Bridging the gap
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CHAPTER 9

Summary and general discussion
RESEARCH SUMMARY

Lipofilling, i.e. transplantation of autologous adipose tissue to correct soft tissue defects and to enhance tissue regeneration is a technique that has already been widely used in clinical practice for several decades. The cellular and molecular mechanisms that govern the regenerative effects of adipose tissue and its components, however, are still poorly understood.

Adipose tissue in the form of lipofilling is used for breast reconstruction or augmentation, aesthetic or reconstructive facial recontouring, rejuvenation of the hands, buttock augmentation and even to induce healing of chronic ulcers or to prevent formation of new pressure ulcers. In combination with a percutaneous needle fasciectomy, lipofilling in Dupuytren's contractures is as effective as a limited fasciectomy, with shorter recovery times and with a lower number of long-term complications. Freshly isolated stromal vascular fraction (SVF) and culture expanded adipose tissue derived stromal cells (ASC) have also been assessed in clinical trials for cardiac, pulmonary and cartilage repair and treatment of chronic ischemic ulcers and hand function impairment in systemic sclerosis patients. Furthermore, adding ASC or SVF to the grafted fat may improve graft take and survival. The variable outcome of these procedures might be due various factors including indication for which lipofilling is used, the amount of ASC added or differences in the protocol for the procedure. Many fat grafting procedures have insufficient standardization of protocols, and have never been tested in placebo-controlled randomized clinical trials or multi-center trials, which are warranted to improve therapeutic outcome.

Adipose tissue-based therapy seems to be a promising treatment to correct dermal scarring. A dermal scar is an inescapable consequence of trauma or surgical procedures. Damage to the skin is healed by a response in which cells proliferate and connective tissue is generated. Damage is resolved and structural integrity of the skin is restored. In adult human wound healing, original morphology and function is often not fully recovered. In wound healing in the skin, pliability and elasticity of scars are decreased as compared to normal skin. Also, hair follicles, sweat and sebaceous glands are often not restored and therefore absent in scarred skin. Therefore, therapies able to improve repair, regeneration and remodeling, may provide new ways to prevent or reverse scarring; lipofilling is a promising method that may be able to reverse skin fibrosis and excessive ECM accumulation, thereby improving the outcome of dermal wound repair.

Therefore, in this thesis, we investigated the use of adipose tissue-based therapy to prevent or treat dermal scars. Furthermore, in clinical and in vitro experiments and in (systematic) reviews of available scientific literature, we assessed where, when and which adipose tissue-based therapy would be favorable to treat dermal scarring. Moreover, we started to investigate the mechanism behind the anti-fibrotic action of adipose tissue-based therapy to be able to build and improve on the effectiveness of this treatment.

Based on the available scientific literature, lipofilling and ASC as a therapy for dermal scarring is still in its infancy (Chapter 2): evidence for applicability and safety of lipofilling procedures in scar areas has been provided by many case reports and small, non-randomized non-controlled clinical studies. Specifically, lipofilling not only reduces scar-related pain but also to improves scar quality and appearance, i.e. scars appeared to normalize towards healthy skin.

In our own prospective, non-placebo controlled clinical therapeutic study, we investigated the effect of two consecutive sessions autologous lipofilling in patients with symptomatic scars (Chapter 3). We showed that a single lipofilling treatment decreases clinical severity of scarring as rated by the validated patient and observer scar assessment score (POSAS). After a second lipofilling procedure, this decreased even further. Simultaneously, we evaluated histological changes in scar tissues from the study patients. After lipofilling, immune cell influx (T lymphocytes, mast cells and M2 macrophages), increased vascularity as well as epidermal proliferation and normalization of scar tissue extracellular matrix (ECM) were observed.

Use of the SVF derived from adipose tissue is another way to deliver therapeutic cells from adipose tissue, more concentrated and with less volume as compared to normal lipofilling (Chapter 4). The SVF contains ASC, as well supra-advental cells, pericytes, endothelial cells, fibroblasts, erythrocytes and immune cells. Efficacy of ASC in early stages of scar formation has been proven in animal models (reviewed in Chapter 2). The use of ASC in wounds or mature scars speeds up wound healing and reduces formation of fibrotic tissue, as demonstrated in animal studies. Yet, to our knowledge, there are no clinical trials yet that use SVF or cultured ASC to prevent scar formation or reduce existing scars.

Therefore, we set up a design for a prospective double-blind randomized placebo-controlled clinical trial where intraoperatively isolated SVF is used directly after mamma reduction surgery to prevent adverse scar formation (Chapter 5). A total of 38 patients will be included. After mamma reduction surgery, the wound of one breast will be treated with SVF, whereas the other side will be placebo treated i.e. with saline solution. To assess scar severity, POSAS questionnaires and photographs are collected at six and twelve months postoperative. Furthermore, a scar biopsy is taken from both scars at these time points. As primary outcome measure, the patient questionnaire of the POSAS is used, whereas the observer questionnaire and histological analyses of the scar biopsies are used as secondary outcome measures. Currently, patient inclusion for this trial has started. In this specific trial, we use fresh SVF that is generated from liposapirates by means of mechanical dissociation. The strength of this study is that it is a placebo-controlled trial to assess the therapeutic efficacy of processed lipoaspirate, in contrast to most other studies that are uncontrolled.

The large number of devices and protocols are available for mechanical or enzymatic, intraoperative isolation of SVF were only poorly compared at best. This prompted us to compile a systematic
In vitro, we explored if ASC CM inhibits myofibroblast differentiation and activation (Chapter 7), and thus could be translated to future use of ASC or ASC CM as anti-scarring treatment. ASC CM was used to culture normal human dermal fibroblasts (HDF) and keloid fibroblasts in presence and absence of the pro-fibrotic cytokine TGF-β1. In TGF-β1 stimulated HDF, ASC CM inhibited hallmark features of fibroblast activation, i.e. proliferation, increase of cytoskeletal components (F-actin and SM22a), functional contraction and accumulation of ECM. In end stage myofibroblasts i.e. keloid fibroblasts, ASC CM suppressed contraction and collagen gene expression. Thus, in vitro ASC are capable of blocking myofibroblast differentiation and activation.

In the final chapter, we investigated the inhibitory function of microRNA-15b (miR-15b) on non-canonical TGF-β signaling during cardiac fibrosis and cardiac fibroblast activation (Chapter 8). We showed that miR-15b is decreased in vivo in mouse hearts during cardiac fibrosis as compared to healthy control hearts, and is also decreased in vitro in TGF-β1-induced cardiac fibroblast activation. The maintenance of miR-15b in cardiac fibroblasts by the delivery of exogenous miR-15b mimics precludes TGF-β1-induced fibroblast activation, as shown by decreased gene expression of mesenchymal markers αSMA (ACTA2) and Calponin (CCN1) and collagens COL1A1 and COL3A1.

We confirmed the small GTPase intermediates Growth Factor Receptor-Bound 2 (Grb2) and Son-of-Sevenless homologue (SOS) 1 and SOS2 as endogenous miR-15b targets. The prevention of TGF-β1-induced fibroblast activation by miR-15b via the inhibition of non-canonical TGF-β signaling might therefore pose a novel therapeutic strategy to reduce fibrogenesis in vivo.

GENERAL DISCUSSION: WHICH, WHEN, WHAT

Treatment of pathological scars, either superficial (skin) or in organs (heart), requires a firm understanding of wound healing mechanisms as well as the knowledge (I) which therapy is best to use and (II) when and (III) where to do so. This will be addressed and discussed in the subsequent sections.

I. Which?

In this thesis, we evaluated the use of adipose tissue-based therapy for prevention of scar formation or reduction of existing scars and part of the underlying mechanisms. As reviewed in Chapter 2 (Fig. 3) every separate component of adipose tissue might have its own ideal applications in different conditions.

Lipofilling

Lipofilling is the optimal adipose tissue-based therapy when scars are accompanied by a condition where correction of volume or soft tissue defects (after e.g. breast amputation or burn wounds and degloving injuries) or cushioning and protection of underlying tissues (e.g. in prevention of pressure ulcers, or coverage of breast implants) are necessary. In Chapter 3 we showed that our lipofilling treatment (percutaneous scar release combined with lipofilling) was more than just

review in which we compared currently published intraoperative isolation procedures for cell yield, viability of cells, composition of SVF, duration, costs and procedure characteristics (Chapter 4). Thirteen studies with eighteen intraoperative isolation procedures (eight enzymatic and ten non-enzymatic procedures) were assessed. In this systematic review of the literature, we showed that none of these intraoperative procedures differed from non-intraoperative (i.e. culture lab-based collagenase protocol) control groups within the same studies in terms of cell yield, viability and SVF composition. Thus, output of intraoperative isolation procedures was comparable to regular lab-based collagenase isolation protocols. However, the intraoperative isolation procedures were less time-consuming. Furthermore, in this systematic review we explored the differences in composition of different SVF isolation procedures. Enzymatic isolation procedures result in a cell suspension, i.e. cellular SVF (cSVF), because the employed proteolytic enzymes disrupt cell-cell interactions and cell-ECM interactions as well as the ECM itself. Non-enzymatic isolation of SVF results in tissue SVF (tSVF), containing largely intact ECM and cell-cell communications between SVF cells. During non-enzymatic isolation, the adipose tissue is mechanically fractionated, but the tSVF fragments contain intact cell-cell interactions and cell-ECM interactions and intact vascular structures can be present.

In general, ASC are obtained from the SVF after enzymatic dissociation followed by culture expansion of the plastic adherent cells in the seeded SVF. Culture expansion of ASC has both positive and negative implications for clinical application. During in vitro expansion with cell culture media in a two-dimensional environment, ASC undergo phenotypical changes, e.g. in cell surface marker expression and acquire a pro-mitotic, pro-angiogenic, anti-apoptotic and anti-inflammatory secretome. A disadvantage of culture expansion of almost any cell type is that xenogeneic, animal serum products such as fetal bovine serum (FBS) are needed, which are undesirable when ASC are intended for clinical application. To avoid this drawback, we tested the use of human platelet poor plasma (PPP) and platelet rich plasma (PRP) for culture expansion of human ASC (Chapter 6). Both PPP and PRP allow for survival and proliferation of ASC in vitro. PRP increases proliferation and changes gene expression of trophic factors (fibroblast growth factor 1 (FGF-1), insulin-like growth factor 1 (IGF-1), interleukin 1β (IL-1β), transforming growth factor β1 (TGF-β1), vascular endothelial growth factor (VEGF) and Angiopoietin 1 (Ang-1)) and of other factors (fibroblast growth factor 1 (FGF-1), insulin-like growth factor 1 (IGF-1), interleukin 1β (IL-1β), transforming growth factor β1 (TGF-β1), vascular endothelial growth factor (VEGF) and Angiopoietin 1 (Ang-1)) and of other factors (fibroblast growth factor 1 (FGF-1), insulin-like growth factor 1 (IGF-1), interleukin 1β (IL-1β), transforming growth factor β1 (TGF-β1), vascular endothelial growth factor (VEGF) and Angiopoietin 1 (Ang-1)).

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a way to correct soft tissue defects. This treatment promoted and maintained a pro-regenerative immune response and augmented vascularization and epidermal proliferation, and induced remodeling of fibrous scar ECM towards normal skin ECM (summarized in Chapter 3, Fig. 9). At present, it is not possible to determine the exact role of the scar release, the lipoaspirate injection or the ASC within the administered lipoaspirate on the observed improved wound healing.

Stromal vascular fraction
SVF or ASC are two prime candidates for adipose tissue-based therapy in conditions where the resident tissue needs to be restructured towards a regenerative microenvironment. Currently, many devices and systems are (becoming) available for fast, intra-operative isolation of cSVF or tSVF (Chapter 4). The use of intraoperative isolation procedures is more suitable for clinical purposes, because intraoperative isolation procedures are less time-consuming and as efficient as the non-intraoperative isolation (i.e. lab-based) protocols. In addition, current and future legislation poses an increasing burden on therapies that require ‘extraoperative’ procedures. Moreover, these non-intraoperative procedures are very expensive because of the need of Good Manufacturing or Laboratory Practice (GMP and GLP) facilities. Differences between tSVF and cSVF provide another opportunity to individually tailor adipose tissue-based therapy. In certain situations tSVF might be more advantageous since it provides a provisional ECM, which is loaded with growth factors and contains intact vascular structures\(^\text{68}\). Therefore, we use of tSVF for prevention of pathological scar formation after mamma reduction surgery (Chapter 5). tSVF is injected directly after surgery in the wound edges. We hypothesized that addition of tSVF, i.e. therapeutic cells as well as ECM and vascular structures into a wound will speed up the second stage (tissue generation) of normal wound healing and thus might prevent adverse outcomes such as excessive scarring. This trial is still ongoing.

Adipose-derived stromal cells and their conditioned media
Due to these recent developments in SVF isolation procedures, use of culture expanded ASC for clinical application may become obsolete. For other purposes, such as tissue engineering of bone, cartilage, muscle, adipose tissue or tendons and ligaments\(^\text{69-71}\), use of ASC remains a first choice if only because adipose tissue is plentiful and easily accessible, with low donor site morbidity. Based on our findings in Chapter 6, the use of appropriate concentrations of human platelet lysates (PRP or PPP) for culture expansion of ASC for tissue engineering purposes seems to be preferable over the use of xenogeneic products like FBS. In addition to culture expansion of ASC for tissue engineering purposes, PRP and PPP can also be used for the production of ASC CM. As described in Chapter 7, ASC CM has anti-fibrotic properties and thus could be used to prevent and possibly even reverse dermal scarring. Which components in ASC CM are responsible for the observed anti-fibrotic effects, is discussed in the paragraph below, regarding ‘Mechanistic mediators’.

II. When?
Scar formation is always the consequence of the three overlapping stages of wound healing (overview in Chapter 1, Fig. 4). Therefore, to prevent scarring and fibrosis, therapies could be applied during all the three different stages of wound healing, i.e. during the inflammatory phase, the tissue generation phase and the remodeling phase.

Inflammatory phase
The obvious approach would be to intervene in the process of scarring as early as possible, i.e. in the inflammatory phase of wound healing. Platelets and immune cells (mainly macrophages) produce pro-fibrotic cytokines such as TGF-β1 and -β2\(^\text{72}\) during the inflammatory phase of wound healing, which in turn leads to activation of resident tissue cells (fibroblasts and keratinocytes)\(^\text{73}\) and results in progression of wound healing, but also in scarring. It would thus be favorable to modulate inflammation towards an immune response that should results in wound healing without symptomatic scar formation.

Which immune response is ideal for scarless wound healing, remains a matter of debate. In the fetus during the first two trimesters of gestation, scarless wound healing occurs in absence of an inflammatory reaction\(^\text{74}\). Moreover, TGF-β1 - the key growth factor in scarring - is not produced prior to the third trimester\(^\text{75}\). Furthermore, low levels of immune cell infiltration and lower grade inflammation characterize adult wound healing of the oral mucosa and result in virtually scarless wound healing\(^\text{66}\). The scar inducing effect of the immune response is further corroborated by studies with PU.1 mice, which are unable to raise an innate immune response in absence of functioning neutrophils and macrophages. After wounding, a wound healing response is initiated, but scar formation is impaired as compared to normal, wild type mice. Concurrently, levels of IL-6 (a key inflammatory mediator) and TGF-β1 (a key pro-fibrotic mediator) are lower in inflicted wounds in these mice\(^\text{66}\). In immunocompetent mice with wounds, the early inhibition of TGF-β1 with neutralizing antibodies inhibits infiltration of macrophages and finally reduces scar formation\(^\text{66,67}\). Hence, it can be concluded that downregulation of the inflammatory response during wound healing is favorable in terms of scar prevention.

In adult mammalian wound healing, the occurrence of an immune response is a given fact. Specific regulatory immune cells, such as macrophages, augment adult wound healing and scar formation. When human normotrophic scars were compared to hypertrophic scars, it became obvious that hypertrophic scar formation is accompanied by prolonged decreased expression of several pro-inflammatory genes and delayed but prolonged infiltration of macrophages\(^\text{66}\). Even though the causality and the exact mechanism are lacking about these observations, immune cell activity is correlated with hypertrophic scar formation. From fibrosis research literature it is known that different cells from the innate and the adaptive immunity could contribute to or prevent fibrosis. The role of macrophages in fibrosis remains elusive. On the one hand, macrophages are pro-fibrotic during the early, induction stage of fibrosis, and have anti-fibrotic properties during
the late, remodeling phase of fibrosis, where they can aid in (partial) resolution of scarring by degradation of ECM\textsuperscript{70,71}. On the other hand, M1 polarized macrophages are considered to be pro-inflammatory and thus pro-fibrotic whereas M2 polarized macrophages are considered to be wound healing macrophages and produce MMP and down-regulate inflammation via secreted IL-10 and prostaglandin E2 (PGE2), and would thus be anti-fibrotic\textsuperscript{72,73}. Natural killer (NK) cells prevent fibrosis by their ability to kill activated myofibroblasts by phagocytosis and by production of interferon γ (IFN-γ) and IL-10\textsuperscript{71}. T cells have many different subsets, of which T-helper (Th) 1, Th2 and Th17 are among the most studied ones in fibrosis. Th1 cells produce the anti-fibrotic factors IL-10 and IFN-γ, whereas Th2 and Th17 cells produce the pro-fibrotic factors IL-4 and IL-13 and IL-17A, respectively\textsuperscript{73,74}. In conclusion, the immune system is an essential component in adult wound healing that either stimulates or prevents scar formation.

For all of the aforementioned growth factors time, place as well as amount and balance of these factors is essential in scar formation. For example, IFN-γ by itself has an anti-fibrotic effect on resident tissue cells, but an also polarize macrophages towards the M1 phenotype, which is considered to be pro-fibrotic. The same holds true for IL-4 and -13, which are pro-fibrotic by themselves, but can also polarize macrophages towards the M2 phenotype, which is anti-fibrotic. Thus, a properly regulated immune response both in place and time is important to resolve wound healing with minimal fibrosis. Modulation of the immune response towards an anti-scarring phenotype (e.g. attraction of NK cells or polarization towards Th1 cells and M2 macrophages) is expected to result in resolution of wound healing without adverse fibrosis but normal scarring.

Mesenchymal stromal cells (MSC), to which ASC belong, have been widely studied for their immunomodulatory effects (overview in Fig. 1). MSC produce many immunomodulatory factors (e.g. IL-10, TNF-alpha-stimulated protein 6 (TSG-6) nitric oxide (NO), indoleamine 2,3dioxygenase (IDO) and PGE2). MSC inhibit the pro-inflammatory phenotype of M1 macrophages (mf), induce M2 mf polarization, downregulate dendritic cell maturation and function and inhibit the function of natural killer T cells (NKT) cells and γδ T cells (effects of MSC on the innate immune system were extensively reviewed by Le Blanc and Mougiakakos\textsuperscript{75}). Furthermore, MSC can inhibit Th1, Th2 and Th17 T cells and can induce differentiation of regulatory T cells (Treg; effects of MSC on T cell populations were extensively reviewed by Duffy and colleagues\textsuperscript{76}). Combined, these effects lead to downregulation of inflammation in presence of MSC.

In the context of scar formation immune modulation by MSC are favorable. Treatment with MSC during wound healing in mice results in less hypertrophic scar formation, due to TSG-6 production by MSC which suppresses inflammation\textsuperscript{77}. Similar immune regulation by ASC has been demonstrated \textit{in vitro} (e.g. suppression of dendritic cell differentiation\textsuperscript{78}) and \textit{in vivo} (e.g. impairment of Th1 driven inflammatory response, downregulation of inflammatory cytokines and increased IL-10 production in a mouse colitis model\textsuperscript{79,80}), confirming an anti-inflammatory effect of ASC treatment.

![Image](https://example.com/image.png)

**Figure 1** | Simplified overview of the main types of immune cells and their cytokines/trophic factors involved in scar wound healing and scar formation and of the immunomodulatory effects of mesenchymal stromal cells.

\textsuperscript{t}SVF may provide a new way to modulate healing during tissue generation, since it not only provides instructive cells in the form of ASC, but also contains ECM and vascular structures\textsuperscript{81}. In other fields of research, it has already been shown that implantation of specific ECM into the dermis can favorably modulate immune responses. After implantation of decellularized cardiac tissue or bone ECM into a traumatic muscle defect in mice, it was demonstrated that these biomaterials induce a pro-regenerative immune response, consisting of influx of CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells, with skewing towards a CD4\textsuperscript{+} T helper cells and was accompanied by increased gene expression of IL-10\textsuperscript{82}. Under these circumstances, ECM modulates the immune response after wounding. The idea of the possibility to modulate the immune reaction by ECM derived from tSVF is intriguing and should definitely be explored further in future research.

Thus, in the inflammatory phase of wound healing, ASC or cSVF and tSVF are ideal candidates to help steer the immune system towards a pro-regenerative phenotype. In other terms, these
processes seem to help avoid excessive resident tissue cell activation and ECM deposition and limit excessive fibrosis, e.g. scar formation in the skin.

**Tissue generation phase**

During tissue generation, the provisional fibrin matrix of the blood clot that has been formed during hemostasis, is first replaced by granulation tissue and later by (more) permanent ECM. In the skin, the granulation tissue is mainly produced by myofibroblasts and consists of procollagen, elastin, proteoglycans, hyaluronic acid, fibronectin and matricellular proteins. This granulation tissue is further modified during the remodeling phase. Thus the quality and quantity of the ECM produced during tissue generation determines the composition of the scar ECM. Excessive myofibroblast differentiation and activation accompanied by excessive ECM deposition during tissue generation, leads to fibrosis at a later stage.

The composition of ECM during tissue generation also affects phenotype and function of resident tissue cells. Myofibroblasts are especially sensitive to ECM composition and stiffness. By means of cell-matrix interactions or focal adhesions (e.g. integrins and vinculin) fibroblasts and myofibroblasts are able to ‘sense’ the composition of their environment, leading to an intracellular response by means of focal adhesion kinases (FAK). Myofibroblast differentiation and activation is caused by TGF-β stimulation, in combination with ECM tension and upon encounter with ED-A splice variant of fibronectin. Fibronectin is produced by (myo)fibroblasts during tissue generation, thus creating a positive feedback loop of fibroblast activation. Thus, it would be interesting to study the effect of addition of exogenous ECM to the wound environment, to investigate if and how this affects generation of endogenous ECM by resident tissue cells such as myofibroblasts. tSVF contains ASC as well as ECM, but the precise composition of this ECM is still under investigation (unpublished data). Therefore, it would be worthwhile to establish if tSVF inhibits e.g. fibroblast activation *in vitro* or scar formation *in vivo*.

**Remodeling phase**

After completion of wound healing, i.e. when the defect has been closed due to tissue generation and re-epithelialization, the remodeling phase commences. During the tissue generation phase, collagen is mainly produced by myofibroblasts. Intracellularly, pro-collagen α-chains are produced and assembled into a pro-collagen triple helix structure and afterwards, the N-terminal pro-peptide is cleaved from the triple helix. After secretion of the pro-collagen triple helix into the extracellular space, the C-terminal pro-peptide is cleaved, converting the pro-collagen into collagen. Then, collagen can be further modified. Either it can be cross-linked and remains as a part of the ECM, or collagen can be broken down by MMP. During wound healing, mainly collagen type III is deposited, which in term can be replaced by collagen type I. There is a strong relation between these remodeling processes and scarring. Firstly, pathological keloid and hypertrophic scars have an increased amount of collagen crosslinks compared to normal skin or normotrophic scars. Secondly, in pathological hypertrophic and keloid scars the balance between collagen production and breakdown by MMP is dysregulated, favoring ECM accumulation. Finally in pathological keloid and hypertrophic scars there is an increased collagen type III:I ratio, compared to normal skin and normotrophic scars. It would be interesting to investigate if adipose tissue-based therapy can influence the ECM composition (e.g. collagen type III:I ratio, changes in the amount of elastin) or the degree of cross-linking.

**III. Where?**

**Patient selection**

Ultimately we would like to use adipose tissue optimally as treatment modality for dermal scars. An important factor in the efficacy of any therapy is accurate selection of the patient group. In the case of adipose tissue-based therapy for scars we would like to clarify what the efficacy of this therapy is for different types of scars, i.e. symptomatic versus non-symptomatic scars, or pathological keloid and hypertrophic scars versus physiological normotrophic scars.

In Chapter 3 we describe the effect of two sessions of lipofilling to treat symptomatic dermal scars. However, in this study we did not investigate in detail if certain patients and/or certain scar types (hypotrophic, hypertrophic or keloid scars) responded better to this treatment than others. Therefore, in the future it would be interesting to investigate if this treatment works better in specific scar types (e.g. hypotrophic scars accompanied by subcutis defects), or if the treatment is equally effective in all types of scars. Here, we provide an algorithm for selection of adipose tissue-based therapy based on clinical indication (Fig. 2).

Furthermore, we did not analyze which aspects of scars, i.e. the different items of the POSAS (patient scale: pain, itch, color, stiffness, thickness and irregularity / observer scale: vascularity, pigmentation, thickness, relief, pliability and surface area) changed after two lipofilling treatments. Based upon our results thus far, we cannot yet draw a definitive conclusion on the efficacy of lipofilling treatment on separate aspects of scar appearance, i.e. color, stiffness, thickness and irregularity.

**Level of evidence**

Regarding the timing of adipose tissue-based therapy, not only phases of wound healing, but also phases of pre-clinical and clinical trials as well all the overall clinical level of evidence for each component of adipose tissue should be taken into consideration. At what time (when) should we apply adipose tissue-based therapy based on the current level of evidence? Can it already be used routinely in the clinical setting or should it (for the time being) be limited to research settings? Further investigations should either prove or rule out adipose tissue-based therapy as anti-scarring treatment, to avoid the hoax often surrounding ‘stem’ cell therapies.

A useful tool in assessing the evidence for a therapy is the Oxford Centre for Evidence-Based Medicine levels of evidence classification, which consists of five levels: level 1 (highest level) of evidence is provided by systematic reviews of clinical trials, level 2 by randomized clinical trials, level
Many clinical studies after lipofilling treatment for dermal scars have been undertaken thus far. However, no systematic reviews (level 1) or randomized clinical trials (level 2) have been conducted for this treatment thus far: only non-randomized studies and case series (reviewed in Chapter 2). However, no systematic reviews (level 1) or randomized clinical trials (level 2) have been conducted for this treatment thus far: only non-randomized studies and case series (levels 3 and 4) (as reviewed by Negenborn et al.19) are available at this moment. Thus although lipofilling seems to be an effective treatment as observed in many clinical studies, a high level of evidence for scientific proven effect is still lacking. To our knowledge, no clinical studies have been performed using SVF or ASC to treat dermal scars have been conducted. The only randomized clinical trials in the field of adipose tissue-based therapies have been in the use of SVF15 or ASC17 to improve fat graft survival.

**MECHANISTIC MEDIATORS**

Taken together, the results described in this thesis support that prevention and treatment of dermal scars with adipose tissue is feasible. The understanding of the underlying mechanisms is growing but in its infancy still. In fact, placebo-controlled randomized clinical trials (such as described in Chapter 5) are needed to confirm the efficacy of adipose tissue-based therapy. We summarized the scar modulating effects of adipose tissue-based therapy as elucidated in this thesis (Fig. 3). To date, the pillars that mediate influence of adipose tissue-based therapy on scarring processes include trophic factors, extracellular vesicles such as micrORNAs-containing exosomes, and extracellular matrix remodeling.

**Trophic factors**

Known trophic factors that influence fibrotic processes in vitro and in vivo which are also secreted by ASC, include FGF-1, FGF-2, TGF-β3 IGF and HGF55,56,95,96. In vivo, neutralizing antibodies against these factors partially abrogate the anti-fibrotic effect of ASC17, whereas over-expression leads to decrease of fibrosis68. Other trophic factors produced by ASC promote angiogenesis (e.g. VEGF and Ang-1) or proliferation of tissue parenchymal cells (e.g. fibroblast growth factor family members FGF-1, FGF-2 and FGF-7). Inadequate resolution of the wound healing process may occur as the consequence of ongoing (chronic) inflammation. The secretion of immunomodulatory factors by ASC, therefore, may exert a dampening influence on the sustenance of the chronic inflammatory triggers. This will augment the resolution of wound healing and may prevent or reverse scar formation. Prime factors that either suppress the adaptive immune system are PGE2, IDO, TGF-β, TSG-6 and anti-CCL218. In cells of the innate immune system such as macrophages, PGE2 induces secretion of the immune suppressive cytokine IL-10. Their interrelation and mode of action was discussed in the previous paragraph under the inflammatory phase of wound healing.

While ASC are typical culture artifacts, it remains challenging to discern their function in vivo. Volume wise, the major cellular constituent of adipose tissue is the adipocyte, which produces several pro-regenerative factors. In vivo, extracts of minced adipose tissue increased the rate of wound healing in a pig wounding model18 which is partly mediated by adipocytes. In a non-placebo controlled clinical study, lipofilling treatment for chronic diabetic ulcers mediated closure of these wounds, which probably is also partly mediated by factors secreted by adipocytes. Adipose tissue produces several anti-inflammatory (e.g. adiponectin) and pro-inflammatory (e.g. PGE2 and IL-10) factors which promote cell proliferation and fibroblast activity.
leptin) factors\(^\text{511}\), as well as pro-angiogenic factors (e.g. IL-6, VEGF\(^\text{505,510}\)) and pro-mitotic factors (e.g. FGF-2, FGF-7)\(^\text{512}\) that might be involved in the regenerative effect of lipoaspirates.

**Extracellular vesicles and microRNA**

Part of the therapeutic effects of adipose tissue-based therapy is mediated by extracellular vesicles (EV)\(^\text{123}\). Among others, EV comprise exosomes (50-200nm) and microvesicles (MV; 100nm-1µm) and apoptotic bodies (1-4µm)\(^\text{105-107}\). Microvesicles are produced by direct shedding from the plasma membrane, whereas exosomes are produced by fusion of intercellular multivesicular bodies with the plasma membrane. To date, little distinction is made between genuine exosomes and genuine microvesicles: vesicles isolated from body fluids or culture media by high speed centrifugation (100,000xg) are referred to as microvesicles\(^\text{127}\). MV are small cell membrane-derived vesicles that support intercellular communication by delivery of cargo (RNAs and proteins) to the cytoplasm if receiving cells\(^\text{104,105,107}\).

In vitro, MSC-derived MV increase the rate of fibroblast proliferation\(^\text{108-112}\), increase collagen and elastin expression\(^\text{112,118}\), stimulate the expression of anti-fibrotic factors HGF and IGF by dermal fibroblasts\(^\text{114}\) and concurrently inhibit myofibroblast features such as α-SMA expression and contractility\(^\text{111}\). In skin regeneration in animals, MSC-derived MV augment wound healing through Wnt4 induced activation of β-catenin nuclear translocation\(^\text{110}\) and to limit scar formation by MV-mediated delivery of miRs that inhibit TGF-β signaling through reduction of Smad2 activation\(^\text{111}\). In animal models for organ fibrosis, MSC-derived MV reduced liver\(^\text{111}\) and kidney\(^\text{113}\) fibrosis.

Especially miR-mediated effects of MSC-derived MV offer opportunities for anti-scarring and anti-fibrotic adipose tissue-based therapy. In Chapter 8 we showed that miR-15b expression is reduced in cardiac fibrosis in mice and in TGF-β1-activated cardiac fibroblasts. In cardiac fibroblasts, fibroblast activation was inhibited by transfection with miR-15b. Thus, in vitro, miR-15b can act as a (partial) rescue mechanism for TGF-β1-induced fibroblast activation. Discovery of anti-fibrotic miR in combination with adipose tissue-based therapy opens the door to new strategies for anti-scarring and anti-fibrotic therapies. ASC can be genetically modified to overexpress anti-fibrotic miR (e.g. miR-15b) which are then enriched into MV using molecular techniques (e.g. by fusion of the miR binding protein Ago with MV cell membrane proteins such as CD63 or tetraspanins\(^\text{129}\)). From the CM of these miR-15b overexpression ASC, MV can then be isolated and purified and are used as a cell-free, non-immunogenic treatment to prevent or revert scarring or fibrosis.

**Extracellular matrix remodeling**

A key phenomenon in fibrosis is the abundant deposition of extracellular matrix. This ECM usually also differs from physiological ECM in terms of mechanical and architectural properties. Besides trophic factors, ASC secrete a plethora of ECM molecules and ECM processing enzymes including proteases\(^\text{51,56,95}\). These ECM molecules may bind the secreted trophic factors and serve as a slow-release reservoir. The secreted remodeling enzymes might facilitate the breakdown of the fibrotic

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**Figure 3** | Summary of mechanisms of action of different components of adipose tissue based therapy for dermal scarring. ASC and ASC CM inhibit scar formation by A) immunomodulation, B) downregulation of fibroblast activation and ECM production and C) by upregulation of matrix remodeling MMP. Since tSVF, and cSVF contain ASC, similar mechanisms of action can be assumed (dashed lines). Lipofilling can alter mature dermal scars by D) re-initiation of an inflammatory phase, with induction of a pro-regenerative immune response.
ECM, in particular the type I and III collagens. The destruction of the fibrotic matrix paves the way to deposit a physiological pro-regenerative ECM. In Chapter 7, we showed that ASC secrete factors that directly preclude activation of dermal fibroblasts, while their secretion of active MMPs might reflect the observation that lipofilling causes softening and reversal of dermal scars.

In a clinical, albeit non-placebo controlled, study by Jasper and co-workers\(^24\), it was shown that a single procedure of autologous lipofilling not only improves scarring clinically, as indicated by a decrease in POSAS scores, but also increased overall scar tissue pliability. If this change in tissue pliability is only due to repletion of subcutaneous tissue, or if there is also a change in dermal stiffness, has not been studied in this study. As known from fibrosis research, biomechanical cues are important mediators of myofibroblast differentiation and activation\(^44\) and increased tissue stiffness could result in fibroblast activation\(^114,115\). Thus, decrease of tissue stiffness by lipofilling might abrogate pro-fibrotic signaling in dermal scars. The relation between changes in scar tissue stiffness and myofibroblast activity as well as ECM organization, provides an exciting new research topic that warrants further investigation.

### FUTURE PERSPECTIVES: ORGAN FIBROSIS

Fibrosis is a result of tissue injury and adverse repair that can occur in every organ of the body. When tissue damage needs to be restored, resident tissue cells such as the myofibroblasts are cued to produce ECM to close the defect\(^5\) (e.g. in dermal wound healing where quick repair ensures the barrier function of the skin\(^17\)) or to strengthen the tissue (e.g. in myocardial infarction where fast healing prevents rupture of the ventricular wall\(^86\)). Here, a quick response leads to fast restoration of structural integrity, but can also be the cause of organ dysfunction in the long run\(^5\). Even though dermal scarring impairs normal function of the skin, this is not a life-threatening dysfunction. In other organs such as the heart, kidney and liver, fibrotic organ failure leads to more morbidity and mortality. Nearly 45% of deaths in the developed world can be attributed to forms of fibrotic disease\(^5\). Therefore, strategies for preventing fibrosis are also of great value in treatment of organ systems. In this thesis, we have focused mainly on the application of adipose tissue-based therapies for prevention and treatment of dermal scarring. However, the use of large volumes of lipoaspirate in organs is usually impossible in organs and may cause fat emboli. But, application of SVF, ASC or ASC CM is feasible.

ASC have been implemented as therapy in animal models after cardiac\(^117,118\), kidney\(^119,120\), liver\(^121,122\) and lung\(^121,122\) injury to prevent fibrosis with good effect. In all organ systems organ function was better and/or the area of fibrosis was smaller as compared to control. In clinical trials, ASC have been and are applied for treatment of myocardial infarction and chronic ischemic cardiomyopathy\(^113\), acute and chronic kidney disease\(^116\), acute and chronic liver failure with different etiologies\(^127\) and idiopathic pulmonary fibrosis\(^131\). MSC and ASC therapies have been used the longest in the field of cardiovascular research and valuable lessons can be learned from clinical trials that have been conducted in this area of research. Several large randomized clinical trials (among others the APOLLO, POSEIDON, TAC-HFT and C-CURE trails, reviewed by Majka et al.\(^129\)) using different cell types (e.g. ASC, bone marrow derived MSC or bone marrow derived mononuclear cells, or combinations of these cell types), amounts (ranging from \(6\times10^5\) to \(200\times10^5\), or with cell amount based on patient body weight) and different approaches for cell delivery (intravenous, intramyocardial, transcoronary or transendocardial) in various cardiac pathologies, have – not surprisingly – yielded differences in clinical outcome\(^125\). Yet, overall we conclude, with caution, that MSC based therapy in fibrotic and ischemic cardiac diseases seems effective.

An important lesson that can be learned from cell-based therapies in cardiovascular disease is that the timing of the treatment is crucial. In most animal studies, animals receive cell treatment (almost) immediately upon injury, whereas in most clinical studies, cell treatment is not started until days, weeks or even months after injury. In animal studies, differences between cell therapy and placebo control are often very striking, whereas in clinical trials, differences in clinical outcome are less obvious. Since fibrosis on a cellular level in the skin is very similar to fibrosis in other organs, it holds true that everything that is learned from application of adipose tissue-based therapy for prevention and treatment of dermal scarring, also has potential for treatment of organ fibrosis.

### CONCLUSIONS

In this thesis, we have investigated the anti-scarring and possibly anti-fibrotic effects of adipose tissue-based therapy and the role of individual cellular fractions, i.e. SVF, ASC or ASC CM using a multi-disciplinary approach. Lipofilling is a safe and simple autologous treatment for dermal scars. Review of the available scientific literature revealed that the general consensus is that lipofilling improves scar appearance and relieves scar-related pain, possibly by an anti-fibrotic effect of lipofilling on the scarred skin (Chapter 2). Still, placebo controlled trials to definitively confirm effectivity of lipofilling for dermal scarring have yet to be carried out. In our own therapeutic clinical trial (Chapter 3), we have demonstrated that lipofilling treatment (scar release in combination with lipofilling) decreases both clinical patient and doctor perceived severity of the scarring. Moreover, histological analysis also demonstrated improvement of the scar tissues, as shown by increased vascularity as well as epidermal proliferation and normalization of scar tissue ECM. The lipofilling treatment induces a pro-regenerative immune response, as demonstrated by influx of T lymphocytes, mast cells and M2 macrophages. However, the optimal timing of the lipofilling procedure as well as the ideal patient population still have to be defined.

Recent developments in intra-operative procedures for fast generation of SVF allows for direct, one-step application of SVF in various clinical conditions. We came to the conclusion that SVF should be subdivided into cSVF and tSVF. Both types of SVF have their own ideal applications in wound healing and scar prevention (Chapter 4). In our recently set up randomized clinical trial,
we will elucidate if and how tSVF can prevent adverse scar formation after mamma reduction surgery (Chapter 5).

Finally, we consider ASC CM to be an optimal ‘off the shelf’ therapy to e.g. improve healing of chronic wounds or to modulate the immune response towards a pro-regenerative, anti-scarring phenotype in the early phases of wound healing. We have shown that culture of ASC with human platelet lysates (PPP and PRP) is effective, even though the pro-angiogenic properties of ASC CM are decreased by culture with PRP in a dose-dependent fashion (Chapter 6). We clearly have demonstrated that ASC CM inhibits fibroblast activation in normal dermal and keloid scar-derived fibroblasts (Chapter 7).

Exosomes or MV from ASC CM emerge as a cell-free, non-immunogenic adipose tissue-based therapy. In the near future ASC-derived MV can be used as a powerful tool to prevent or revert scarring, by overexpression of specific, anti-fibrotic proteins or miR and enrichment of these constituents into MV. We have shown that miR-15b is decreased in fibrotic hearts and in TGF-β-activated cardiac fibroblasts. Moreover, we demonstrated that overexpression of miR-15b decreases non-canonical TGF-β signal transduction via inhibition of the small GTPase intermediates Grb2 and SOS and inhibits in vitro cardiac fibroblast activation (Chapter 8). Thus, miR-15b overexpression in ASC-derived MV, could be used therapeutically in fibrosis.

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