nanofat procedure.[13] Nanofat procedure uses only decanted lipoaspirate and thus a large amount of protective infiltration fluid is still present. Consequently, a large number of adipocytes are still intact present in the final product.[14] A study of Mashiko et al. compared the Nanofat procedure with the FAT procedure and showed a larger number of adipocytes of 83.3% in the Nanofat fraction compared to 45.8% in the FAT procedure fraction. Moreover, the extracellular matrix portion was lower in Nanofat than in tSVF isolated by the FAT procedure (16.7% vs 54.2%).[15] These findings show that the Nanofat procedure is an emulsification and filtration procedure to increase injectability by mixing infiltration fluid with lipoaspirate rather than an mechanical isolation procedure of tSVF.

In contrast to enzymatic isolation procedures, mechanical isolation procedures disrupt only adipocytes while maintaining all cell-cell communications including extracellular matrix. This difference might be of significant importance because individual cells in a single cell suspension of SVF tend to quickly migrate from the tissue, e.g. into the draining lymph nodes, directly after injection. The extracellular matrix in mechanically-derived SVF, literally retains the cells on the site of injection resulting in a larger regenerative effect. Additionally, extracellular matrix itself has an important and often underestimated regenerative role because it functions as a natural instructive scaffold for both cells and growth factors. In this thesis, extracellular matrix from SVF obtained by the FAT procedure was isolated through an extensive decellularization protocol and using mild enzymatic proteolysis with pepsin converted into a self-assembling hydrogel.[16] This hydrogel was loaded with conditioned medium from cultured ASCs containing the complete secretome of ASCs in order to stimulate angiogenesis as well as fibroblast proliferation and migration *in vitro*.[16] These findings showed that

extracellular matrix isolated from FAT procedure-obtained SVF plays an important role in binding and releasing growth factors released from ASCs in a controlled fashion. Moreover, these factors are still biologically active over a long release time *e.g.* stimulate angiogenesis as well as fibroblast proliferation and migration.[16] In contrast to the FAT procedure, many mechanical isolation procedures focus only on the cellular fraction of SVF trying to eliminate extracellular matrix in order to improve injectability.[10,13,17] Probably a significant part of the regenerative capacity of SVF is thereby reduced by reducing the extracellular matrix with pericytes and supra-adventitial cells attached around vessels.

To date, the definition of SVF comprising both enzymatic and mechanical isolated SVF should be redefined. According to definitions by pathologists and histologists, stromal tissue is the connective and structural component of every organ throughout the human body. Connective tissue consists of cells and extracellular matrix containing proteins embedded in ground substance.[18] After enzymatic digestion of adipose tissue, all connective tissue *i.e.* stromal tissue including vascular structures are resolved leaving just the cellular component. To our opinion, mechanically isolated SVF should therefore be named tissue SVF (tSVF) whereas enzymatically isolated SVF should be named cellular SVF (cSVF).[12]

In 2006, Coleman was the first to mention regenerative effects of lipofilling.[19] Coleman described less wrinkles and an increased skin quality of the ageing face *e.g.* reduced pigmentation and reduced number of pores after autologous lipofilling. Though, no formal scientific proof was given because of a lack of statistics and objective validated measurement outcomes. Besides, the addition of subcutaneous volume by autologous lipofilling causes wrinkles to disappear. Since 2006, multiple case series as well as retrospective and prospective studies have investigated the effects of autologous lipofilling to ageing facial skin showing positive results.[20-25] In comparison with Coleman's first report, most of these studies lack a good study design with validated measurement outcomes as well as a control group. In contrast, in this thesis, two well-designed prospective randomized placebo-controlled double-blinded clinical trials showed that lipofilling with and without additives *i.e.* tSVF or platelet rich plasma (PRP) does not improve skin elasticity of ageing facial skin nor patient satisfaction.[26,27] On the other hand, the addition of PRP to lipofilling reduced postoperative recovery time. A possible explanation for the reduced recovery time is that lipofilling is an invasive procedure causing many minor (sub)cutaneous traumas. Recovery from these iatrogenic traumas *e.g.* small wounds might benefit from released growth factors from additional platelets.

The lack of skin quality improvement in ageing facial skin might be caused by the absence of a 'trigger' to stimulate adipose tissue or tSVF to regenerate damaged tissue. Aging of the skin is a physiological process of a gradual decrease of elasticity, primarily caused by reduced elastin deposition in the extracellular matrix by skin fibroblasts. This can be due to hormonal changes, genetic factors as well as environmental influence *e.g.* cigarette smoke and ultraviolet radiation.[28] Our hypothesis is that 'ordinary' aging of the skin is a normal physiological process in which certain components of the extracellular matrix, such as elastin gradually disappears. In other aspects, such as epidermal regeneration, perfusion, and neurosensation, no markable changes occur due to ageing. Hence, tSVF has little to repair. Moreover, *de novo* generation of dermal elastin is often considered absent or inefficient whilst this would neither lead to tightening of the expanded aged tissue. In this way, ageing-related skin changes are not considered damaged tissue and do not result in clinically identifiable changes of the skin as a result of facial lipofilling. In literature, only two studies have reported histological improvement of facial skin after autologous lipofilling enriched with tSVF, adipose derived stromal cells (ASCs) or PRP.[21,22] Three months after injection, the reticular dermis showed a decrease and more dissociated fiber network containing smaller as well as smoother fibers in comparison with before injection. Moreover, in the ASC as well as tSVF enriched lipofilling group a higher number of superficial oxytalan elastic fibers in the papillary dermis were noted. Skin consist of a complex organization of three types of elastic fibers: oxytalan, eluinin and elastic fibers.[29] Oxytalan fibers are immature elastic fibers containing mainly microfibrils devoted of elastin crosslinking, while the extent of elastin crosslinking increases in elauinin and elastic elastic fibes. This indicates that the three types of elastic fibers represent different stages of elastogenesis of the skin.[30] A larger amount of oxytalan elastic fibers might indicate elastogenesis. However, changes in eluinin and elastic elastic fibers were not mentioned.[21,22] Another histological study demonstrated an increase in dermal thickness after lipofilling in comparison with no treatment after 69 days.[23] Yet, all studies lack a control group with microneedling only. It is well-known that needling of the skin results in histological changes of the skin including finer and more organized dermal elastic fibers. For this reason, histological improvement after autologous lipofilling might be a needling effect. In this case, microneedling alone would be sufficient to improve skin quality and thus autologous lipofilling unnecessary. Furthermore, no clinical effects were measured while histological improvement might not always be clinically significant.

Compared to physiological ageing of the skin, pathophysiological processes *e.g.* dermal fibrosis or chronic wounds might be accelerated by tSVF. Pathological processes go along with a disbalance of extracellular factors resulting in inflammation, excessive extracellular matrix deposition and crosslinking, or a lack of angiogenesis. Lipografting

re-educates damaged tissue by tSVF of adipose tissue. Although, no significant improvements have been described as a result of tSVF or lipofilling applied to 'ordinary' ageing skin, significant improvement of skin quality have been described in cases where skin changes occurred due to a disbalance of extracellular factors as in dermal scarring and wound healing.[31-34] Yet, formal proof derived from randomized prospective trials is lacking thus far. In this thesis, a prospective randomized placebo-controlled double blinded clinical trial was performed to accelerate postoperative wound healing by tSVF in order to reduce postoperative scarring. tSVF was injected in the wound after a breast reduction, while the contralateral breast received a placebo injection with 0.9% NaCl. After six months, a significant improvement in scar appearance measured with the validated patient and observer scar assessment scale (POSAS) was shown by both patient as well as observer. However, twelve months postoperative scar appearance was comparable between the tSVF and control group. Many of the included patients noticed a faster healing of the wound in the first weeks postoperative. This indicates that tSVF might play a more significant role in accelerating wound healing than as anti-scarring treatment. Unfortunately, this trial used an one-time injection, while a second administration might have had a more profound long-term influence. To date, many studies have investigated if lipofilling or any regenerative component *i.e.* ASCs or SVF can accelerate wound healing. Yet, all of these studies lack a proper designed study with validated outcome measurements to reliably conclude that (or substrate of) lipofilling accelerate dermal wound healing.

Since evidence of the skin rejuvenating effect of lipofilling on 'ordinary' aged skin seems to be lacking, lipofilling for skin rejuvenation purposes solely should ideally only be offered in prospective randomized and placebo-controlled trials. Controlled randomized clinical trials are study types that can only show, with the highest predictability, whether a treatment, that seemed to be beneficial as apparent in initial case reports or retrospective studies, indeed is evidence-based working and effective. In general, both patients as well as clinicians expect an elective non-life saving and often self-paid treatment delivers an effective, efficient, satisfactory end result that was originally aimed for. Without well-defined readouts, such as third person analyses on blinded samples, claims in case reports and retrospective studies suggesting that lipofilling is effective for the use of skin rejuvenation are actually non-evidence based and thus not true nor valid. These claims, however, contribute to the hype that ASCs in lipografts are responsible for the questionable rejuvenating effect on skin. Moreover, the terminology of ASCs in communication between clinicians and patients contribute to this aforementioned hype as well. Till recently, adipose derived stromal cells (ASCs) were named adipose derived stem cells. However, ASCs are not stem cells for several reasons. First, stem cells are capable of self-renewal and have an unlimited proliferation

potential both *in vivo* as well as *in vitro*, such as embryonic stem cells, induced pluripotent stem cells as well as satellite cells, neuronal stem cells and intestinal stem cells.[35-38] Second, stem cells have the ability of multipotent cell differentiation.[37] In contrast, stromal cells have a limited lifespan, with a limited proliferation potential and at higher passages ASCs undergo senescence. Thus, the term mesenchymal stem cells is in fact incorrect; it should be named mesenchymal stromal cells. Nevertheless ASCs, irrespective of proliferation, are able to differentiate (be constructive), secrete growth factors and cytokines (be instructive), and can remodel the extracellular matrix (be reconstructive).[39] Although, recently, in literature the word "stem" cell has been replaced by "stromal" cell, clinicians still use the word "adipose stem cell" in their communication with their patients. Reason for this continuing use of the word "stem cells" probably is because people have more confidence in the word "stem" cells rather than "stromal" cells. Moreover, stem cells are associated with the beginning of life and therefore a rather "magical" meaning is attributed to this word.

Basically, we all start as a single totipotent stem cell *i.e.* the fertilized oocyte, which develops towards a human being. Actually, embryonic stem cells are a culture artefact because these are derived from the inner cell mass of a blastocyst, which normally develops to form the three embryonic germ layers (mesoderm, endoderm and ectoderm).[40] In this process of development, all cell types in the body arise from stem cells that comprise the inner cell mass. Because stem cells play a key role in the development of early life, it is easy to believe that so called "stem cells" (*i.e.* actually stromal cells) are also able to extent life by regeneration of damaged tissue (damage of pathological nature like osteoarthritis or dermal scarring and damage of physiological nature such as aging of the skin). Patients as well as clinicians and researchers might magically think and hope that ASCs are the solution to every medical problem and hopefully extent life. However, clinicians and researchers should understand and realize (based upon their access to the knowledge) that if the application of ASCs or lipofilling is indeed beneficial, it is merely based upon thoughts and believe. In contrast, patients do not have access to such knowledge and are not well-grounded and therefore fully rely on their clinicians.

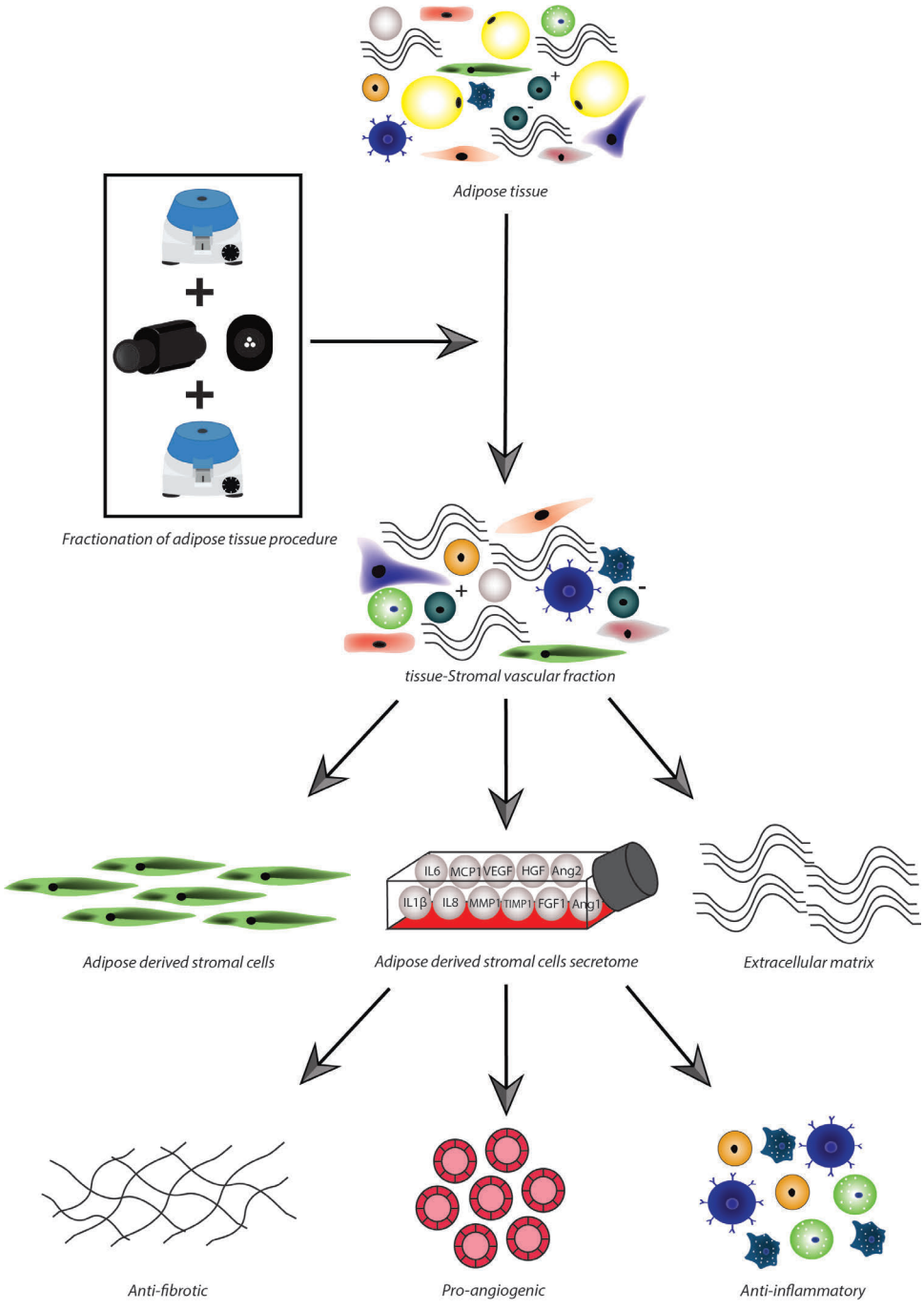

Figure 1. Schematic overview of therapeutic components of tissue-stromal vascular fraction obtained by the fractionation of adipose tissue procedure and its therapeutic mechanism.

Consequently, the clinicians should protect the patient from their own overestimated believe that stem cells are the solution for every medical issue and should not nurture this belief. Clinicians, irrespective of their specialism, are all taught to act according to the four basic medical ethical principles: patient autonomy, beneficence, non-maleficence and respect for human rights. In this perspective many clinicians, who treat patients for skin rejuvenation purposes beyond clinical trials, fail to act according to one or more of the aforementioned principles. Also, researchers could and should play an important role in this respect by generating awareness among clinicians and patients about the facts and fables of ASCs and stem cells as well as lipofilling for skin rejuvenation and other clinical purposes. Progress in the field of regenerative medicine can only be accomplished if all parties *i.e.* clinicians, researchers, patients and industry work together in a respectful manner and learn to communicate in an understandable way. Moreover, protection of the patients can only be offered when treatments are fully understood and are evidence-based: this is not the case when evidence is based on case reports, (too) small clinical studies (often without control groups) and retrospective studies and this is not in line with the Declaration of Helsinki. The Declaration of Helsinki states that individuals have the right to make self-determined informed decisions regarding the participation in research. When the evidence is based on case reports, small clinical studies and retrospective studies, individuals have the right to be informed about it. Thus, randomized clinical placebo-controlled clinical trials are definitely warranted, when feasible, before treatments are offered as being evidence-based and real problem solving in regular and commercial medicine.

It is clear that tSVF is not the solution to all (non)-medical problems. Clearly, physiological processes, such as ageing of the skin, cannot be reversed by a single injection of tSVF. Further research should focus on diseases and disorders that would potentially benefit from (multiple) tSVF injections. This thesis showed that tSVF is able to interfere in pathological processes, especially processes with excessive collagen deposition, a lack of angiogenesis or being pro-inflammatory. Moreover, tSVF obtained by the FAT procedures contains a high concentration of all regenerative components in a small volume: cellular fraction as well as extracellular matrix (Fig. 1).[11,12] Hence, tSVF might be a future therapeutic option where only small amounts of volume can be injected *e.g.* osteoarthritis of carpometacarpal or temporomandibular joints. An important part of the pathophysiology of osteoarthritis is the pro-inflammatory state of the joint causing an excess of pro-inflammatory mediators *e.g.* cytokines, prostaglandin E2 and nitric oxide production resulting in disabling pain.[41] An *in vitro* study showed a decrease of prostaglandin E2 production by inflamed synoviocytes co-cultured with cSVF.[42] Reduction of prostaglandin E2 production by synoviocytes might be causing reduction of pain after injection of (c- or t-)SVF clinically as mentioned in case series.

Another potential indication of using tSVF where inflammation as well as lack of angiogenesis plays a crucial role is diabetic wound healing. Both pathological processes can be positively affected by tSVF. However, some diabetic wounds might be too large to cover entirely with tSVF obtained by the FAT procedure. The low production yield of the FAT procedure is one of the main limitations. Another limitation is the necessity of liposuction prior to performing the FAT procedure. Liposuction is an invasive procedure, which might not always be favorable in patients with a disturbed wound healing. Moreover, isolated autologous adipose tissue might be affected by systemic diseases such as diabetes mellitus.[43-45] For these specific cases, the extracellular matrix based hydrogels with loaded factors from therapeutic cells *e.g.* ASCs or immortalized pluripotent cells might be ideally as an off the shelve product for several pathologies to replace autologous lipofilling or SVF administration.[46]

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English summary

ENGLISH SUMMARY

In **chapter 2**, a systematic review of all commercially available intra-operative enzymatic and mechanical isolation procedures of stromal vascular fraction (SVF) was performed. Pubmed, EMBASE (OvidSP) and the Cochrane central register of controlled trials databases were searched until September 2016. Studies validating SVF isolation procedures based on cell yield, viability of nucleated cells, composition of SVF, duration, cost and characteristics of procedures were included. In total, thirteen studies evaluating eighteen procedures were included. None of the procedures in terms of cell yield, viability of nucleated cells or composition of SVF could be designated as preferred method due to a large heterogeneity in validation methods. However, major differences in duration and costs between enzymatic and mechanical procedures exist. In general, enzymatic isolation procedures are time-consuming and expensive due to the use of non-autologous material *e.g.* enzymes or animal derived products and disposable components. Mechanical isolation procedures are less time-consuming and less expensive because only shear stress is used as dissociation method. Moreover, mechanical isolation procedures result in a tissue like SVF (tSVF) with intact intercellular communications including the extracellular matrix, while enzymatic isolation results in a single cell suspension of SVF (cellular, cSVF). Therefore, mechanically isolated tSVF has been used in our prospective randomized clinical trials.

In **chapter 3** a mechanical isolation procedure, the so-called Fractionation of Adipose Tissue (FAT) procedure was developed and validated to isolate tSVF from harvested lipoaspirate. The FAT procedure consists of three consecutive steps: centrifugation followed by fractionation and subsequently centrifugation. The obtained tSVF was validated based on viability, histological composition and presents of adipose derived stromal cells (ASCs). ASCs were characterized based on function, phenotype and ability to form colony forming units. All these aforementioned protocols to perform and validate the FAT procedure were presented in an overview in **chapter 6**. The FAT procedure was able to concentrate tSVF tenfold containing mainly extracellular matrix and vasculature by destructing adipocytes. Viability of tSVF was up to 100%. ASCs isolated from tSVF were not affected by the FAT procedure and showed a comparable multilineage differentiation capacity, CD expression as well as ability to form colonies in comparison with non-processed ASCs. Hence, the FAT procedure was able to isolate tSVF preserving all regenerative components in a small volume within twenty minutes and is therefore suitable for intra-operative use.

Although the FAT procedure was efficient in isolating tSVF, some improvements could be made to improve its usability. The original fractionator contained an off-centered

internal disk with three holes of 1.4 mm. The absence of a central hole frequently resulted in regular blocking due fibrotic tissue in lipoaspirate. Furthermore, the original fractionator was reusable which is, with respect to patient safety, less safe as a disposable device. In **chapter 4**, a disposable one-hole fractionator was developed and compared to the original reusable three-hole fractionator regarding histological composition, composition based on CD marker expression, the ability of cells to form colony forming units and the number of cells isolated as well as their viability. No differences were observed in histological composition as well as composition based on CD marker expression between tSVF isolated by both types of fractionators. Moreover, the number of cells enzymatically isolated from tSVF as well as their viability and ability to form colony form units was comparable between both types of fractionators. Additionally, the number of procedures that blocked during process was lower when the disposable one-hole fractionator was used. This study demonstrated the improved usability of the disposable one-hole fractionator without losing its efficiency in isolating tSVF compared to the original reusable three-hole fractionator.

One of the main advantages of tSVF isolated by means of the FAT procedure was the increased number of regenerative cells in one ml of tissue in comparison with unprocessed lipoaspirate. In this way, a higher number of regenerative cells can be injected in small volume areas such as small joints suffering from osteoarthritis. Recently published studies show that SVF or ASCs can be effective in reducing inflammation of chondrocytes, one of the key players in osteoarthritis. In **chapter 5**, we have investigated if tSVF isolated by means of the FAT procedure is able to reduce the pro-inflammatory state of chondrocytes *in vitro*. In this study, the proliferation of chondrocytes and production of sulphated glycosaminoglycans (sGAG) by chondrocytes co-cultured with various ratios of enzymatically isolated tSVF cells was studied. Moreover, the expression of interleukin-1β (IL-1β) and cyclo-oxygenase 2 (COX2) during co-culture of tumor necrosis factor-α (TNF-α) stimulated chondrocytes with enzymatically isolated tSVF cells was investigated. A significant increase in number of chondrocytes was noted after twenty-one days of culture when chondrocytes were co-cultured with tSVF in various ratios, especially in ratios 50/50 and 25/75. Chondrocytes co-cultured with tSVF in ratio 25/75 were functional because the amount of sGAS produced was comparable to single cultured chondrocytes. Moreover, expression of IL-1 β and COX2 by TNF-α stimulated chondrocytes was significantly reduced by tSVF. These *in vitro* results show the anti-inflammatory and proliferative effect of tSVF on chondrocytes.

The main goal of this thesis was to study skin rejuvenation by tSVF on skin alterations caused by ageing and dermal scarring. Skin quality decreases over time due to ageing, fibroproliferative diseases of the skin or scarring. These causes can be divided

into two types of processes: physiological process (ageing) or pathological process (fibroproliferative diseases or scarring). Many studies have described a potential effect of lipofilling on skin rejuvenation whether decrease in skin quality has a physiological or pathological cause. In **chapter 7**, an extensive overview is given of the literature regarding the anti-scarring effect of lipofilling in both clinical and animal studies. This review underlines the lack of well-designed prospective randomized clinical trials in the field of regenerative plastic surgery, especially when lipofilling or any component of adipose tissue *e.g.* ASCs, SVF is used as anti-scarring treatment. However, many case reports and retrospective studies suggest a clinical effect of lipofilling on dermal scars in terms of softening of the skin, improvement in color and thinning of the skin. Although formal proof derived from randomized prospective trials is lacking, data from these case reports and clinical studies has led to the acceptance of lipofilling as a treatment of burn scars nowadays. The underlying mechanism can be ascribed to three main processes, which are involved in dermal wound healing as well: stimulation of angiogenesis, remodeling of extracellular matrix and immune modulation. These cellular processes are stimulated by ASCs through secretion of growth factors, cytokines and proteins.

Due to a lack of well-designed prospective randomized clinical trials, we designed a prospective randomized double-blinded placebo-controlled multi-center clinical trial investigated the effect of tSVF on early wound healing in order to prevent or reduce scar formation. In **chapter 8**, tSVF was produced by means of the FAT procedure and injected after a breast reduction, while the contralateral breast served as placebo control. In total, 34 female subjects were treated (34 versus 34). The results of this study showed a significant improvement in scar appearance after six months. Many of the included patients noticed a faster healing of the wound in the first weeks postoperative. Twelve months postoperative scar appearance was comparable between the tSVF and control group. This indicates that tSVF probably plays only a significant role in early wound healing rather than have a role as an anti-scarring treatment.

In **chapter 9**, the effect of autologous lipofilling in combination with platelet-rich plasma (PRP) on ageing related skin changes was studied in a prospective randomized placebo-controlled clinical trial. In this study, PRP was added to stimulate ASCs present in lipofilling in order to enhance the regenerative effect by autologous lipofilling. Skin improvement was measured using the cutometer to measure elasticity and a questionnaire to evaluate the opinion of participating subjects as well as their return to work in days. In total, 32 female subjects were treated with either autologous lipofilling with platelet rich plasma or autologous lipofilling with placebo control. After twelve months of follow-up, no significant difference was noted in skin elasticity

nor patient satisfaction between both groups. Although, a regression analysis of the 'true skin elasticity' parameter as a function of age reversed from negative to positive, especially after treatment with lipofilling in combination with PRP. Yet, this reversal was not significant and only notable on one of the two measurement locations in the face. The addition of PRP to lipofilling resulted in a significant faster return to work in comparison with autologous lipofilling alone. In conclusion, lipofilling with or without PRP does not contribute to skin rejuvenation. To further increase the regenerative capacity of lipofilling with PRP, tSVF was used as additive in order to improve skin alterations due to ageing. A comparable prospective clinical trial, as mentioned in **chapter 9,** was subsequently performed in **chapter 10**. In this study, 28 female subjects were treated with either lipofilling with PRP in combination with tSVF or lipofilling with PRP in combination with placebo control. Skin improvement was measured using the cutometer to measure elasticity, tewameter to measure transepidermal water loss, VISIA camera to analyze superficial spots, wrinkles, skin texture, pores, vascularity and pigmentation as well as the FACE-Q questionnaires to evaluate the opinion of participating subjects. No significant improvement of skin rejuvenation nor improvement in patient satisfaction was seen after lipofilling and PRP with or without tSVF. Results in **chapter 10** confirmed our findings of the previous published trial in **chapter 9** and showed that the addition of tSVF does not improve skin alterations caused by ageing related factors.

In contrast to the results published in **chapter 9** and **10**, many studies in literature suggest that lipofilling or any regenerative component of adipose tissue *e.g.* SVF or ASCs contribute to facial skin rejuvenation. The aim of **chapter 11** was to systematically search in literature to all available studies regarding facial skin rejuvenation by lipofilling, SVF or ASCs. Medline, EMBASE (OvidSP) and the Cochrane central register of controlled trials databases were searched until May 2018. Studies evaluating the effect of lipofilling on facial skin quality were included. Skin quality improvement was defined as improvement of skin elasticity, texture and color or histological improvement. In total, nine studies treating 301 patients were included. Most of the studies reported improvement of skin elasticity or texture or a more homogenous color of the skin after facial lipofilling, however the majority of studies lacked a well-designed study protocol with validated outcome measurements. One study with a proper study design showed no improvement of skin elasticity after facial lipofilling. All histological studies failed to correlate histological changes to clinical improvement and thus it remains unknown whether histological changes result in clinically relevant improvements. Hence, facial lipofilling, SVF or ASC injection does not significantly improve skin quality.

The key therapeutic components of tSVF consist of a cellular fraction *i.e.* ASCs and tissue fraction *i.e.* extracellular matrix. ASCs are one of the regenerative key players in the cellular fraction of tSVF and are able to stimulate other cell types by released paracrine factors. In **chapter 12**, extracellular matrix was isolated through an extensive decellularization protocol and enzymatically micronized using pepsin to subsequently form a self-assembly hydrogel. This hydrogel was loaded with conditioned medium from cultivated ASCs containing the complete secretome of ASCs in order to stimulate angiogenesis as well as fibroblast proliferation and migration *in vitro*. This study showed that extracellular matrix isolated from tSVF plays an important role in binding and releasing growth factors released from ASCs in a controlled fashion. Moreover, these factors are still biologically active over time *e.g.* stimulate angiogenesis as well as fibroblast proliferation and migration. No differences were observed between extracellular matrix isolated from diabetic patients and non-diabetic patients. Besides in **chapter 5,** we have showed the anti-inflammatory effect of tSVF on pro-inflammatory chondrocytes *in vitro*. These findings indicate that tSVF is able to stimulate angiogenesis, remodel extracellular matrix and acts immunomodulative *in vitro*.

The extracellular matrix derived hydrogel loaded with released factors from ASCs might be a therapeutic option itself in the future as well. This hydrogel is suitable for allogenic transplantation and contains all regenerative key components of tSVF. Yet, the production of the extracellular matrix derived hydrogel consist of an extensive decellularization process requiring cytotoxic reagents. Hence, in **chapter 13** we have studied the cytotoxic effects of these hydrogels on connective tissue *i.e.* fibroblasts. Cytotoxicity was studied using a metabolic activity assay (MTT) and show that the extracellular matrix derived hydrogels were non-cytotoxic. Therefore, clinical use of these hydrogels might be an interesting possibility in the future.

NEDERLANDSE SAMENVATTING

In **hoofdstuk 2** is een systematische review uitgevoerd welke alle beschikbare commerciële en intra-operatieve procedures analyseert om de stromale vasculaire fractie (SVF) te isoleren. Pubmed, EMBASE, (OvidSP) en het Cochrane centraal register van gecontroleerde studies zijn hiervoor doorzocht tot september 2016. Studies die zich bezighielden met de validatie van isolatieprocedures van SVF op basis van aantal cellen, vitaliteit van cellen met kernen, compositie van SVF, duur, kosten en karakteristieken van procedures zijn geïncludeerd. Geen enkele van deze geïncludeerde studies kon aangewezen worden als geprefereerde methode op basis van aantal cellen, vitaliteit van cellen met kernen of compositie van SVF door een grote mate van heterogeniteit in validatiemethodes. Echter, er bestaan grote verschillen in duur en kosten tussen enzymatische en mechanische isolatieprocedures. In zijn algemeenheid kosten enzymatische procedures meer tijd en zijn duurder door het gebruik van niet-autoloog materiaal zoals enzymen of dierlijk materiaal en disposable componenten. Mechanische isolatieprocedures zijn minder tijdrovend en minder duur omdat er alleen gebruik wordt gemaakt van scheuringskrachten als dissociatiemethode. Verder resulteren mechanische isolatieprocedures in een tissue achtige SVF (tSVF) met een intacte intercellulaire verbindingen zoals extracellulaire matrix, terwijl enzymatische isolatieprocedures resulteren in een suspensie van enkele cellen (cellulaire SVF (cSVF)). Dit is de reden dat we voor een mechanische isolatieprocedure om tSVF te isoleren hebben gekozen in onze prospectieve gerandomiseerde klinische studies.

In **hoofdstuk 3** is een mechanische isolatieprocedure, de zogenaamde Fractionation of Adipose Tissue (FAT) procedure ontwikkeld en gevalideerd om tSVF te isoleren van geoogst vetweefsel. De FAT procedure bestaat uit drie opeenvolgende stappen: centrifugeren gevolgd door fractioneren en aanvullend wederom centrifugeren. De geproduceerde tSVF is gevalideerd op basis van vitaliteit, histologische compositie en de aanwezigheid van vet mesenchymale stromale cellen (ASCs). ASCs zijn gekarakteriseerd op basis van functie, fenotype en de mogelijkheid om kolonies te vormen. Alle protocollen van de hiervoor genoemde experimenten om de FAT procedure uit te voeren en te valideren zijn opgenomen in een overzicht in **hoofdstuk 6**. De FAT procedure was in staat om tSVF tienvoudig te concentreren en bestond voornamelijk uit extracellulaire matrix en de vaatvoorziening door middel van het afbreken van vetcellen. De vitaliteit van tSVF was nagenoeg 100%. ASCs geïsoleerd uit tSVF waren niet aangedaan door de FAT procedure en lieten in vergelijking met onbewerkte ASCs dezelfde differentiatie capaciteit in meerdere cellijnen, CD expressie

en de mogelijkheid om kolonies te vormen. Kortom, de FAT procedure was in staat tSVF te isoleren binnen 20 minuten met daarmee behoudt van alle regeneratieve componenten en is daarom geschikt voor intra-operatief gebruik.

Ondanks dat de FAT procedure efficiënt was in het isoleren van tSVF waren er toch een aantal zaken ten aanzien van de bruikbaarheid welke verbeterd konden worden. De originele fractionator bevatte intern een disk die niet geheel in het midden zat met daarin drie gaten van 1.4mm. De afwezigheid van een centraal gat zorgde regelmatig voor een blokkade, voornamelijk wanneer fibrotisch vetweefsel werd gebruikt voor de procedure. Verder was de originele fractionator herbruikbaar wat minder veilig is voor patiënten dan disposable instrumenten. In **hoofdstuk 4** is een disposable één gats-fractionator ontwikkeld en vergeleken met de originele herbruikbare drie gats-fractionator in het kader van histologische compositie, compositie gebaseerd op CD marker expressie, de mogelijkheid tot het vormen van kolonies door losse cellen en de hoeveelheid geïsoleerde cellen alsmede hun vitaliteit. Er werd geen verschil gevonden in geobserveerde histologische compositie of compositie gebaseerd op CD marker expressie tussen tSVF geïsoleerd door beide typen fractionators. Verder was het aantal enzymatisch geïsoleerde cellen uit tSVF alsmede hun vitaliteit en mogelijkheid tot het vormen van kolonies vergelijkbaar tussen beide fractionators. Daarnaast blokkeerde de procedure uitgevoerd met de disposable één gats-fractionator minder vaak. Deze studie liet zien dat het gebruiksvriendelijkheid van de FAT procedure was verbeterd door middel van de één gats-fractionator zonder verlies van functie.

Eén van de grootste voordelen van de tSVF geïsoleerd door middel van de FAT procedure is het verhoogde aantal regeneratieve cellen in één ml weefsel in vergelijking met één ml onbewerkt lipoaspiraat. Op deze manier kan een hoger aantal regeneratieve cellen geïnjecteerd worden in een gebied met een klein volume zoals gewrichten met osteoartritis. Recent gepubliceerde studies laten zien dat SVF of ASCs effectief zijn in het reduceren van ontsteking bij ontstoken chondrocyten, één van de belangrijkste componenten van osteoartritis. In **hoofdstuk 5** hebben we onderzocht of tSVF geïsoleerd door middel van de FAT procedure in staat is om de pro-inflammatoire staat van chondrocyten te reduceren *in vitro*. In deze studie was de proliferatie van chondrocyten en productie van gesulfateerde glycosaminoglycanen (sGAG) door chondrocyten in co-kweken met enzymatische geïsoleerd tSVF in verschillende ratio's onderzocht. Verder is ook de expressie van interleukine-1β (IL-1β) en cyclo-oxygenase 2 (COX2) onderzocht tijdens co-kweken van tumor necrose factor-α (TNF-α) gestimuleerde chondrocyten met enzymatisch geïsoleerde tSVF. Er werd een significante verhoging in het aantal chondrocyten gezien na eenentwintig dagen kweken wanneer chondrocyten samen gekweekt werden met tSVF in verschillende ratio's, met

name in de ratio's 50/50 en 25/75. De met tSVF samen gekweekte chondrocyten in een ratio van 25/75 waren functioneel gezien de hoeveelheid geproduceerde sGAG was vergelijkbaar met alleen gekweekte chondrocyten. Verder was de expressie van IL-1β en COX2 door TNF-α gestimuleerde chondrocyten significant verminderd door tSVF. Deze *in vitro* resultaten laten zien dat tSVF een anti-inflammatoire en proliferatief effect heeft op chondrocyten.

Het belangrijkste doel van deze thesis was het bestuderen van de mogelijke huidverjongingseffecten door tSVF veroorzaakt door veroudering en verlittekening van de huid. De huidkwaliteit bij mensen verminderd door de jaren heen ten gevolgen van veroudering, fibroproliferatieve ziekten van de huid of door littekens. Deze oorzaken kunnen in twee groepen ingedeeld worden: fysiologische proces (veroudering) of pathologische processen (fibroproliferatieve ziekten of verlittekening). Veel studies beschrijven een potentieel effect van lipofilling op de huidkwaliteit ongeacht of de vermindering in huidkwaliteit initieel veroorzaakt wordt door een fysiologische of pathologische oorzaak. In **hoofdstuk 7** is er een uitgebreid overzicht weergegeven over de literatuur omtrent het anti-litteken effect van lipofilling in zowel klinische als dierlijke studies. Dit overzicht onderstreept de tekortkoming van goed opgezette prospectieve gerandomiseerde klinische studies op het gebied van regeneratieve plastische chirurgie, met name wanneer lipofilling of één van de componenten van vetweefsel zoals ASCs of SVF wordt gebruikt als anti-litteken behandeling. Echter, meerdere case reports en retrospectieve studies laten een mogelijk klinisch effect zien van lipofilling op dermale littekens zoals zachter worden van de huid, verbetering van kleuring en het dunner worden van de huid. Ondanks dat formele bewijzen uit prospectieve gerandomiseerde studies ontbreken, zorgt de data uit case reports en klinische studies er wel voor dat lipofilling alom geaccepteerd is als behandeling van brandwonden. Het mechanisme wat hieraan ten grondslag ligt kan toebedeeld worden aan drie belangrijke processen welke ook allemaal betrokken zijn bij dermale wondgenezing: stimulering van angiogenese, remodellering van extracellulaire matrix en immuunmodulatie. Deze cellulaire processen kunnen allemaal gestimuleerd worden door uitgescheiden groeifactoren, cytokines en eiwitten door ASCs.

Door het ontbreken van goed georganiseerde prospectieve gerandomiseerde klinische studies hebben wij een prospectieve, gerandomiseerde, double blinde, placebo gecontroleerde en multicenter studie opgezet om het effect van tSVF op de vroege wondgenezing te onderzoek. Hiermee proberen we uiteindelijk littekenvorming te voorkomen dan wel te verminderen. In **hoofdstuk 8** is tSVF wederom geproduceerd middels de FAT procedure en daarna geïnjecteerd in de wonden na een mammareductie waarin de contralaterale mamma als placebo controle fungeerde. In totaal zijn

vierendertig vrouwelijke patiënten behandeld (34 vs 34). De resultaten van deze studie laten een significante verbetering zien in de esthetiek van het litteken zes maanden postoperatief. Vele geïncludeerde patiënten bemerkte een snellere wondgenezing in de eerste weken postoperatief van het litteken wat behandeld is met tSVF. Twaalf maanden postoperatief is de esthetiek van litteken vergelijkbaar tussen de tSVF en de controle groep. Dit indiceert dat tSVF waarschijnlijk een significante rol speelt in de vroege wondgenezing maar een minder belangrijke rol heeft als anti-litteken behandeling wanneer het direct postoperatief wordt geïnjecteerd.

In **hoofdstuk 9** is het effect van autologe lipofilling in combinatie met bloedplaatjes verrijkt plasma (PRP) op veranderingen van de huid door veroudering onderzocht in een prospectieve, gerandomiseerde, placebo gecontroleerde, klinische studie. In deze studie was PRP toegevoegd aan lipofilling om ASCs te stimuleren en daarmee het regeneratieve effect van autologe lipofilling te vergroten. Huidkwaliteitsverbeteringen werden gemeten met de cutometer (huidelasticiteit) en een vragenlijst werd gebruikt om de mening van deelnemende patiënten vast te leggen alsmede de snelheid waarmee ze weer terug naar werk konden. In totaal zijn tweeëndertig vrouwelijke patiënten behandeling met of autologe lipofilling met PRP of autologe lipofilling met een placebo controle. Na twaalf maanden follow-up werden er geen significante verschillen gezien in huidelasticiteit of patiënttevredenheid tussen beide groepen. Echter, een regressie analyse van de 'ware huidelasticiteit' parameters als functie van de leeftijd veranderde van negatief naar positief na behandeling met lipofilling in combinatie met PRP. Deze verandering van negatief naar positief was alleen niet significant en alleen zichtbaar op één van de twee meetlocaties in het gelaat. De toevoeging van PRP aan lipofilling resulteerde wel in het feit dat patiënten sneller terugkeerde naar het werk in vergelijking met patiënten die alleen behandeld werden met lipofilling. De conclusie is, is dat lipofilling met of zonder PRP niet bijdraagt aan verbetering van huidkwaliteit. Om de regeneratieve capaciteit van lipofilling met PRP verder te verhogen is tSVF toegevoegd om de verminderde huidkwaliteit ten gevolgen van veroudering te verbeteren. Een vergelijkbare prospectieve klinische studies, als benoemd in **hoofdstuk 9**, was uitgevoerd in **hoofdstuk 10**. In deze studie zijn achtentwintig vrouwelijke patiënten behandeld met lipofiling met PRP in combinatie met tSVF of lipofilling met PRP in combinatie met een placebo controle. Huidkwaliteit verbetering werd gemeten middels de cutometer (huidelasticiteit), de tewameter (transepidermaal verlies van water), VISIA camera om oppervlakkige vlekken, rimpels, huidtextuur, poriën, vaatvoorziening en pigmentatie te meten en de FACE-Q vragenlijsten om de mening van deelnemende patiënten te evalueren. Er werd geen significante verbetering in huidkwaliteit of patiënttevredenheid gezien na behandeling met lipofilling en PRP met of zonder

tSVF. De resultaten in **hoofdstuk 10** bevestigen onze bevindingen uit onze eerder gepubliceerde studie in **hoofdstuk 9** en laten zien dat de toevoeging van tSVF niet leidt tot verbetering van de door veroudering aangedane huidkwaliteit.

In tegensteling tot de resultaten in **hoofdstuk 9** en **10** menen vele studies in de literatuur dat lipofilling of een regeneratieve component van vetweefsel zoals SVF of ASCs bijdraagt aan huidkwaliteit verbetering in het gelaat. Het doel van **hoofdstuk 11** was om systematisch de literatuur door te zoeken naar alle beschikbare studies welke het effect van lipofilling, SVF of ASCs onderzocht hebben op de huidkwaliteit in het gelaat. Medline, EMBASE (OvidSP) en het Cochrane centraal register van gecontroleerde studies werden doorzocht tot mei 2018. Huidkwaliteit verbetering was gedefinieerd als verbetering van elasticiteit, textuur en kleur of histologische verbeteringen. De meeste studies lieten verbetering zien van huidelasticiteit, textuur of een meer homogene kleur van de huid na lipofilling in het gelaat. Echter, bij de meerderheid van de studies ontbrak het aan een goed studiedesign met gevalideerde meetmethodes. Eén studie met een goed studie design liet geen verbetering zien van de huidelasticiteit na lipofilling in het gelaat. Alle histologische studies faalden om een correlatie te laten zien tussen de beschreven histologische verbeteringen en eventuele klinische verbeteringen. Hierdoor blijft het onduidelijk of deze histologische veranderingen leiden tot klinische relevante veranderingen. De conclusie is dat lipofilling, SVF of ASCs injecties in het gelaat niet leidt tot verbetering van de huidkwaliteit.

De belangrijkste therapeutische componenten van tSVF zijn de cellulaire fractie zoals ASCs en weefselfractie zoals extracellulaire matrix. ASCs zijn één van de belangrijkste regeneratieve cellen in de cellulaire fractie van tSVF en in staat om andere celtypes te stimuleren door middel van uitgescheiden paracriene factoren. In **hoofdstuk 12** is extracellulaire matrix geïsoleerd middels een uitgebreid decellularisatie protocol en enzymatisch verkleind met behulp van pepsine om vervolgens een zelf-uithardende hydrogel te worden. Deze hydrogel werd geladen met geconditioneerd medium van gekweekte ASCs welke alle uitgescheiden groeifactoren, cytokines en eiwitten door ASCs bevat om angiogenese alsmede fibroblast proliferatie en migratie te stimuleren *in vitro*. Deze studie laat zien dat extracellulaire matrix geïsoleerd uit tSVF een belangrijke rol speelt in het binden en vrijlaten van groeifactoren uitgescheiden door ASCs op een gecontroleerde manier. Daarnaast zijn deze factoren nog steeds biologisch actief gedurende een langere tijd (stimulatie van angiogenese en fibroblast proliferatie en migratie). Er werd geen verschil gezien tussen extracellulaire matrix geïsoleerd uit tSVF van patiënten met en zonder diabetes. Verder hebben we in **hoofdstuk 5** laten zien

dat tSVF een anti-inflammatoir effect heeft op pro-inflammatoire chondrocyten *in vitro*. Deze bevindingen indiceren dat tSVF in staat is om angiogenese te stimuleren, extracellulaire matrix te remodeleren en om de immuun status te beïnvloeden *in vitro*.

De op extracellulaire matrix gebaseerde hydrogel geladen met uitgescheiden factoren uit ASCs kan wellicht in de toekomst een therapeutische behandeling zijn. Deze hydrogel is namelijk geschikt voor allogene transplantatie en bevat alle regeneratieve factoren uit tSVF. Echter, de productie van op extracellulaire matrix gebaseerde hydrogelen bestaat uit een uitgebreid decellularisatie proces waarvoor cytotoxische reagentia nodig zijn. Kortom, in **hoofdstuk 13** hebben we onderzocht of de hydrogelen cytotoxisch zijn op fibroblasten. Cytotoxiciteit is onderzocht middels een metabolische activatie experiment (MTT) en liet zien dat de op extracellulaire matrix gebaseerde hydrogelen niet cytotoxisch zijn. Daarom kan het therapeutisch klinisch gebruik van deze hydrogelen in de toekomst een interessante mogelijkheid zijn.

DANKWOORD

De afgelopen jaren zijn ontzettend snel voorbij gevlogen waarin ik met veel plezier gewerkt heb aan mijn promotieonderzoek. Dit promotieonderzoek heeft de basis gelegd voor mijn verdere wetenschappelijke en klinische carrière. Echter, zonder de mensen die hieronder vermeld staan had ik deze promotie nooit kunnen afronden.

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Beste dr. Stevens, beste Jeroen,

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Waar Jeroen de aandrijvende kracht was van de drie-eenheid, was prof. dr. Harmsen degene die ervoor zorgde dat we niet uit iedere bocht vlogen.

Beste prof. dr. Harmsen, beste Marco,

Ik weet nog goed dat ik voor het eerst bij jou in het lab kwam. Ik moest van Jeroen ‘even’ aantonen dat er stamcellen in (wat later bleek) SVF zou zitten. Dat ‘even’ is een regelmatig terugkerend woord geworden waarvan jij en ik weten dat ‘even’ in laboratoriumland helemaal niet bestaat. In het begin wist ik helemaal niets van het doen van onderzoek in het lab. De laatste keer dat ik een pipet had vast gehouden was tijdens biologie in 5 VWO. Door jouw enthousiasme voor het vak en jouw uitmuntende skills als mentor, heb ik het vak van onderzoeker snel eigen kunnen maken. Iedere keer als we een meeting hadden gehad, dan liep ik weer met tal van nieuwe ideeën

naar buiten. Dit heeft uiteindelijk geleid tot de vele publicaties die we samen hebben behaald. Initieel, was de regel om bij iedere publicatie een whisky te drinken, maar op een gegeven zijn we de tel 'gelukkig' kwijt geraakt. Ik ben je dankbaar voor de lessen die je me hebt geleerd. Jij bent degene die deze promotie naar een hoger wetenschappelijk niveau heeft getild en mij enthousiast heeft gekregen over basaal wetenschappelijk onderzoek.

Het opstarten van een goed wetenschappelijk onderzoek is 1 ding, maar het vervolgens zo op papier zetten dat geen enkel tijdschrift het meer kan weigeren is het tweede.

Beste prof. dr. B. van der Lei, beste Berend,

Achteraf gezien kan ik mij geen betere promotor wensen dan jij. Ieder moment van de week stond je paraat om vragen te beantwoorden, ideeën door te spreken of manuscripten te reviewen. Op het moment dat ik een manuscript op zondagochtend toestuurde, had ik het over het algemeen op zondagavond om 23:00u terug, in het rood. Jouw mate van productiviteit en de manier waarop je een artikel kon schrijven heeft ervoor gezorgd dat de productiviteit van de bovenstaande drie-eenheid op een zeer hoog niveau lag en nog steeds ligt. Het was daarom ook niet de eerste keer en het zal ook niet de laatste keer zijn, dat jij hoogstpersoonlijk ervoor gezorgd hebt dat een reeds afgewezen manuscript, alsnog geaccepteerd werd in een niet nader te noemen blad. Naast mijn bewondering voor jouw productiviteit, heb ik ook enorm genoten van de kopjes koffie op vrijdagmiddag, diners bij jouw thuis of 'werkbesprekingen' bij Prinsenhof.

Beste dr. Tuin, beste Jorien,

Het is alweer even geleden dat we hebben lopen zwoegen op het lab, maar ik herinner me het als de dag van gisteren. We hebben samen mooie avonturen op het lab beleefd zoals onze mislukte RNA isolaties of de late SVF isolaties welke ook niet altijd even succesvol bleken te zijn. Maar desondanks hebben we mooie projecten opgezet en hebben we ons van onze beste kant laten zien op menig IFATS congres. Ondanks dat ik al even weg ben uit Groningen hebben we nog steeds regelmatig contact en staan er nog mooie nieuwe plannen op de agenda. Samen gaan we de wereld veroveren met SVF (team A&A), maar wellicht dat we binnenkort ook gewoon een keer een rondje kunnen fietsen in je nieuwe jersey!

Beste Linda Brouwer,

Ik heb het een beetje met je te doen, al die SVF samples en huidbiopten die je voor mij hebt moeten verwerken in paraffine en moeten kleuren. Honderden zijn het uiteindelijk geworden de laatste maanden. Desalniettemin was dit wel één van de belangrijkste onderdelen van dit proefschrift. Zonder jouw hulp had ik er wel tien jaar over gedaan. Dank voor je tomeloze inzet en je hulp bij de experimenten op het lab.

Dear (ex)-CAVAREM members,

Thank your for the amazing years in Groningen. I changed Rotterdam for Groningen without knowing anybody in Groningen. However, The moment I arrived I felt home thanks to your hospitality. It was my pleasure to be your colleagues for several years!

Dear Brazilian colleagues, dear dr. Camargo, prof. dr. Felipe, dr. Liguori and dr. Tavares,

Thank you for your hospitality during my scientific internship in Sao Paulo. It was a great experience and I hope I can visit you one day again.

Beste dr. Krenning, beste Guido,

Ik zal de vele biertjes op het terras in Groningen of bij jouw thuis missen. Dank voor alle gezelligheid op het lab en buiten het ziekenhuis. Je haar zit na al die jaren nog steeds voortreffelijk!

Beste Joep Willemsen,

Het is even geleden dat we elkaar gesproken hebben, maar dank voor je hulp bij twee prospectieve studies en de gezelligheid toen je weer even terug was op het lab. De hulp die je me hebt geboden aan het begin van mijn promotie werd zeer gewaardeerd.

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Dank voor jullie hulp tijdens de prospectieve trials die zijn uitgevoerd bij Bergman Clinics. Met jullie hulp is het gelukt om de follow-up van alle cliënten uit de prospectieve studies in goede banen te leiden.

Beste leden van de SVF werkgroep, Marco Harmsen, Berend van der Lei, Jeroen Stevens, Johan Jansma, Rutger Schepers en Jorien Tuin,

We hebben een mooie club opgericht waarmee we ons hard gaan maken voor gedegen onderzoek met SVF. Dank voor jullie energie en tijd die jullie besteden aan deze werkgroep.

Beste Joeri van Boxtel, Jan-Aart Schipper en Linda Vriend,

Jullie zijn inmiddels lang en breed onderweg in de fantastische onderzoekswereld van SVF. Ik ben blij dat Jorien en ik zulke gedreven en goede opvolgers hebben. Jullie tillen het onderzoek weer naar het volgende niveau. Daarnaast was het geweldig om te zien hoe we allemaal ons praatje klaar hadden staan op het congres in Marseille met een mooi stapavond als afsluiter.

Beste miljardairs, beste Jerôme Dirven, Luc Dirven, Emiel van Dongen, Guus Onnink en Steven Plat,

Al meer dan 20 jaar zijn we bevriend met elkaar en delen we lief en leed. Het begon allemaal als vriendengroep die carnavalswagens bouwden in het plaatselijk dorp Oudenbosch. Ik denk dat hier de basis is gelegd voor de plastische chirurgie, alleen zijn de piepschuim poppen vervangen voor levensechte gezichten en lichaamsonderdelen! Inmiddels zijn de carnavalswagens vervangen voor racefietsen en ski's, maar gelukkig komen we nog ieder jaar terug om carnaval samen te vieren (als dat weer mogelijk gaat worden).

Beste paranimfen, beste Steven & Ben,

Jullie ken ik al van ver voor mijn Groningen avontuur.

Steven, volgend jaar vieren we ons 5de lustrum en ik ben benieuwd naar welk restaurant we dit keer gaan. De Prinsenhof was een groot succes tijdens het vorige lustrum, met een mooie afsluiter in de 9de cirkel. Ik ben blij dat we elkaar al zo lang kennen en je bent altijd iemand geweest met wie ik alles kan bespreken, zelfs als het over wetenschap ging! Daarnaast zorg je al jaren voor de ontspanning (fietsen, hardlopen, skiën, feesten) die nodig is om iedere week weer fris te kunnen beginnen in de kliniek of aan wetenschap.

Ben, waar moet ik over jou beginnen. Ik denk dat onze carrière is begonnen bij de Wiener melanges bij het koffiezet apparaat in het oude onderwijsgebouw van het Erasmus MC. Hier waren we sporadisch te vinden als we verplicht practicum hadden, om vervolgens onze medestudenten van commentaar te voorzien. Hier bespraken we dan ook onze eerste ideeën over het doen van het onderzoek. Samen zijn we begonnen bij dr. Stevens om borsten te beoordelen en hier een fotografische schaal van te maken. We hebben

een serieuze poging gewaagd om van onze hobby ons werk te maken. Gelukkig zijn we daarna ieder ons eigen pad op gegaan, jij naar de slokdarmchirurgie en ik naar de stamcellen. Ik wacht met smart op jouw verdediging.

Beste Vincent de Meijer,

Je bent mij inmiddels achterna gegaan naar Groningen en staflid geworden alhier. Het aantal nieuwe publicaties van jou vliegen mij iedere week om de oren en het zal niet lang meer duren voor je professor bent. Ik wil je bedanken voor het feit dat je de afgelopen jaren als mijn mentor hebt gefungeerd. Ik hoop dat ik in de toekomst, bij vragen, nog steeds bij je terecht kan.

Beste Arie Onnink,

Dank voor het voortreffelijk ontwerp van de kaft, geheel passend bij de inhoud.

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Beste Leescommissie,

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Lieve Dion en Ilona Goderie,

Ik wil jullie bedanken voor het feit dat jullie altijd voor Sophie en mij klaarstaan en ons de afgelopen jaar al velen malen hebben geholpen. De klusjes in het huis in Utrecht zijn voorbij, maar inmiddels staat er een nieuwe project in 't Ginneken voor de deur! Reden genoeg om vaak over de vloer te komen.

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Lieve Sophie,

Deze promotie was er simpelweg niet geweest zonder jou en daarom is deze promotie net zoveel van jou als van mij. Al vanaf het begin van mijn geneeskunde studie ben jij erbij. Alle ups heb je samen met mij gevierd en bij alle downs heb je me weer op het rechte pad gezet. Jij bent degene die mij in balans houdt. Verder ben ik enorm trots op jou hoe jij je eigen pad bewandeld en hoe je, je creatieve vaardigheden combineert met het recent behalen van je studie toegepaste psychologie. Gelukkig heb ik ook gebruik kunnen maken van je creatieve brein en heb je de titel en lay-out van dit boekje ontworpen. Ik had dit avontuur niet met iemand anders aan kunnen of willen gaan.

CURRICULUM VITAE AUCTORIS

Joris van Dongen was born on February 25th 1993 in Roosendaal, the Netherlands. After graduating from Markland College Oudenbosch in 2011, he started studying Medicine at the Erasmus University of Rotterdam in September 2011. During his study, he was an active member of student communities and started participating in research projects in his second year of Medical School. From his third year, he started participating in several research projects in the field of Plastic Surgery under the supervision of dr. H.P.J.D. Stevens. After finishing the bachelor, he put his Medical study on hold to move to Groningen and start his PhD in the field of Regenerative Plastic Surgery focusing primarily on adipose mesenchymal stromal cells under the supervision of dr. H.P.J.D. Stevens, prof. dr. B. van der Lei and prof. dr. M.C. Harmsen. Following two and a half year of fulltime research in Groningen, Joris continued with his Medical study with his clinical rotations while continuing his research. In between clinical rotations, he also performed a scientific fellowship between May and June 2018 at the Department of Plastic and Reconstructive Surgery as well as the Department of Cardiovascular Surgery of the University of São Paulo, Brazil under the supervision of dr. C.P. Camargo and prof dr. L.F.P. Moreira. Joris received several grants (Jan Cornelis de Cock Stichting (three times) and KNAW van Waalre Beurs) and presented his research at many national and international conferences receiving several awards. During his final clinical rotation, he was selected for the Plastic Surgery residency at the University Medical Center Utrecht. In September 2019, Joris graduated from Medical School and started working as surgical resident at the Department of Surgery of the St. Antonius Hospital in Nieuwegein and Utrecht as part of his Plastic Surgery resident program.

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