Dissertation

Autologous Fat Grafting in Reconstructive Breast Surgery

submitted by

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The future belongs to those who believe in the beauty of their dreams.

ELEANOR ROOSEVELT

Statutory Declaration

"I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organizations that have contributed to the research for this thesis.
Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the "Guidelines of the Medical University of Graz on Good Scientific Practice".

Graz, 8th August 2022 Dr.med.univ. Hanna Luze

Disclosures

It is hereby confirmed that (a) all co-authors agree to the use of their published data in the present doctoral thesis and that (b) permission to reproduce figures and tables from their own publications (Luze H, Schwarz A, Nischwitz SP, Kolb D, Bounab K, Zrim R, Winter R, Kamolz LP, Rappl T, Kotzbeck P. Autologous Fat Grafting in Reconstructive Breast Surgery: Clinically Relevant Factors Affecting the Graft Take. Aesthet Surg J. 2022 Jun 20:sjac166. doi: 10.1093/asj/sjac166.) has been granted by Oxford University Press on July 11, 2022, License Number: 5346020180876.

Clinical Trial Registration, Institutional Review Board, and Informed Consent Statement

This clinical study was registered in the U.S. National Library of Medicine (clinicaltrials.gov; unique identifier: NCT05286424). The Ethics Committee of the Medical University of Graz approved the clinical trial protocol with the code 32-487 ex 19/20 on July 27, 2020. The trial and thus the doctoral thesis were conducted according to the Declaration of Helsinki. Written informed consent was obtained from all study participants enrolled.

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Contributions

The following people have supported this doctoral thesis through their expertise in alphabetical order:

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Presentations

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  59. Jahrestagung der ÖGPÄRC; OCT 07-09, 2021; Vienna, Austria

- H. Luze, S.P. Nischwitz, R. Winter, S. Spendel, L.P. Kamolz, P. Kotzbeck, T. Rappl
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Hanna Luze

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Abbreviations

ADIPOQ	Adiponectin
ADSCs	Adipose-derived stem cells
AFG	Autologous fat grafting
asp	Lipoaspirates
BMI	Body mass index
CEBPA	CAAT/enhancer binding protein
DNA	Deoxyribonucleic acid
FABP4	Fatty acid binding protein 4
GLUT4	Glucose transporter type 4
H&E	Hematoxylin & eosin
ICFs	Informed consent forms
ID	Identification
IL	Interleukin
MAP4K4	Mitogen-activated protein 4 kinase 4
md	Missing data
n	No
No.	Number
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PLIN1	Perilipin 1
PPARG	Peroxisome proliferator-activated receptor gamma
PECAM1/CD31	Platelet endothelial cell adhesion molecule
PTPRC/CD45	Protein tyrosine phosphatase, receptor type C
py	Pack years
RNA	Ribonucleic acid
rpm	Revolutions per minute
SEM	Scanning electron microscopy
SD	Standard deviation
SVF	Stromal vascular fraction
SAT	Subcutaneous adipose tissue
TA	Tumescent anesthesia

VEGF	Vascular endothelial growth factor
W	Washing
y	Yes
ys	Years

Abstract in German (Zusammenfassung)

Hintergrund und Zielsetzung: Neben zahlreichen Vorteilen der minimal-invasiven Eigenfetttransplantation stellt die unvorhersehbare Resorptionsrate, welche nur bei ca. 40% des ursprünglich infiltrierten Volumens liegt, einen wesentlichen Limitationsfaktor in der rekonstruktiven Brustchirurgie dar. Für ein zufriedenstellendes Ergebnis sind meist mehrere Eingriffe erforderlich, was für betroffene Patientinnen und Gesundheitssysteme eine enorme Belastung darstellt. Bisher konnte aus zahlreichen Absauge-, Aufbereitungs-, sowie Infiltrationstechniken des autologen Fettgewebes kein Goldstandard für eine optimale Resorptionsrate definiert werden. Ziel dieser Arbeit ist es daher, mögliche negative Einflussfaktoren auf die Resorptionsrate zu eruieren, um zukünftig zu einer Optimierung und Standardisierung dieser wertvollen Methode beitragen zu können.

Material und Methoden: 10 Patientinnen, welche sich einem elektivem Brustaufbau nach subkutaner, nippelsparender Mastektomie mittels Eigenfetttransplantation unterzogen, wurden in diese klinische Studie inkludiert. Mittels nicht-invasiver Lipometrie wurde die Resorptionsrate im transplantierten Bereich eruiert. Im Zuge der Liposuktion wurden außerdem Proben zur histologischen, laborchemischen und elektronenmikroskopischen Analyse entnommen. Daneben wurden diverse Charakteristika der Patientinnen sowie operationsbezogene Faktoren erfasst, um einen möglichen Zusammenhang mit der Resorptionsrate festzustellen.

Resultate: Eine statistisch signifikante Korrelation zwischen der Resorptionsrate und der am Empfängerareal vorhandenen Fettschichtdicke, sowie ein möglicher Zusammenhang mit einem höheren Körpergewicht und BMI sowie mehreren vorangegangenen Eigenfetttransplantationen konnte festgestellt werden. Umgekehrt zeigte sich eine höhere Resorptionsrate bei niedrigerer Expression der Adipozytenmarker FABP4 und PLIN1. In rasterelektronenmikroskopischen Aufnahmen konnten Unterschiede hinsichtlich der Zellschädigung bei verschiedenen Aufbereitungstechniken des Fettgewebes gezeigt werden.

Schlussfolgerung: Durch dieses Projekt konnten einige grundlegende Einflussfaktoren auf die Resorptionsrate detektiert werden. Um diese zukünftig optimieren und die Anzahl der notwendigen Sitzungen minimieren zu können, ist die Durchführung größerer Kohorten Studien essentiell.

Abstract

Background: Autologous fat grafting can be used as an effective tool for soft-tissue augmentation for various indications throughout the body and is particularly popular in reconstructive breast surgery. In spite of the great advantages of this minimally-invasive approach, the unpredictability of graft survival, which is only about 40% of the original infiltrated volume, is a challenge. This inadequate take rate often requires multiple grafting sessions for an optimal, yet unpredictable final result, causing a tremendous burden for patients and healthcare systems. Despite excessively researched, no clear consensus on the optimal technique has been published to date, and well-defined prospective studies on potential factors limiting the graft take are lacking. The present clinical study aims to generate valuable baseline data to help optimize and standardize this valuable method in the future.

Methods: 10 female breast cancer patients undergoing scheduled autologous fat grafting after termination of oncological procedures and subcutaneous, nipple-sparing mastectomy were enrolled. Punch biopsies and lipoaspirates were obtained from the harvest site for histological, gene expression and scanning electron microscopic analysis. The take rate was calculated through sub sequential non-invasive Lipometer measurements determining the thickness of the subcutaneous adipose tissue at the grafted breast. Patient- and surgery-related data were collected and correlated with the take rate.

Results: The take rate correlated statistically significant with the existing mean subcutaneous adipose tissue thickness at the grafted breast before surgery. Furthermore, an approximate correlation between the take rate and the number of prior grafting sessions, body weight and BMI were observed. No statistical significance was reached regarding the Take Rate and patient age, harvest site, or the mean adipocyte size. Furthermore, a strong indirect correlation was observed with the expression of the adipocyte markers FABP4 and PLIN1. Scanning electron microscopy revealed lower levels of cellular damage in samples of washed lipoaspirates.

Conclusions: We were successful in identifying factors correlating and affecting the take rate. Future studies focusing on the clinical relevance of each potential factor impairing the outcome and determined through this trial are essential to help in the definition of a gold standard.

1 Introduction

1.1 History and application areas of autologous fat grafting

Subcutaneous adipose tissue (SAT), as a soft and deformable, autologous tissue, is usually present in large amounts in the human body and represents ideal filling material for the correction as well as remodeling of profile and volume defects (1). The first attempt of adipose tissue transfer was performed by Van der Meulen in 1889, who grafted autologous fat to treat a diaphragmatic hernia (1,2). In the following decades, autologous fat grafting (AFG) became popular among various medical specialties, and SAT was transplanted to a variety of cutaneous and subcutaneous defects, while establishing different techniques (1). Early work by the authors Neuber, Czerny, and Holländer report satisfactory, natural-looking results in facial and breast reconstruction (3–6). However, later reports indicated difficulties with varying degrees of asymmetry caused by fat resorption (6,7). A radical change in the history of AFG is due to the publications of Coleman *et al.,* who modified and corrected the methods of his predecessors and emphasized the technique of AFG, including the harvesting, processing, and reinjection of autologous fat, as substantial to a successful and satisfactory outcome (8– 12).

Today, AFG has become a popular and common technique in aesthetic as well as reconstructive surgery as an effective tool for soft-tissue augmentation (13). Host compatibility, favorable safety profile with low morbidity, low cost, longevity, and good availability of adipose tissue are just some of the many advantages of this minimally invasive technique (14). However, the unpredictability of the outcome remains a major challenge for healthcare providers (15).

Areas of application include a broad spectrum of tissue augmentation at a variety of anatomic sites such as:

- Face: facial lipoatrophy (e.g. Parry Romberg syndrome), scleroderma, nasal deformities, facial rejuvenation,
- Breast: micromastia, postaugmentation refinements, postoperative breast deformities, postmastectomy reconstruction, congenital breast or chest defects (e.g. Poland syndrome, tuberous deformity),
- Hand: Dupuytren's contracture, Raynaud's syndrome and hand rejuvenation
- Skin and soft tissue: pathological scars (e.g. burn injuries), body contouring and skin rejuvenation
- Other applications: laryngoplasty, mastoidectomy defects, gluteoplasty, etc. (6,14)

1.2 Principles of autologous fat grafting

1.2.1 Composition of autologous fat grafts

Fat cells or adipocytes account for only 20% of cell amount in fat grafts, but their volume account for more than 90% of the volume of adipose tissue (16,17). The remaining 80% of cells are accounted for by the stromal vascular fraction (SVF), a heterogeneous mixture of lymphocytes, fibroblasts, macrophages/monocytes endothelial cells, pericytes in an extracellular matrix, and, more importantly, adipose-derived stem cells (ADSCs) (16,18). Although adipose tissue was initially mistaken for an inert material to store energy, recent findings have demonstrated various regenerative capabilities (17). The regenerative potential is attributed to the content of ADSCs and includes peripheral nerve regeneration, angiogenesis, improvement of dermal elasticity and thickness, or reversal of fibrosis among others (17). More importantly for AFG, SVF cells are able to proliferate and differentiate into new adipocytes after grafting into a new area, and also tolerate harsh conditions at the recipient site such as inflammation or ischemia (16). On the other hand, mature adipocytes tend to be very fragile, leading to apoptosis or necrosis of this cell fraction during or immediately after grafting (16). The potential loss of mature adipocytes contributes the volume loss, which is typically observed in the initial phase after AFG (16). Both initial volume loss by adipocyte apoptosis as well as adipocyte differentiation by SVF cells are key mechanisms for volume maintenance in the long term (16).

1.2.2 Limitations of AFG

Despite several advantages of this minimally invasive technique, there are still issues such as the unpredictable graft survival and adipose tissue stability, that limit the use of AFG (15). Since necrosis and reabsorption processes may account for a volume loss of 40-60%, several AFG sessions may be required in order to achieve optimal results (15,19,20). The final take rate is largely dependent on the balance between adipose tissue regeneration and reabsorption and may be affected by a variety of factors, including the specific procedure used, the amount of fat grafted, and the graft microenvironment which are discussed below (21).
Inadequate take rate often requires multiple grafting sessions to achieve an optimal, yet initially unpredictable outcome, which is particularly evident in reconstruction after mastectomy (22).

Despite the increasing use of AFG and the excessive research on the subject, the actual mechanism of how adipose tissue grafts survive is not fully understood (23). To reach long-lasting and desirable results, understanding the biology and scientific principles underlying adipose tissue, as well as corresponding adjustments of methods in adipose tissue transfer are crucial (21).
Currently, three main theories of fat graft survival after avascular surgical transplantation have become accepted and available evidence assumes, that each theory may be involved in the process of graft survival (24).

1.2.3 Cell survival theory

Cell survival theory is a well-established older theory of autologous fat graft survival after *in-vivo* transplantation introduced by Peer in the early 1950s (25). It still is referenced in many trials today, it is by far the most popular theory (23) and reads as follows: "*In humans the cells in free autogenous grafts tend to survive and retain their normal tissue structure when transplanted as complete cell entities in favorable transplantation sites. When the cells in free grafts fail to survive, the graft is replaced by connective tissue but this replacement is not a duplicate of the original graft."* (25)
This theory assumes, that transplanted grafts initially survive by nutrient diffusion from plasma at the recipient site, until neovascularization occurs (21). The grafted adipocytes can survive only by a sufficient new blood supply (21). A mouse study by Zhao *et al.* in 2012 proved this theory by demonstrating direct survival of autologous fat after transplantation through neovascularization in the recipient site (23,26).
According to this theory, small-volume grafts result in better survival than large-volume grafts because complete diffusion and perfusion may be easier to achieve (25,27).
Viable endothelial cells lining blood vessels in autologous fat grafts are considered relevant in establishing early circulation and perfusion of the grafted tissue (25). Furthermore, a higher amount of transplanted viable adipocytes is considered relevant for a higher retained volume of autologous fat grafts in the long-term (25,27). To ensure a larger amount of viable adipocytes and SVF cells, a harvesting and injection technique that is as atraumatic as possible should be preferred (23). In order to generate a concentrated and uniform mass, the authors recommend removing oil, water and erythrocytes when processing autologous fat grafts (23).
Despite its logic and popularity, not all effects of AFG observed in clinics can be explained solely by this theory, indicating additional mechanisms of graft survival (23).

1.2.4 Graft replacement theory

According to the graft replacement theory studied in detail by Suga *et al.,* a small number of donor adipocytes survive the full transplantation process because of the hypoxic environment of the host tissue at the recipient site.(21,28,29). A large amount of grafted adipocytes is replaced by more stable donor ADSCs, which are attributed to the SVF (21,28,29). Because adipogenesis and angiogenesis can be attributed to the SVF (16), multiple trials have investigated the effect of fat graft enrichment with SVF and especially with ADSCs to date (21). Most of these studies have shown auspicious results regarding an improved graft retention, however, the authors emphasize the great demand for additional clinical trials to substantiate this claim (21,30).

1.2.5 Host replacement theory

The host cell replacement theory of Yoshimura *et al.* states that grafted cells do not survive but are completely replaced by recipient cells (21). The authors demonstrate early adipocyte death and sub sequential replacement by fibrous tissue and new adipocytes by activation and regeneration of ADSCs and progenitor cells, and by neovascularization from the recipient site (23,31).

Therefore, several authors consider recipient site integrity and environment as important determinants of graft survival (21,32). To date, a variety of recipient site preparation methods have been evolved and have shown promising results in terms of graft retention (32).

1.3 Technique of autologous fat grafting and potential surgery-related compromising parameters

Although AFG is now a common procedure, the unpredictability of outcome remains a major challenge for health care providers (15). This unpredictability can largely be attributed to the technique of AFG, which typically involves harvesting adipose tissue, procession and reinjection to a different site (1). To date, a variety of different techniques have been presented, whereas no clear consensus on a gold standard has been reached yet (15,22). This may be due to inconclusive results on numerous procedural variables that may affect the quality of AFG and thus the outcome (14). Some impairing (mainly mechanical) variables have been extensively studied, whereas other parameters potentially affecting outcome have been less studied or even neglected (16,18). The following sections discuss current techniques of AFG and what is known about risk factors that affect outcome.

1.3.1 Harvesting

Reviewing and comparing the various harvesting techniques presented thus far is challenging given the enormous outcome variables and numerous factors that must be considered in each method described (33). Despite ongoing discussions on how to obtain the most viable and functional cells for grafting, no superior harvesting technique has been defined to date (34). Details such as the donor-site, usage of tumescent anesthesia, cannula diameter, or the use of power- or ultrasound-assisted liposuction may have a significant impact on both the initial survival of adipocytes during the harvesting process and the differentiation ability of SVF cells after harvesting and transplantation (16). These additional factors need to be considered to define a gold standard and achieve the highest take rate possible (33).

Harvest site

Abdomen, flanks, thighs, and knees are the most commonly used harvest sites (33). Several authors reported no significant differences between autologous fat grafts harvested from different donor sites regarding weight, volume, or histologic characteristics such as integrity, inflammation, fibrosis, cysts and neovascularization in preclinical (35,36) as well as clinical trials (37). Although the abdomen is considered the best harvest site due to its higher yield (13,20,38,39), clinical studies have specifically not demonstrated a significant difference in the take rate of grafts harvested from the abdomen or other donor sites (40).

However, an age-related difference in adipocyte viability was observed by Geissler *et al*: The authors reported a higher viability of adipocytes in the lower abdomen compared to the flanks in younger patients, whereas this difference was not observed in older patients (41). In contrast, adipocytes harvested from the flanks showed a higher viability in older patients whereby no difference was observed for the inner thighs (41). Depending on the age of the patients, harvesting from the lower abdomen and flanks could optimize the take rate because of the higher viability of adipocytes, whereas harvesting from the inner thighs can always be considered (39), but further trials are required to confirm this theory.
Li *et al.* stated that currently no ideal harvest site can be defined and the selection should be made depending on the ease and safety of access followed by patient preference (35). Nevertheless, the quality of autologous tissue may be affected by prior local radiation or surgery, including prior liposuction and must always be considered by the surgeon (14).

The use of tumescent anesthesia

The original harvesting technique introduced by Coleman *et al.* (8), although modified several times, is associated with less trauma during liposuction and is still recommended as the method to be preferred for harvesting autologous fat (34). The establishment of "wet" and "super wet" harvesting approaches aimed to reduce the substantial damage to adipocytes and SVF cells during the harvesting process (34,42). The super wet liposuction technique introduced by Fodor *et al.* uses larger volumes of tumescent solutions in a 1:1 infiltration to aspiration ratio, (43) and is considered advantageous and safe in larger liposuction volumes because of the lower blood loss (42). Although typically smaller harvesting volumes are required for AFG purposes, the graft retention may still be improved using super wet techniques due to potentially lower mechanical stress (42) during the harvesting process. On the other hand, there is controversy as to whether the use of local anesthetics in the harvesting process, either with or without a vasoconstrictor, impairs (6,44) or enhances adipocyte and SVF cell viability (45). In 2012, Weichman *et al.* reported no significant effects of local anesthetics on long-term graft survival (46), contradicting the original hypothesis of Moore *et al.* in 1995, who suggested an inhibition of adipocyte growth through lidocaine (47).

In clinical practice, lidocaine is the most widely used local anesthetic to anesthetize the donor and in some cases also the recipient site (48). Apart from its use in general liposuction, concerns have been raised about its suitability for AFG because of other reports of cytotoxic effects (49).

However, these results are mainly from *in-vitro* studies showing for example a dose-dependent negative impact of lidocaine on the proliferation capacity and viability of fibroblasts as well as ADSCs (48,50). Despite the current generally accepted presumption that a single injection of local anesthetics does not affect the effective dose of ADSCs in a clinical setting (50), the expression of adipocyte and other cell markers could be altered (51). Harvesting-related differences in cell markers may in turn serve as an indicator of the vitality and quality of autologous fat grafts (51).

Harvesting cannula

Various characteristics of the cannula, such as diameter, arrangement and number of holes, may affect graft survival and have been extensively studied (33).

Apart from the finding that the inverse relationship between cellular damage and cannula diameter is a widely accepted assumption (33,52), multi-perforated, large-bore cannulas may be beneficial to avoid structural destruction (53,54) and preserve the natural structure of autologous tissue (55). An enhanced cell viability in grafts harvested with a 6-mm cannula compared to smaller diameters such as 4- or 2-mm was for instance demonstrated by Erdim *et al.*, while other authors even reported an increase in histologic integrity and decrease of immune infiltration and fibrosis under the usage of larger diameters (6,56). Although there is still no clear consensus on the qualities of a harvesting cannula, the authors conclude that a sufficiently large cannula should be used to avoid shear forces while preserving the viability and vitality of both, adipocytes and SVF cells (33).

Power- and/or ultrasound- assisted liposuction

Applied negative pressure during the harvesting process may theoretically affect the viability of adipocytes as well as SVF cells through shear stress (14). However, the current literature suggests a wide range of pressure values ranging from mild pressure through the use of manual syringes (14,57,58) to approximately 750 mmHg (14,57,59,60).

Several studies examining the effects of manual syringe aspiration, power-assisted or ultrasound-assisted liposuction also suggest differential effects on cell viability and adipocyte functionality (6). Although hand-held liposuction is often considered superior to the power-assisted technique, Keck *et al.* did not find a significant difference in cell number or viability comparing both techniques (61).

In addition to the widely used power-assisted liposuction, the present findings on ultrasound-assisted liposuction provide promising results for application in AFG: *In-vitro* analyses of human SAT, harvested through the power- or ultrasound-assisted liposuction demonstrated equivalent results regarding cell viability, yield, surface markers, phenotype as well as proliferation and differentiation capacity of ADSCs (62). These results were confirmed by another trial quantifying the viability of adipocytes in lipoaspirates harvested with ultrasound-assisted liposuction, suggesting that this technique is potentially suitable for AFG (63). Nevertheless, a clinical impact on the graft survival and predictability of outcome as well as potential superiority over other techniques currently used, remains to be elucidated.

1.3.2 Processing

Processing of lipoaspirates is crucial because they consist not only of beneficial adipocytes and SVF cells but also of collagen fibres, debris and blood (34,64). Such components can cause inflammation and subsequent degradation of the transplanted tissue at the recipient site (34,64). In particular, injection of debris is problematic, since it gives a false impression of the volume potentially achieved, but is reabsorbed after several hours (34,64).

Removal of these components can be achieved by processing of lipoaspirates, whereby a variety of techniques such as gravity separation, centrifugation, washing and/or filtration have been presented to date (6). A large survey among members of the American Society of Plastic Surgeons in 2013 revealed, that 45% of surgeons, performing AFG in breast surgery use gravity separation while 34% use centrifugation as well as filtration and 11% use gauze rolling (20). 7% performed other techniques not specified and only 3% did not process lipoaspirates at all (20).

Verifiably preservation of higher concentrations of SVF cells, particularly ADSCs during processing of autologous fat grafts is assumed to enhance the graft retention (65). Establishing of a gold standard for autologous fat graft processing is therefore essential for optimized outcomes (6).

<u>Gravity separation, decantation and sedimentation</u>

Gravity separation, decantation, and sedimentation as the most common processing technique (20), leads to a division of the autologous fat into its three phases of fat, oil and water over time (66,67). After disposal of the oil and aqueous layers, the only the fatty layer is extracted for reinjection at the grafting site (66,67). To date sterile, closed systems are available in which the harvested fat and oil layer remain on top while the aqueous layer can be removed though a tube at the bottom of the container (67). These devices are easy to handle and intended for single use, which speeds up the AFG process (67).

Critics of this method point to possible erroneously higher fat injection volumes diluted by unwanted components if sufficient separation is not maintained (67). In addition, studies on different processing methods found the lowest percentage of fat tissue in gravity separation, decantation, and sedimentation, although lipoaspirates obtained with this approach have the highest volume yield (68). The higher volume yield may be due to the great amount of oil, liquid and debris remaining in the lipoaspirates (68).

Centrifugation

Centrifugation is now a very common technique (20) and was first introduced by Coleman *et al.* in 1987 (8). The original technique involves the separation of lipoaspirates by centrifugation at high speeds (3,000 revolutions per minute (rpm)) for 3 minutes, draining tumescent solution and blood from the bottom layer (8,67) Afterwards the oil is decanted and soaked up with a cotton pad for 3 minutes (8,67).

Although centrifugation is less popular, clinical studies have demonstrated favorable outcomes compared to gravity separation (6), possibly due to a higher concentration of ADSCs in the processed fat (67,69). Evidence suggests, that higher concentrated lipoaspirates lead to slower reabsorption processes and increased take rates over time through vasculogenic mechanisms dependent on ADSCs and vasculogenic mediators (67,70).

However, similar to negative pressure applied during power-assisted liposuction, the positive pressure occurring during centrifugation may also affect the quality of harvested autologous fat (6,71,72). Multiple studies address different centrifugation settings (67), and interestingly, one *in-vitro* study found the same amount of viable cells in the 500- and 1,300 rpm setting, despite an increase in peripheral damage at higher speeds (73). The authors preferred a setting of 1,300 rpm *in-vivo*, which did not show any reabsorption signs one year after grafting (73). Several other trials have also shown no effect of different centrifugation settings on the quality and viability of lipoaspirates (37,74), but comparison of these data is difficult due to the lacking standardization of centrifugal speed, force, duration, and setting reports (67).

Washing and filtration

Autologous fat grafts can be processed by washing in physiological solutions and/or following filtration, for which a closed system is normally used (67). According to the survey among members of the American Society of Plastic Surgeons, washing and filtration is as frequently used as centrifugation (20) and appears to be a feasible, more attractive option, particularly for large-volume grafting (75).

Similar to previous techniques, washing of lipoaspirates aims to eliminate unwanted components such as oil, blood or debris (34,64,67). Washing with lactated Ringer's solution or normal saline or is usually performed multiple times, whereas filtration of lipoaspirates is performed through membranes with different pore sizes, depending on the product used (67). Several closed systems that combine washing and filtration are commercially available, such as the PureGraft (Cytori Therapeutics Inc., San Diego, California, USA), REVOLVE (LifeCell

Corp., Branchburg, New Jersey, USA) or LipiVage (Genesis Biosystems, Lewisville, Texas, USA) (67) but they are often criticized as very expensive (76).

Previous trials have investigated the composition of lipoaspirates processed with the PureGraft and REVOLVE system (77). The results showed that both systems can achieve higher and more consistent adipose tissue content with a significant lower amount of compromising components than gravity separation, decantation and sedimentation, or centrifugation (77). The REVOLVE system also preserves a significantly higher amount of viable adipocytes as well as SVF cells compared with other processing methods (68). Similar results were obtained in a comparative analysis with the LipiVage system: a higher amount of viable adipocytes as well as higher intracellular enzyme activity was observed compared with centrifugation (59). The higher yield of viable cells might be accredited to the less traumatic nature of this technique (1). Recent data by Valmadrid *et al.* also show that the use of a closed washing system for processing autologous fat grafts was associated with a lower rate of fat necrosis, also indicating a less traumatic application (78).

Gauze rolling

Cotton gaze rolling may be used as a method to isolate harvested fat grafts and absorb undesired components of the lipoaspirate with different cotton gauzes available (67). Harvested autologous fat is usually placed on the cotton gauze before being rolled back and forth over the gauze with, for example a scalpel, absorbing excess oil and aqueous layers and leaving behind the cellular components (67). Similar to centrifugation, this process takes approximately 2-4 minutes, therefore limiting ischemia time (69). Other advantages include minimal trauma to adipocytes and SVF cells as well as low costs (67). However, especially for large-volume grafting, cotton gauze rolling can be more time-consuming than centrifugation (67,75).

Interestingly, preclinical *in-vivo* studies of processing techniques have shown that SVF cell yield and graft retention are highest with the cotton gauze rolling technique compared with centrifugation and filtration (65). However, the exact method by which cotton gauze rolling improves lipoaspirate quality and subsequent outcome, as well as a potential superiority over other processing methods remain to be determined (65).

1.3.3 Preparation of the recipient site

Various authors consider the tissue integrity and environment of the recipient site a major determinant of fat graft survival (21,32), but existing evidence is inconclusive (6). A recent review by Oranges *et al.* identified multiple recipient site preparation methods: external volume expansion,, administration of cell-proliferating factors such as vascular endothelial growth factor (VEGF), SVF or interleukin (IL)-8), iatrogenic ischemia, microneedling, or implantation of alloplastic material (32). Most of these techniques aim to maximize oxygen tension as well as nutrition at the recipient to create favorable conditions before grafting of autologous tissue (79).

External volume expansion

External volume expansion involves the application of external expanders such as the BRAVA system (Brava LLC, Miami, USA) (80,81). This bra-like device creates a low negative pressure of up to -80 mmHg and is usually worn for 10-24 hours/day 2-4 weeks before surgery (82). The application of negative pressure leads to stretch, edema, and inflammation of the tissue, which is related to cell proliferation and neoangiogenesis (32). As a result, a proadipogenic effect with an increased density of subdermal adipocytes and increase of tissue blood vessel density can be observed (32). In addition, an increase in parenchymal space, a decrease in interstitial pressure in the breast, and a decrease in contour irregularities are noted (81). Most of these effects are stronger expressed with prolonged application of negative pressure (82,83).

The results on outcome after external volume expansion followed by AFG were promising, with take rates ranging from 53% to 82% (82). Moreover, external volume expansion appears to be suitable even for mega volume grafting and to be a valuable alternative to the use of implants for reconstructive or aesthetic purposes (82). However, Oranges *et al.* criticize the difficult comparability of different study settings and the low level of evidence and emphasize the need for future studies (82).

Administration of cell-proliferating factors

Several preclinical studies showed promising results following the administration of cell-proliferating factors, such as improvement of the take rate, vascularity and cell proliferation, as well as a decrease in cyst formation (32,84–86). In most trials, VEGF was administered as a strong stimulator for vascularization to improve fat graft viability (86,87). The authors also postulated that enrichment with SVF could improve the microenvironment at the recipient site

through dynamic reassembly of blood endothelial cells (85). Despite potential beneficial effects, administration of cell-proliferating factors was not associated with significant, clinically relevant increases in graft weight or viability after AFG (79). Furthermore, no clinical trials investigating this technique exist to date (79).

Ischemia

Iatrogenic remote ischemia can reduce graft ischemia and reperfusion injury, and has already shown promising effects in organ transplantation (88). According to Gassman *et al.* inducing ischemia at the recipient site before AFG may improve the take rate and limit subsequent liposclerosis (89). The authors studied the effects of intermittent, temporary hindlimb tourniquet application at the recipient site within three cycles of 5 minutes in a rat model (89). The best overall adipose cellular viability and native architecture were observed when recipient site preconditioning was performed (89). However, to date there are no clinical trials confirming this effect in humans.

Microneedling

In preclinical trials of recipient site preparation with microneedling, a device where hundreds of microneedles or 18-gauge needles were attached was used to ablade the subcutaneous tissue in a crisscross pattern to generate a maximized surface area before AFG (79,90,91).
The authors demonstrated increased vascularity and a significant decrease in inflammation following AFG; but despite these supposedly beneficial effects, cell proliferation and fat graft viability did not improve significantly (79,90,91).

Implantation of alloplastic material

According to a preclinical study in rabbits, insertion of silicone sheets and induction of capsule formation may result in higher vascularization of the recipient site and potential higher graft retention (92). Baran *et al.* observed a higher take rate in groups with prior implantation of silicon sheets (92), but there are no studies investigating the efficacy of this technique in humans to date. Moreover, this approach requires an additional session, whereas the aim of current research is to reduce the number of sessions required for an optimal outcome by optimization of the technique in AFG (92).

1.3.4 Grafting

Reinjection of (processed) lipoaspirates requires great care in order to achieve aesthetically and functionally pleasing outcomes (6) and is usually performed through a small skin incision (79). It is recommended to inject small aliquots of lipoaspirates in a fanning out, crosshatch pattern during withdrawal of the cannula (8,11,79). Lee *et al.* further recommend a slow injection speed of 0.5 to 1.0 ml/second resulting in smaller aliquots in comparison to faster injection speed of 3.0 to 5.0 ml/second (71). Slower injection of smaller aliquots maximizes oxygenation and perfusion of the graft (79) and reduces damage on a cellular level caused by sheer forces (71). Smaller gauge cannulas for grafting autologous fat are considered beneficial to minimize the trauma at the recipient site, as complications such as hematoma formation may affect graft oxygenation and thus the take rate (79). In contrast, other authors suggest the use of cannulas with larger diameters for both aspiration and injection in order to minimize damage on adipocytes and SCF cells and improve their viability (55). Further studies investigating potential effects of different cannula diameters on cell viability are of paramount importance to establish a standard technique of reinjection.

Grafting can be performed subcutaneously, intramuscularly or in a multiplane technique to maximize the distribution area and avoid excessive interstitial pressure at a given point on the recipient site (79). Early reports by Nguyen *et al*. demonstrated increased vascularization and better outcomes following grafting to the muscle (93). However, depending on the recipient site, outcomes may be compromised by increased mobilization of lipoaspirates in muscle compared with SAT (94).

The volume of lipoaspirates injected depends mainly on the existing volume and space at the recipient site (79). However, it is generally assumed that a large volume transplanted into a confined space may compromise capillary perfusion and that densely packed graft droplets coalesce into lakes with poor graft-to-recipient interface, ultimately impairing the graft take (95). Safe injection of larger volumes may be feasible via recipient site preparation with external volume expansion as described in section 1.3.3.

1.4 Patient-related compromising parameters

Despite the discovery of several surgery-related risk factors for impaired graft survival, the remaining variability in outcomes suggests a more prominent dependence of viability and functionality of ADSCs and SVF cells on individual patient characteristics (96). The existing evidence primarily shows significant discrepancies in the number of ADSCs and SVF cells between patients, which may account for the varying results (6,97). A variety of compromising factors such as patient age and BMI, diabetes mellitus type I and II, HIV status, as well as cancer treatment including radiotherapy, chemotherapy, and tamoxifen use have been previously noted (96) and are discussed in the following sections.

However, most studies on potential patient-related risk factors have been of small sample size and have rarely examined a clinical relevance. Further large-cohort studies are necessary to examine whether these findings can be translated into clinical application and impact long-term graft survival. In a clinical setting, other individual impairing variables related to the recipient site, such as previous trauma, burns, scars, or structural defects must always be considered when planning elective AFG (82,98,99).

Age

Early trials examining age-related changes in ADSC and SVF yield were inconclusive (41,100) but current studies quantifying levels of gene expression have reported a significant decrease in overall cell yield with age (96,97,101). Clinical studies by Madonna *et al.* and Zhu *et al*. also report decreased angiogenetic capacity and osteogenic potential with age (102,103). In comparison, other authors have not found a significant connection between the proliferation capacity of ADSCs and the patient age (104). Apart from affecting the yield of nucleated cells, age may also be associated with poorer revascularization, possibly affecting the take rate, so that AFG results may be superior in younger individuals (6).

Body mass index

Obesity is associated with an increased risk of developing a number of comorbidities associated with the metabolic syndrome (105). Yet, the question of whether adipocyte differentiation and proliferation capacity is affected by BMI is still debated (105).

The majority of available studies report an impact of increased BMI on the viability and function of adipocytes and SVF cells (96).

In a clinical study by van Harmelan *et al.* of 30 obese women (BMI > 30kg/m^2), the number of viable adipocytes and the differentiation capacity of ADSCs were significantly reduced compared to standard values (106). These findings are supported by several *in-vitro* studies showing a decrease in both the proliferation and differentiation capacity of adipocytes with increasing BMI (96,105,107). In addition, alterations in telomerase activity and telomere length of deoxyribonucleic acid (DNA) have been observed in obese individuals, suggesting decreased differentiation capacity and early apoptosis of enlarged adipocytes (108). This process can be related to an increase in the expression of mitogen-activated protein 4 kinase 4 (MAP4K4) which in turn can inhibit peroxisome proliferator–activated receptor (PPARG) activation and thus adipogenesis (96,109,110).

Interestingly, other studies report no significant correlation between BMI and adipocyte viability and function (96). Geissler *et al.* did not find a significant difference in cell viability for any given donor site between obese and normal weight subjects (41), while other trials even suggest an impact on ADSC yield and proliferative capacity (104).

Nevertheless, it is recommended to improve the diet and overall health of patients before elective AFG to ensure adequate nutrition and oxygenation of autologous fat grafts (79). Some authors even recommend the use of micronutrients approved for wound healing, such as bromelain, magnesium, selenium, or vitamin B, suggesting better graft viability after surgery (79).

Diabetes mellitus

Adverse effects of chronic diseases such as diabetes mellitus on stem cells are well known, and results from preclinical studies also demonstrate adverse effects on the function and differentiation capacity of ADSCs (111,112). An *in-vitro* study comparing the gene expression profiles of ADSCs in diabetic, BMI- and age-matched control subjects demonstrated a significant lower differentiation capacity of ADSCs and on the other hand an upregulation of genes associated with inflammation and apoptosis in diabetic samples (96,112). Preclinical studies support these findings and suggest a lower take rate when AFG is performed in a diabetic setting (113,114). Ultimately, additional trials in humans are necessary to confirm this theory and its clinical relevance.

Radio-, chemotherapy and tamoxifen

Radio- and chemotherapy are increasingly used to treat a variety of malignant diseases in humans, but in addition to the favorable effects on cancer control, undesirable adverse effects

such as DNA damage to normal tissue may occur (96,115). Although ADSCs are known for their enhanced DNA damage repair mechanisms and lower metabolic requirements as a protection from hypoxia and subsequent apoptosis, radiotherapy can adversely affect them (115,116).

A preclinical study by Poglio *et al.* revealed a significant reduction in both number and proliferative capacity of ADSCs following radiation (117). In contrast to radiotherapy, other authors have shown no effect on the differentiation capacity of ADSCs when exposed to three commonly used chemotherapeutics *in-vitro*: cisplatin, camptothecin, and vincristine (118).

Other aspects of prior radiotherapy at the recipient site include hypoxia and chronic inflammatory conditions which lead to an unfavorable microenvironment for autologous fat grafts and potentially affect the outcome of AFG (96).

In-vitro experiments have shown, that administration of immunosuppressive drugs such as tamoxifen, a selective estrogen receptor modulator commonly applied for breast cancer treatment, is associated with a dose-dependent decrease in viability and proliferative capacity of ADSCs (96,119). To avoid an impaired graft survival, AFG may be performed after completion of tamoxifen administration, as it adversely affects ADSCs (96).

1.5 Autologous fat grafting in reconstructive breast surgery

According to recent statistics, breast cancer accounts for approximately 30% of all malignancies making it the most common cancer in women (120,121). Encouragingly, early detection and significantly improved treatment options are contributing to a decreasing mortality rate (121). To date, postoperative restoration of an acceptable breast appearance regarding form and functionality has become an integral component of the treatment process and may avoid some of the psychological effects associated with carcinoma therapy (122,123). Significant improvements in recent outcomes after breast reconstruction through developments in autologous and implant-based surgical techniques have ultimately raised patient expectations (122).

Although practiced for multiple decades and various indications, AFG has only recently became increasingly popular in reconstructive breast surgery to optimize and refine the aesthetic outcome after cancer therapy (8,124), treat radiation damage or capsular contracture (6). A national survey by the American Society of Plastic Surgeons in 2013 revealed, that 62% of plastic surgeons regularly use AFG for reconstructive breast surgery in their practice, verifying the increasing use of this technique in this field (20).

Prospective evidence on the specific time to perform AFG after carcinoma therapy is lacking, but authors recommend waiting until oncologic treatment is completed in order to avoid complications (124). For example, secondary radiation therapy required may particularly affect the take rate of a primarily performed AFG , leaving the newly transplanted tissue vulnerable to ischemia, fibrosis, and necrosis (124).

Similar to other application sites, fat grafts are usually injected into the subcutaneous space and prepectoral plane using a multiplane technique, as well as into the breast tissue itself, when available (79). However, apart from primary aesthetic augmentation, breast reconstruction with AFG is more challenging because the recipient site is smaller and has less vascularity and compliance (17). In addition, surgical scars or previous oncologic procedures such as radiotherapy may create a difficult initial situation (17) and require additional procedures such as rigotomy (124). In patients undergoing mastectomy, AFG can be used in addition to autologous tissue- or implant-based reconstruction or as the sole AFG reconstruction (124).

When mastectomy skin flaps are thin, the authors recommend direct preparation of the recipient site with the reinjection cannula (124). In this patient population, multiple AFG sessions are usually required with a minimum interval of 3 to 6 months (124).

1.5.1 Oncological safety

Despite good aesthetic and functional results, underlying biological mechanisms responsible for the filling and regenerative effects of AFG are still not completely understood (125). Therefore, grafting into or near a potential tumor bed is considered particularly problematic (125). Some *in-vitro* and preclinical models question the oncologic safety of AFG (126,127), but clinical data have not shown any association with increased risk of cancer development or recurrence (124,128). Especially the recent analysis by Wang *et al.*, in which 5,550 patients from 11 eligible trials were pooled did not find an increased risk of locoregional recurrence of invasive cancer or *in situ* disease with AFG compared to the control group (128). Another study even reported lower recurrence rates in patients who underwent secondary AFG (1.3%) compared with the control group (2.4%) ($p = 0.455$) (129). Although there is no clinical evidence of increased oncologic risk, long-term data are not available (79), and further confirmation by multicenter randomized clinical trials is warranted (125).

1.5.2 Impact of AFG on imaging

Imaging surveillance examination after breast cancer diagnosis and surgery are of paramount importance because of the increased risk of recurrence and improved survival with early detection (130).

Surgical procedures on the breast, both oncologic and reconstructive inherently alter the breast parenchyma and may result in divergent imaging (124). In particular, when autologous fat grafts are used, postoperative inflammation and ischemia can lead to palpable irregularities, adipose tissue necrosis, oil cysts or calcifications —all of which can potentially appear on radiographic imaging (124). In AFG in the oncologic setting, oil cysts are reported as the most common radiologic finding (130). Transplanted adipose tissue appears translucent on postoperative mammograms but may lead to the development of calcifications associated with fat necrosis, necessitating biopsy or shorter intervals of follow-up visits (131). However, trials on radiologic safety in aesthetic breast augmentation with AFG have found, that micro- and macrocalcifications were observed in less than 10% of cases and can be clearly distinguished from those indicative of malignancy (124,132). Therefore, it is generally believed that AFG is not associated with the inability to adequately assess radiographic changes throughout the breast (124,128).

1.6 Aim

Although several surgery- and patient-related risk factors for an impaired take rate in AFG have been discussed in the current literature, the overall evidence remains scarce and results are often inconclusive. Therefore, this exploratory study aims to identify additional, surgery- and patient-related factors with a clinically relevant impact on the take rate. The findings are expected to contribute to a more comprehensive understanding that will allow a better predictability and planning of interventions using AFG approaches. In addition, this project is expected to contribute to the standardization of AFG and the establishment of a gold standard for clinical practice.

In this context, the impact of patient-related factors such as age, smoking status, BMI or adipose tissue composition, as well as surgery-related factors such as differences in harvest sites, lipoaspirate processing techniques or the amount of injected fat, on a possible association with the take rate was investigated.

1.7 Hypothesis

According to preliminary studies, the outcome in AFG could be affected by several patient- and surgery-related risk factors. Harvest site and technique as well as adipose tissue composition, bodyweight, BMI, and age influence the graft take in autologous fat grafting.

2 Methods

The study was conducted at the Medical University of Graz, Department of Surgery, Division of Plastic, Aesthetic and Reconstructive Surgery, Graz, Austria in cooperation with JOANNEUM RESEARCH Forschungsgesellschaft mbH, COREMED – Cooperative Centre for Regenerative Medicine, Graz, Austria and has been reviewed and approved by the associated ethics' committee (EK: 32-487 ex 19/20). This study was registered at the trial registry "Clinical Trials" (Unique Identifier: NCT05286424) (https://clinicaltrials.gov/ct2/show/NCT05286424?term=NCT05286424&draw=2&rank=1).

2.1 Study cohort

Ten female patients undergoing elective AFG at the Division of Plastic, Aesthetic and Reconstructive Surgery, Department of Surgery, Medical University of Graz, Austria were enrolled. Inclusion and exclusion criteria were defined as follows:

Inclusion criteria:

1. Women, age 18-70 years (limited upwards due to the indication for AFG)
2. History of BRCA1 and BRCA2 - positive breast cancer with subsequent subcutaneous, nipple-sparing mastectomy and completed oncologic procedures
3. Scheduled elective AFG on the mastectomized breast
4. Currently healthy individual, willing to participate in this study
5. Signed informed consent form

Exclusion criteria:

1. Previous surgery at the harvest site (e.g. caesarian section)
2. Pregnancy or planned pregnancy
3. Current or previous chemotherapy
4. Previous radiation
5. Inability to fully understand study procedures or to give informed consent

Written informed consent forms (ICFs), created in accordance with the ethical standards of the institutional and national research committee and with the Helsinki declaration of 1964 and its later amendments were obtained from all subjects prior to any trial-related activities.

2.2 Experimental design

The duration of the study was set at 42 days/6 weeks. Elective, scheduled AFG was performed on the respective mastectomized breast on day 1. Non-invasive Lipometry (Lipometer, Möller Messtechnik, Graz, Austria) was conducted to calculate SAT thickness at the recipient site before and after the procedure. Further measurements were taken during study visits on day 7 and 42 post-surgery. The respective take rate was calculated after 6 weeks, whereby this time frame was chosen on the basis of previous data by Salinas *et al.* (133).

During the procedure of AFG, -10 ml lipoaspirate as well as punch biopsies from the harvest site were collected for laboratory assessments. A variety of patient-related characteristics (e.g. age, weight, BMI, smoking status, etc.) as well as surgery-related factors (e.g. harvest site, usage of a washing system, grafted volume, etc.) were acquired. *Figure 1* depicts a schematic overview of the study procedures.

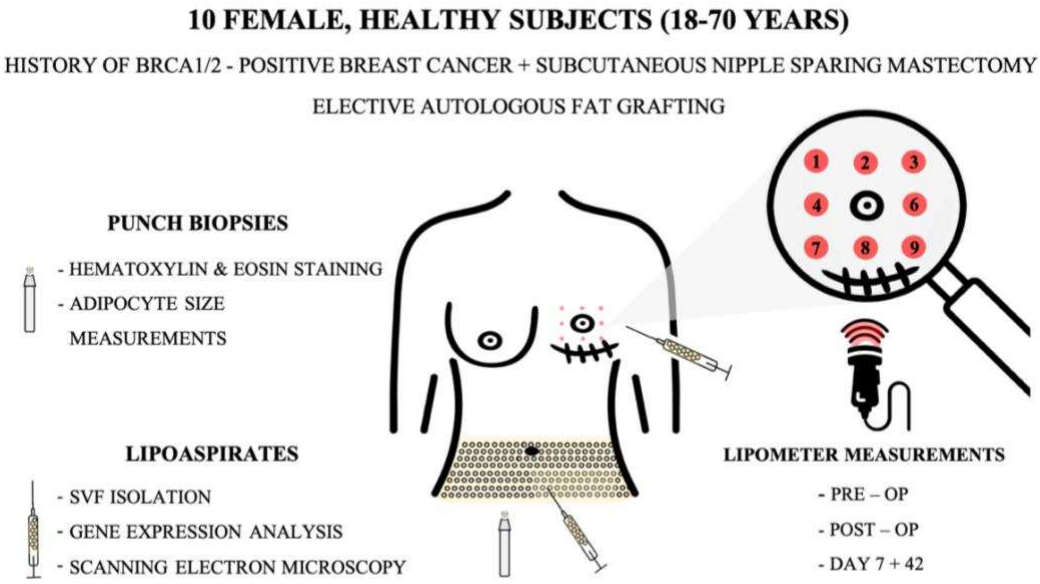


Figure 1: Schematic study design. 10 healthy, female subjects with a history of BRCA1/2 breast cancer, and following subcutaneous nipple sparing mastectomy as well as scheduled AFG were enrolled. During the procedure, lipoaspirates and punch biopsies were collected. Non - invasive Lipometer measurements at the grafted breast were performed before and after AFG, on day 7 and 42 to elucidate SAT thickness and calculate the take rate. Measurements were performed using 9 defined points throughout the breast, whereby measurement point 5 was located above the nipple. *Permission to reproduce this figure has been granted by Oxford University Press, License Number: 5346020180876.* (134)

2.3 Measurements

2.3.1. Lipometry

Fat topography and SAT thickness [mm] of the mastectomized and subsequently grafted breast was measured with a patented, optical, non-invasive device referred to as Lipometer (European Patent Number: 0516251) (135,136). The Lipometer as a computerized optical device can measure the thickness of a subcutaneous fatty layer in a non-invasive, safe, precise and quick way (135,136). The SAT layer is illuminated through light-emitting diodes, forming certain geometrical patterns varying with the thickness of the tissue (135,136). A photodiode subsequently measures the light intensity radiated back from the SAT. The system amplifies and digitalize these light signals which can subsequently be viewed and saved to computer (135,136).

For the present study, a standardized template was used to define nine measurement points around the nipple areola complex. Measurement point 5 in the middle of the template was located above the nipple in every measurement in order to ensure a safe reproduction of the same measurement pattern during several study visits. (See *Figure 1*) Non-invasive Lipometry at the mastectomized breast was conducted prior to AFG and immediately after, as well as after 1 week (Day 7) and 6 weeks (Day 42). Calculation of the take rate after 6 weeks were calculated from differences in the SAT thickness on Day 1 prior to surgery and Day 42.

2.3.2 Punch biopsies

In each patient, one 4 mm punch biopsy was taken at the harvest site during AFG. Punch biopsies were fixed in 4% neutral buffered formalin, dehydrated and ultimately embedded in paraffin. 2-4 µm sections were processed with conventional hematoxylin and eosin (H&E) staining to evaluate the average size of adipocytes within 3 independent sections. Quantification of the average adipocyte size was performed using the Adiposoft Plugin from ImageJ (Version 1.16., Imaging Unit of the Center for Applied Medical Research, University of Navarra, Spain), an automated, accurate, open source software to analyse white adipose tissue cellularity in histological, H&E stained sections (137). *Figure 2* displays an exemplary H&E image of selected adipocytes for following size evaluation via Adiposoft Plugin from ImageJ.

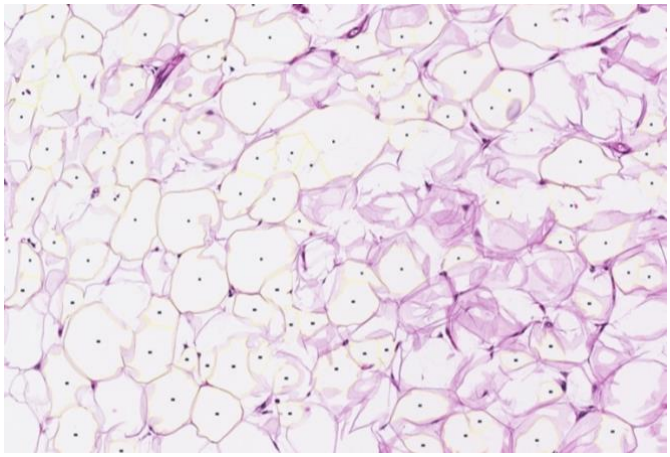


Figure 2: Hematoxylin and eosin stained sample obtained from a harvest site. Selection of adipocytes (see black dots) and evaluation of their size was performed within 3 independent sections in each patient. Adiposoft Plugin from ImageJ was used to quantify the average adipocyte size.

2.3.3 Lipoaspirates

Lipoaspirates were manually harvested from the abdomen, upper and/or lower legs. The super wet approach with lidocaine wetting solution (1ml infiltrate:1ml aspirate) was used. The same surgeon performed the harvesting, processing and reinjection process in all participating patients, using 3mm Tulip® Premium Single-Use GEMS cannulas (BONDIMED Aesthetics GmbH, Ohlsdorf, Austria). Subcutaneous reinjection of small aliquots of autologous fat grafts was performed in a fanning out pattern and during withdrawal of a small gauge cannula.
To assess potential processing-related differences in adipocyte viability, the LipiVage (Genesis Biosystems, Lewisville, USA) System and Ringer`s solution (Fresenius Kabi Austria GmbH, Graz, Austria) was used in 6 patients. Samples harvested separately for this project were aliquoted for further assays as follows:

Scanning electron microscopy (SEM)

Adipocytes were fixed in 2,5 % glutaraldehyde and 2 % Paraformaldehyd pH 7.4 at room temperature for 2h. Samples were subsequently fixed with 2 % Osmiumtetroxid for 2 h at room temperature following dehydration in graded ethanol series (30-96 % and 100 % (vol/vol) EtOH). After application of critical point drying (BalTec CPD, BalTec, Pfäffikon, Switzerland) and sputter coating (Baltec Sputter Coater 500, BalTec, Pfäffikon, Switzerland), samples were placed on stubs covered with a conductive double coated carbon tape. Images of various areas were taken with a Sigma 500VP FE-SEM with a SEM

Detector (Carl Zeiss Industrielle Messtechnik GmbH, Oberkochen, Germany) operated at an acceleration voltage of 5 kV.

3 representative images of 100 µm and 20 µm per lipoaspirate were chosen to grade the damage to the fat cell suspension in different subjects and processing techniques. Two individual, blinded investigators independently graded the selected images according to the following cell damage grading system. The mean value and differences between subgroups were calculated in the further course. Since no standardized visual grading system for cellular damage in SEM images is available to date, this system was created by the authors. *Figure 3* shows an example of a damaged adipocyte and parameters on which the grading system is based.

- *Grade 1:* Absent to minor cell damage, intact fibers of reticular collagen and cell membrane
- *Grade 2:* Minor cell damage (macroscopically non-visible cell membrane damage with detachment of lipid droplets from the cell body) in < 50% of adipocytes
- *Grade 3:* Minor to major cell damage (macroscopically non-visible to visible damage with detachment of lipid droplets from the cell body and burst adipocytes) in > 50% of adipocytes (134)

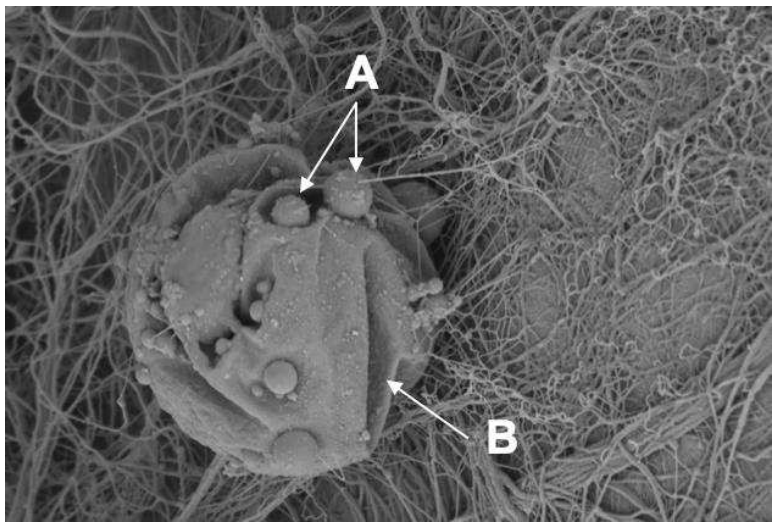


Figure 3: Adipocyte cell damage in scanning electron microscopy. Visual grading of cellular damage was calculated according to the above-mentioned grading system including the following parameters: A: Detachment of small lipid droplets from the adipocyte; B: burst cell membrane.

Stromal vascular fraction (SVF) cell isolation

Washing of 5- 10 mL aspirate was performed with an equal amount of 1x phosphate-buffered saline (PBS) pH 7.4 by centrifugation at 300 g for 7 min. After completed phase separation, the watery red phase was discarded and the yellow adipose fraction incubated in 10 mL 0.2% w/v Collagenase IV (Gibco, Life Technologies, Carlsbad, CA, USA) and 0.5% Bovine Serum Albumin solution for 45 – 60 min. Through addition of an equal volume of Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 containing 10% fetal calf serum (Gibco, Life Technologies, Carlsbad, CA, USA), the digestion of collagenase was stopped. The digested aspirate was subsequently filtered through a 100 µM cell strainer and centrifuged for 3 min at 300 g. The resulting SVF cell pellet was resolved in 5 mL erythrolysis buffer (eBioscience/Affymetrix, Santa Clara, CA, USA) and incubated for 10 min at room temperature to remove erythrocytes. To remove additional cellular debris, the pellet was washed by centrifugation as described above in 10 mL PBS. To extract ribonucleic acid (RNA) the SVF cell pellet was finally resolved at -80°C.

Real-time polymerase chain reaction (PCR)

RNA was isolated from 300 µL lipoaspirate and frozen SVF cell pellets using QIAzol Lysis reagent, RNAeasy extraction kit and RNase-Free DNase (Qiagen, Düsseldorf, Germany) as per the manufacturer's manual. Reverse RNA transcription was achieved on 250 ng of total RNA using iScript™ Reverse Transcription Supermix (Biorad, Hercules, CA, USA). Real-time PCR was performed on the CFX384 Touch Real-Time PCR System (Biorad) with TaqMan™ Gene Expression Master Mix and the following TaqMan™ Gene Expression Assays (Thermo Fisher Scientific, Waltham, MA, USA):

PPARG Hs01115513_m1; CEBPA Hs00269972_s1; CD36 Assay Hs00354519_m1; FABP4 Hs01086177_m1; PLIN1 Hs00160173_m1; ADIPOQ Hs00605917_m1, PECAM1 Hs00169777_m1; SLC2A4 Hs00168966_m1; CAV1 Hs00971716_m1; PTPRC Hs04189704_m1; CD34 Hs02576480_m1; BECN1, Hs01007018_m1; SQSTM1 Hs00177654_m1 and TBP Hs00427620_m1. In total, 40 cycles of real-time PCR were conducted (initial denaturation 95°C, 10 min; denaturation 95°C, 15 sec; annealing/extension 60°C, 1 min). Tri-n-butyl phosphate was used as housekeeping gene and relative quantification was determined using the $2^{-\Delta CT}$ method.

2.4 Statistical analysis

Since the present project is an explorative clinical trial with a small sample size, no formal sample size calculation was performed. The reason for conducting a study exclusively with female study participants is due to the fact that reconstructive breast surgery after carcinoma therapy using AFG is mainly used in women. Data was documented on paper as well as in Microsoft Excel while statistical evaluation was performed with GraphPad Prism Software (version 9.0.2; GraphPad Software, Inc., San Diego, CA, USA). Mean value and standard deviation was calculated in numeric data, while surgery- and patient-related data were correlated to the take rate 6 weeks postoperative using Spearman correlation and Student's t - test. Gene expression was evaluated by using a Spearman correlation test. Since data of cellular damage were not normally distributed, harvesting technique-related differences were analyzed via non-parametric Mann Whitney U tests. All statistical tests were two-tailed and statistical significance was considered if $p < 0.05$.

2.5 Actual trial milestones

The dissertation topic was specified in 2019, December, subsequently first drafts of the study protocol were created and discussed with internal and external supervisors as well as potential contributors. Submission of the final study protocol version agreed upon by all investigators, informed consent form and other key documents to the ethics committee of the Medical University of Graz was performed in 2020, May. Final approval after minor adaptations of the protocol and ICFs was received on the 27 July 2020. All 10 female subjects were included according to the study protocol, with the last subject recruited in August 2020. No study visits were cancelled due to the COVID-19 pandemic. Study progress reports were submitted to the ethics committee annually to obtain continuing approval.

Table 1: Clinical trial milestones

Finalized study protocol (1st protocol version)	2-MAY-2020
First submission to the ethics committee	25-MAY-2020
Ethics committee approval (4th protocol version)	27-JUL-2020
Recruitment start	1-OCT-2020
First patient visit	15-FEB-2021
Last patient visit	8-SEPT-2021
Study termination	10-JUL-2022

3 Results

10 female patients at the age from 29 to 68 years (mean age of 46.6 years, standard deviation ((SD)) ± 12 years) were included in this clinical trial between October 2020 and September 2021. All patients enrolled underwent elective, scheduled AFG at least 1 year after completion of oncologic procedures and nipple-sparing mastectomy as cancer surgery. Several patients underwent prior AGF sessions, whereby the number of previous AGR sessions ranged from 0 to 8 with a mean grafting session number of 3.2 (See *Table 2)*. Depending on the previously undergone AFG sessions, different SAT thicknesses were observed, which were analyzed for a potential correlation with the take rate.

Patients expressed a mean bodyweight of 63,7 kg (SD ± 7.84kg) and a respective mean BMI of 23.5 kg/m^2 (SD ± 2.73 kg/m^2) 3 patients (30%) were frequent smokers with 10, 15 and 45 pack years.

Harvesting was performed from the following sites: Abdomen (6), Upper Legs (3) and Lower Legs (1). In 6 patients (60%) the LipiVage System was used to process lipoaspirates prior to reinjection.

The mean size of adipocytes was 2,010.86 µm (SD ± 70.98 µm). The mean volume of autologous fat reinjected per session was 154.7 ml (SD ± 56.17 ml) and the mean grafted mm of adipose tissue were 3.9 mm (SD ± 1.0 mm). The mean remaining thickness of grafted adipose tissue after 6 weeks was 1.8 mm (SD ± 0.5 mm) resulting in a mean take rate of 46.72 % (SD 11.7 %). To assess potential adverse effects, a 3-month follow-up period was set in each study participant, but no minor or major adverse events have occurred. *Table 2 and 3* display a descriptive overview of surgery- and patient-related data obtained.

Table 2: Patient- and surgery-related data

Subject ID	Age	Bodyweight	BMI	Nicotine Abuse	Grafting Session	Usage of LipiVage	Harvest Site	Grafting Site	Mean Adipocyte Size	SEM Grading
	[ys]	[kg]	[kg/m^2]	(y/n)	[No.]	(y/n)			[µm]	
S01	50	60	21,11	n	4	y	Upper Legs	Right	2973	3
S02	32	80	28,34	n	4	n	Abdomen	Left	1406	2
S03	54	60	20,76	y (15 py)	3	n	Upper Legs	Right	1626	2
S04	49	72	24,91	y (45 py)	1	y	Abdomen	Left	md	1
S05	40	65	26,71	n	2	y	Abdomen	Right	1882	1
S06	59	59	21,41	n	9	n	Upper Legs	Right	1608	3
S07	68	70	25,71	n	5	y	Abdomen	Right	2160	1
S08	29	60	22,31	n	1	n	Abdomen	Left	2136	2
S09	52	60	24,03	n	2	y	Abdomen	Left	md	1
S10	33	51	19,68	y (10 py)	1	y	Lower Legs	Left	2296	1
Mean	46.6	63.7	23.5		3.2				2010.86	

Abbreviations: body mass index (BMI); missing data (md); no (n); number (No.); pack years (py); scanning electron microscopy (SEM); years (ys); yes (y);

Table 3: Mean SAT thickness over the study course of 6 weeks, grafted volume and take rate

Subject ID	SAT Preoperative [mm]	SAT Postoperative [mm]	SAT Day 7 [mm]	SAT Day 42 [mm]	Grafted Volume [ml]	Grafted SAT [mm]	Remaining grafted SAT after 6 Weeks [mm]	Take rate [%]
S01	8.6	14.62	14.33	11.21	225	6.02	2.61	43.36
S02	17.19	20.62	20.11	19.21	208	3.43	2.02	58.90
S03	9.2	11.96	11.61	10.07	98	2.76	0.88	31.85
S04	7.08	11.07	10.75	8.65	120	3.99	1.58	39.55
S05	12.27	16.43	15.99	14.21	126	4.17	1.94	46.67
S06	35.13	38.57	38.13	37.36	170	3.43	2.23	65.05
S07	26.12	28.9	28.26	27.83	90	2.78	1.71	61.60
S08	6.37	11.54	11.16	8.63	260	5.18	2.27	43.78
S09	4.23	7.03	6.62	5.22	100	2.8	0.99	35.32
S10	9.79	13.92	13.33	11.48	150	4.13	1.7	41.13
Mean	13.6	17.47	17.03	15.39	154.7	3.9	1.8	46.72

Abbreviations: identification (ID); subcutaneous adipose tissue (SAT);

3.1 Patient- and surgery-related data correlated to the take rate

The take rate correlated to the mean SAT thickness present at the grafting site before surgery ($p = 0.01$, $r = 0.77$). No statistically significant connection was shown between the take rate and the number of previous grafting sessions ($p = 0.07$), but interestingly, an approximate correlation similar to the mean SAT thickness was detected. These findings indicate a higher mean SAT thickness and subsequent improvement of the take rate in subjects who underwent more previous grafting sessions (*Figure 4*). While no statistical significance was reached regarding an interaction between take rate and body weight ($p = 0.67$, $r = 0.16$) or BMI ($p = 0.20$, $r = 0.44$), an approximate correlation was also noted, indicating a better graft take in patients with increased bodyweight and BMI (*Figure 5*).

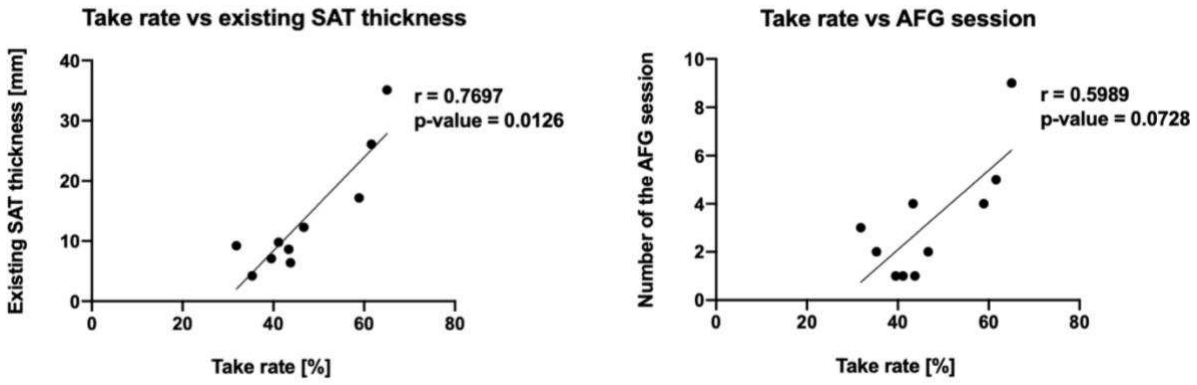


Figure 4: Spearman correlation of the take rate with the SAT thickness before surgery and the AFG session. A statistically significant correlation of the existing SAT thickness at the grafting site and the take rate is noted ($p = 0.01$). A trend towards a higher take rate is shown in patients with a higher number of previous AFG sessions. *Permission to reproduce this figure has been granted by Oxford University Press, License Number: 5346020180876.* (134)

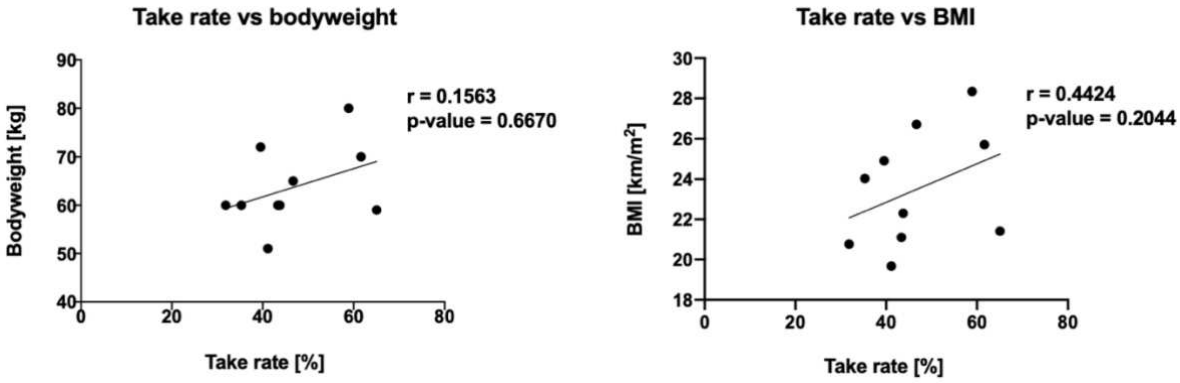


Figure 5: Spearman correlation of Bodyweight and BMI with the take rate after 6 weeks. A trend towards a higher take rate is shown in patients with a higher bodyweight and BMI. *Permission to reproduce this figure has been granted by Oxford University Press on July 11, 2022, License Number: 5346020180876.* (134)

No statistical significance was established between take rate and the mean adipocyte size ($p = 0.33$, $r = -0.40$), patient age ($p = 0.84$, $r = 0.08$), harvest site ($p = 0.65$, $r= -0.17$), usage of a washing system ($p = 0.61$, $r = 0.21$) or the amount of fat grafted ($p = 0.41$). Although statistical significance was also not reached regarding a connection between the smoking status and the take rate, non-smokers tended to a higher take compared to smokers ($p = 0.07$, $r = -0.65$). Excluding smokers, a mean take rate of 50.67% (SD 10.33%) was achieved in our study collective. These findings need to be further elucidated in larger cohort studies. Spearman correlations of these parameters are depicted in *Figure 6* and *7*.

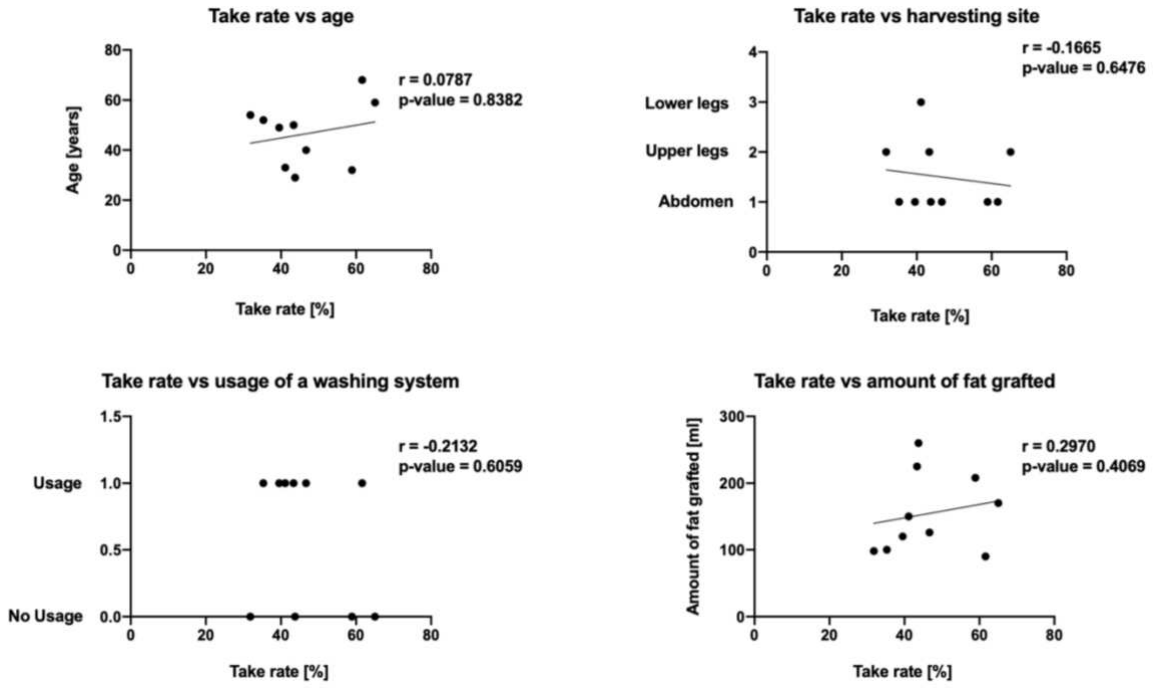


Figure 6*:* Spearman correlation of various patient- and surgery related factors with the take rate 6 weeks after AFG. No statistically significant connection was noted between the take rate and the patient age (p = 0.84) harvest site (p = 0.65), usage of a washing system (p = 0.61 ot the amount of fat grafted (p = 0.41). *Permission to reproduce this table has been granted by Oxford University Press, License Number: 5346020180876.* (134)

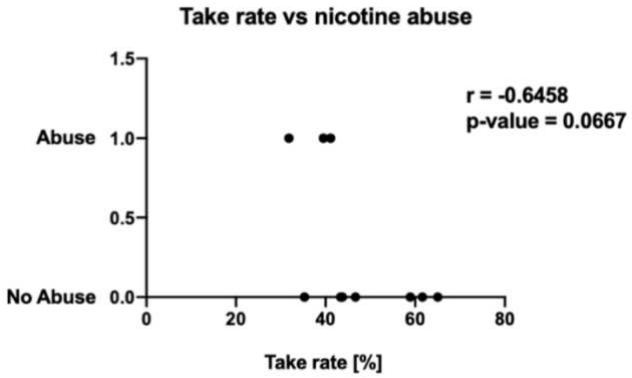


Figure 7: Correlation of the nicotine abuse with the take rate after 6 weeks. No statistical significance could be reached due to the small number of patients in each group. Nevertheless, frequent smokers (n=3) tended to a lower take rate than non-smokers (n=7) and vice versa.

3.2 Grading of cellular damage in scanning electron microscopy (SEM)

Cellular damage in lipoaspirates harvested exclusively with the super-wet technique (n=4) was calculated 2.25 (SD ± 0.49); while a lower cellular damage with a mean grade of 1.33 (SD ± 0.69) was noted in lipoaspirates processed with the LipiVage System. Differences between these processing techniques were statistically significant (p = 0.05), indicating less cellular damage with the use of the LipiVage System (See *Figure 8*). However, no significant impact on the take rate was found (p= 0.46, r = 0.26). Exemplary SEM images of lipoaspirates harvested with these two approaches are displayed in *Table 4*.

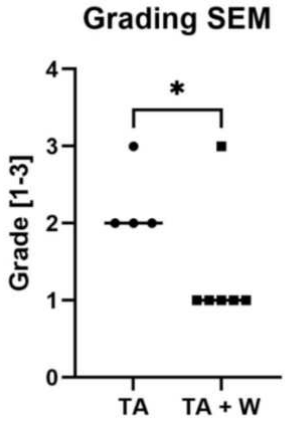
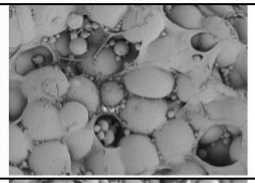
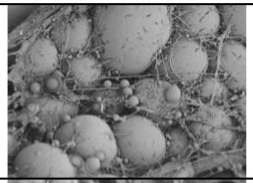
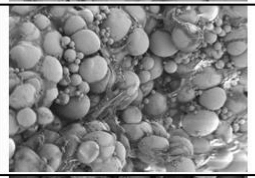
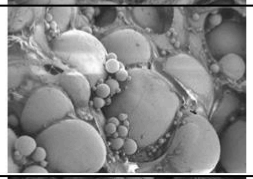
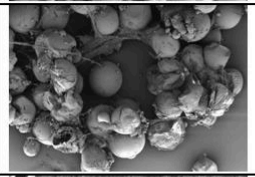
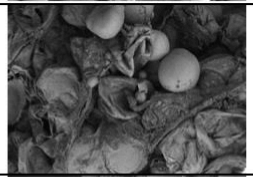
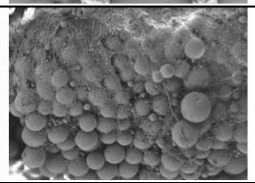
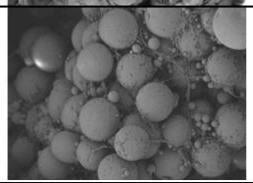
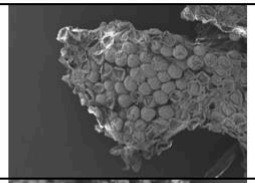
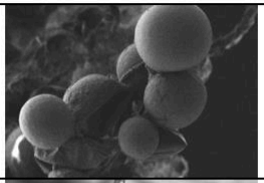
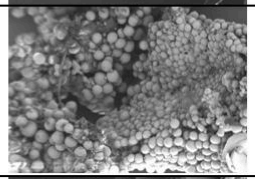
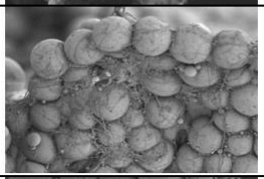
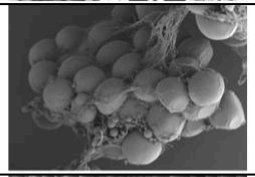
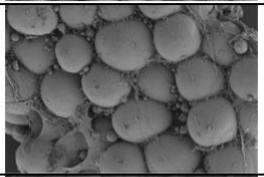
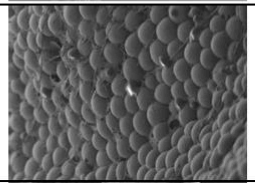
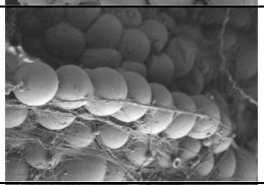
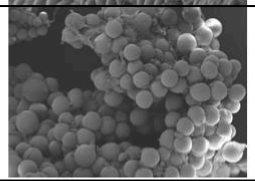
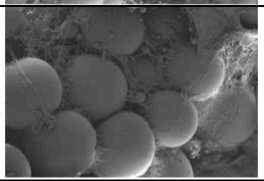
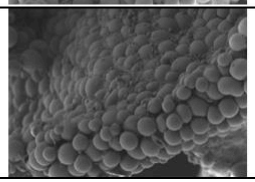
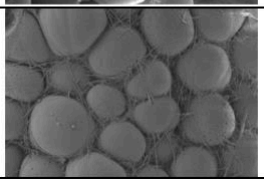


Figure 8: SEM grading of lipoaspirates harvested with the super-wet technique exclusively or in combination with a washing and filtration system. A statistically significant, processing-related difference in the grading of cellular damage was found (p = 0.05), indicating a reduction of cellular damage with the LipiVage System.

Table 4: Scanning electron microscopy and grading cellular damage in lipoaspirates harvested with different techniques

Subject No.	Technique	Site	100 µm	20 µm	G
S02	TA	Abdomen			**2**
S03	TA	Upper Legs			**2**
S06	TA	Upper Legs			**3**
S08	TA	Abdomen			**2**

Abbreviations: Grade (G); Number (No.); Tumescent Anaesthesia (TA); Washing (W);

Subject No.	Technique	Site	100 µm	20 µm	G
S01	TA + W	Upper Legs			**3**
S04	TA + W	Abdomen			**1**
S05	TA + W	Abdomen			**1**
S07	TA + W	Abdomen			**1**
S09	TA + W	Abdomen			**1**
S10	TA + W	Lower Legs			**1**

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3.3 Gene expression of adipose tissue and cell markers in lipoaspirates and stromal-vascular cell fractions

Gene expression of adipocyte and adipose tissue markers PPARG, CEBPA (CAAT/enhancer binding protein), CD36, FABP4 (fatty acid binding protein 4), PLIN1 (perilipin 1), ADIPOQ (adiponectin), SLC2A4/GLUT4 (glucose transporter type 4), and the cell surface markers for PECAM1/CD31 (Platelet endothelial cell adhesion molecule) and PTPRC/CD45 (Peroxisome proliferator-activated receptor gamma) were analyzed from both the lipoaspirate and SVF fraction, but no significant correlation of the take rate and adipocyte markers (PPARG ($p = 0.29$), CEBPA ($p = 0.2$)) or other cell markers (CD34 ($p = 0.49$), CD36 ($p = 0.43$), CD45 ($p = 0.43$), PECAM ($p = 0.23$)) was noted.

However, the expression of FABP4 ($p= 0.0734$, $r = -0.42$) and PLIN1 ($p= 0.1786$, $r = -0.14$), showed an indirect connection towards the take rate as displayed in *Figure 9*.

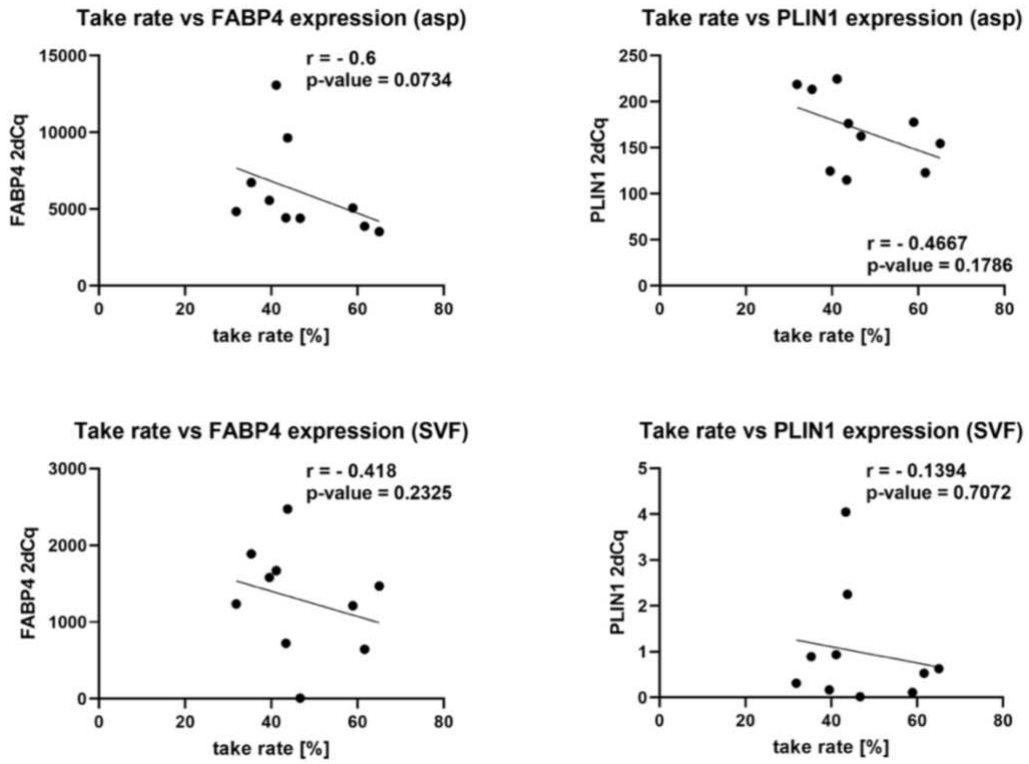

Figure 9: Correlation of the take rate and the expression of adipocyte markers FABP4 and PLIN1 in lipoaspirates (asp) and the SVF fraction. Although not statistically significant, FABP4 ($p= 0.0734$, $r = -0.42$) and PLIN1 ($p = 0.1786$, $r = -0.14$), indirectly correlated to the take rate in lipoaspirate analysis. Effects were less pronounced in the SVF fraction. *Permission to reproduce this figure has been granted by Oxford University Press, License Number: 5346020180876.* (134)

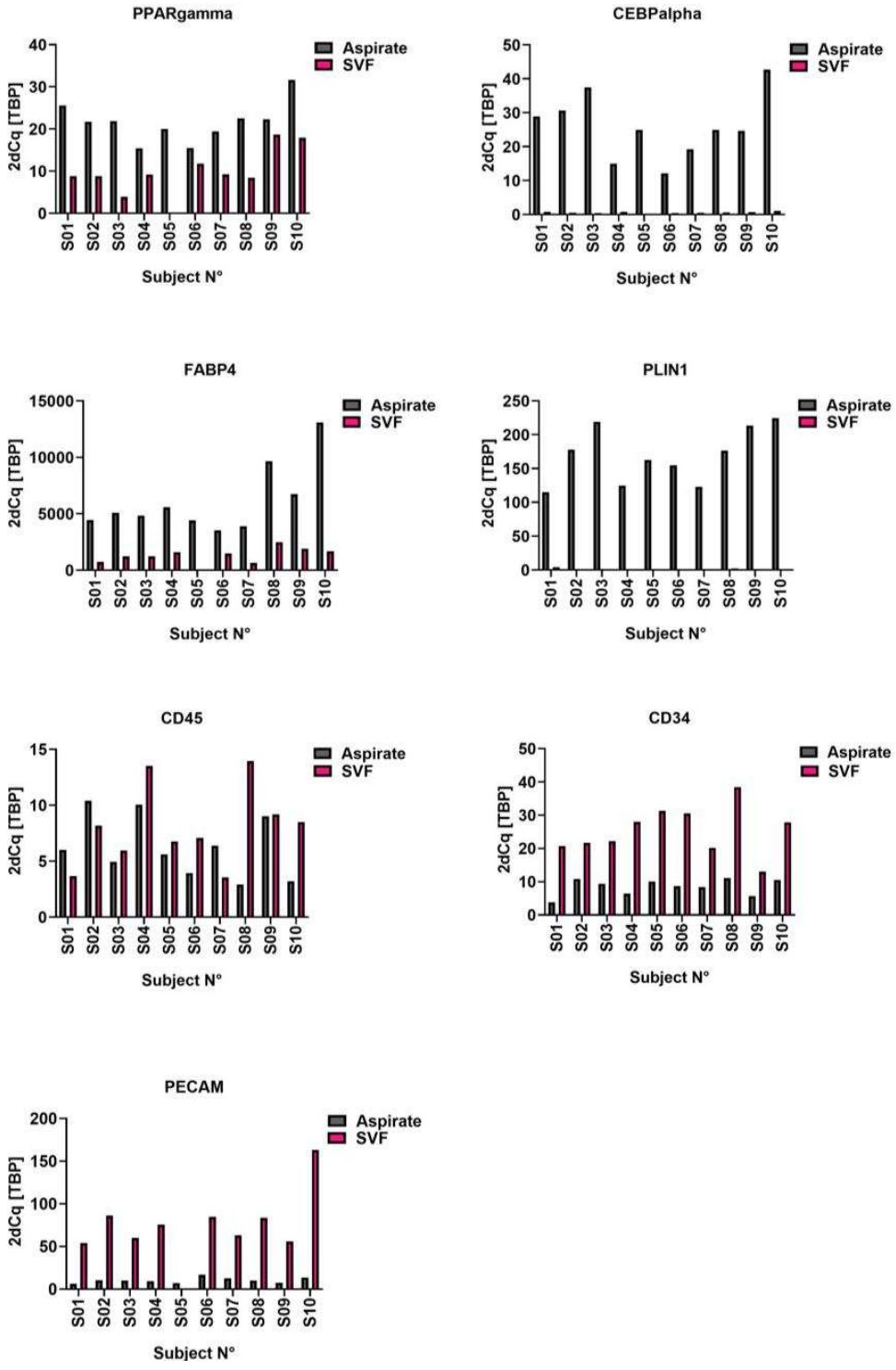

Figure 10: Gene expression of adipocyte and cell markers of the aspirate and the SVF fraction. Gene expression of the adipocyte and adipose tissue markers PPARG, CEBPA, FABP4, PLIN1 as well as cell surface markers for PTPRC/CD45, CD34 and PECAM1/CD31 were investigated from both the lipoaspirate and SVF fraction. No statistically significant correlation between the take rate and gene expression of those markers was reached. *Permission to reproduce this table has been granted by Oxford University Press, License Number: 5346020180876.* (134)

4 Discussion

AFG is a rapidly evolving technique that has attracted attention in reconstructive breast surgery in recent decades (27). Lacking of consensus and standardization of this approach along with the still unpredictable, unsatisfactory outcome, limit its application (27,138). In postmastectomy reconstruction with AFG, optimal outcomes are only achievable with a staged approach including multiple sequential grafting sessions, resulting in a significant burden on patients and the healthcare system (27). The present project provided valuable baseline data that could be helpful in planning large-cohort studies and contribute to the development of a more precise and accurate predictive model for AFG in the following.

4.1 Surgery-related factors affecting the take rate

Surgeons performing AFG believe that the unpredictability of graft take is largely due to the technique (1). Some impairing variables are already well known, but because other potential risk factors are less studied or even neglected (16,18), our project contributed significantly to an increase in knowledge.

4.1.1 Harvesting

Despite the ongoing debate about the optimal, least invasive harvesting method to obtain the largest possible number of functional and viable adipocytes as well as SVF cells, no superior technique has yet been defined (34) and the technique originally introduced by Coleman *et al.* (8) is still recommended method to be preferred in AFG (34). To date, many authors favor hand-held syringes over power-assisted liposuction, but no significant difference in cell number of viability has been found in trials comparing these methods (61). In our department, the standard method is manual harvesting with large-bore cannulas using the "super-wet" approach to avoid sheer forces causing structural destruction (53) and preserve natural adipose tissue structure (55). Compared with other reports (15,19,20), achieved a satisfactory mean take rate of 46.7 % (SD 11.7 %)). Due to the small sample size other harvesting techniques not were investigated in this project. However, promising results were obtained from studies on the use of ultrasound-assisted liposuction for AFG purposes (62,63), which were also observed in another study by our group (139).

Interestingly, we detected the lowest cellular damage with the super-wet, ultrasound-assisted liposuction compared to the classic, super-wet approach or the dry harvesting technique (139). The use of ultrasound during liposuction may therefore be appropriate in AFG, but future large-cohort studies to determine the superiority of one harvesting technique over another are absolutely required.

According to available clinical evidence, the abdomen should be favored for harvesting because of its higher yield of adipose tissue (13,20,38,39) and was also preferred in 6 of 10 patients of this explorative project. Since each patient has an individual fat distribution and requires a different volume of grafted fat, harvesting can alternatively be performed from the flanks, upper and lower legs or medial knees (13,20,38,39). In our study population, we did not observe a statistically significant difference of the mean adipocyte size at different harvest sites ($p = 0.33$). Furthermore, no correlation between the mean adipocyte size and the take rate ($p = 0.33$, $r = -0.40$) and thus no advantage of the harvest site on outcome was observed. Our results are supported by several preclinical studies demonstrating no influence of the harvest site on the take rate of grafted adipose tissue (35,36). For example in an *in-vivo* mouse study investigating long-term survival of human fat harvested from a 48-year-old woman, weight, volume, and various histologic parameters of grafted fat such as integrity, cyst formation, vascularization, fibrosis, inflammation and necrosis were examined (36). The authors did not find statistically significant differences in the parameters studied among the three harvest sites examined (36).

In addition, findings by Rohrich *et al*. did not report any differences in cellular viability at different harvest sites such as the abdomen, flanks, thighs or medial knee (37).

On the other hand, findings on age-related differences in adipocyte viability by Geissler *et al.* suggest that, depending on the age of the patients, harvesting from the lower abdomen and the flanks may optimize the take rate due to higher viability (39). Furthermore, preclinical evidence indicates a higher yield of ADSCs in the lower abdomen and medial thighs compared with the upper abdomen, knees, flanks and the trochanteric region (96). These findings were confirmed in a prospective cross-sectional trial of 25 women who underwent liposuction in the upper and lower abdomen, flank, trochanteric region, inner thigh, and knee (140). In addition, Tsekouras *et al*. recently found a significantly higher amount of ADSCs in the inner and outer thigh compared with other harvest sites (141).

Therefore, both the lower abdomen and the thigh seem to be favorable harvest options (41), but clinical evidence on a potential impact on the take rate is lacking.

Based on our data and similar to other authors (35–37), we therefore assume, that no superior harvest site can yet be defined and the selection should be made based on the individual SAT distribution pattern (6), preference of the surgeon and the patient.

4.1.2 Processing

Centrifugation, gravity separation, washing, and filtration are among the most common techniques of adipose tissue processing (6), and recent results by Valmadrid *et al.* indicate, the rate of fat necrosis can be reduced with the aid of a closed washing system, such as the one used in the present project (78). The LipiVage system preserves a large amount of viable adipocytes and maintains high levels of intracellular enzyme activity in harvested adipose tissue (59). Because of these advantages, the use of this system is particularly recommended for grafting of large volumes, as often required for complete breast reconstruction after mastectomy (59).

In our study population, processing of lipoaspirates with the LipiVage system did not show any statistically significant impact on the take rate ($p= 0.61$); however, the level of cellular damage was statistically significant lower than in processing with gravity separation exclusively ($p = 0.05$). Similarly to findings of Fergusen *et al*. (59), lower levels of damage at the cellular level and a higher overall cell viability were found across SEM compared with other samples. Cellular damage grading revealed a mean grade of 2.25 (SD ± 0.49) for gravity separation and 1.33 (SD ± 0.69) for processing with the LipiVage system. However, statistical significance was reached regarding a correlation between the take rate and grading of cellular damage in SEM.

The presence and diameter of lipid droplets were used as indicators of cellular damage, with fewer and smaller droplets considered better than the presence of many large lipid droplets. Previous trials on the mechanisms of fat graft survival have shown that lipid droplets are mainly absorbed by macrophage phagocytosis, however this process tends to be very slow and its duration significantly depends on the droplet diameter (23). In large-diameter droplets, a cyst wall forms before complete absorption, which may calcify over time (23).

In the center of transplanted adipose tissue, even ADSCs or progenitor cells undergo apoptosis, which inhibits further adipogenesis and leads to the formation of scar tissue or

cysts (23). According to the widespread cell survival theory, final long-term volume retention after AFG is determined by the amount of successfully replaced adipocytes (23,27). If only small lipid droplets are present in grafted adipose tissue, absorption is thought to be complete after 3 months and volume is not expected to change significantly after this period (23). If a larger amount of large lipid droplets remains 3 months after AFG, the graft is expected to atrophy between 3 and 12 months (23).

Despite lacking statistical significance regarding a correlation of the take rate and the processing technique in our study population, a higher amount of viable adipocytes and less cellular damage as observed in lipoaspirates processed with the LipiVage system, may still be relevant for an improved long-term graft retention (27). Furthermore, reinjection of a more uniform and higher autologous fat tissue content with less compromising components (77) may be considered beneficial.

In spite of these quality improvements, one clinical trial reported a long-term take rate of only 41% for fat processed with the PureGraft washing system (142). These findings suggest, that further factors beyond the composition of the lipoaspirates, possibly including the function of adipocytes, ADSCs, and SVF may be involved in long-term outcome (1,68). When wetting solutions are used in liposuction, lipoaspirates are exposed to local anesthetics such as lidocaine, which may inhibit cell function (47). However, Moore *et al.* reported that this effect lasts only as long as lidocaine is present and that adipocytes can fully recover their growth and function after washing with for example the LipiVage System, regardless of the duration of exposure (47). Despite inconclusive evidence of long-term results, the use of a washing system has become the current standard in our department because of several favorable properties. Additional studies investigating whether higher quality grafts have a clinically relevant impact and focusing identifying an optimal technique for processing autologous fat grafts are essential in determining a gold standard in AFG (77).

4.1.3 Grafting

As suggested by previous authors, reinjection of small aliquots was performed in a fanning out pattern during withdrawal of small blunt cannulas (approximately 17-gauge) (8,11,79) connected to a Luer-Lock syringe. Despite potentially increased vascularization during the reinjection of lipoaspirates into the muscle (93), we opted for subcutaneous grafting because of an increased mobilization of the Musculus pectoralis major and minor compared with the SAT (94).

Existing studies that examined the effect of the condition at the recipient site on the take rate were inconclusive and suggest that parameters such as age, the severity of structural damage or previous trauma to the overlying layers are potential adverse factors in graft take (6). In our study collective, results showed a statistically significant correlation between the take rate and the mean SAT thickness at the graft site before surgery ($p= 0.01$) and an approximate correlation with the sequence number of the current AFG session. Therefore, higher take rates can be expected in subjects with a higher pre-existing SAT layer thickness, which may be due to previously undergone multiple AFG sessions or a higher BMI. The highest take rate in this collective (65.05%) was observed in a patient with the highest mean SAT thickness before AFG (35.15 mm), whereas lower take rates less than 40% were found in patients with significantly lower mean SAT thicknesses of less than 10 mm. Our results are in agreement with those of other authors such as Chen *et al.* who speculate that loss of fat graft volume occurs (and possibly increases) when grafted to a recipient area where less fat is present (27). These effects at a recipient site with lower SAT can be explained by a decreased function of reinjected autologous fat grafts to store lipids in adipocytes, leading to a decrease in cell and tissue size over time (27).
To ensure maintenance of the patient compliance during AFG-based postmastectomy reconstruction within several sessions, these findings should be taken into account for the preoperative planning as well as for medical briefing.

We did not observe a statistically significant correlation between the take rate and the amount of autologous fat transplanted ($p= 0.41$), but higher take rates tended to be observed in patients with larger volumes grafted. However, the general presumption is that higher volumes transplanted into a tight, limited space may affect the take rate (95), but an optimal grafting volume in relation of the recipient site has not yet been defined. Regardless of which theory of graft survival applies, donor lobules of autologous fat should be dispersed into the recipient site tissue and volumetric limits should be considered (143). Limitations on graft capacity are found, for example in skin or hair transplantation, both of which are based on the principle of diffusion angiogenesis (143). In these respective two- and three-dimensional systems, the outer sides of the transplanted tissue are in contact with recipient's tissue during and after reinjection (143). This graft- to-recipient dynamic limits the volume that can be transplanted within a single session, and grafting according to the capacity of the recipient site is known as the graft-to-capacity concept (143).

Therefore, for mega volume grafting (ranges of 250 ml and above), as frequently performed in postmastectomy reconstruction, some authors suggest external expansion, for instance with the BRAVA system, to increase vascularity and volume of the recipient area (81).
In our study cohort, reinjection of lipoaspirates was done according to the graft-to-capacity concept (143). The mean volume of reinjected fat was 154.7 ml, with a single patient receiving over 250 ml while an acceptable take rate of 43.78% was still achieved. In this case however, the prior to AFG existing mean SAT thickness only 6.37 mm, which could be considered a compromising parameter. Ultimately, patient variability is challenging in planning appropriate graft volume. In our opinion it is essential to avoid grafting beyond what the recipient site can accommodate, as tissue compliance decreases rapidly over certain grafting volumes (95). The upper limit of the volume that can be safely injected has yet to be determined.

4.2 Patient-related parameters affecting the take rate

The still remaining variability in outcome after AFG indicates a significant impact of patient-related characteristics on the functionality and viability of adipocytes, SVF cells and particularly and ADSCs (96). Effects of patient age on their ADSC viability has been extensively researched, and findings indicate a reduction in overall yield of nucleated cells with age as well as a significant decrease of differentiation and proliferation capacity (96). Age can also lead to poorer revascularization, which in turn may be associated with reduced graft perfusion rates and therefore lower overall graft take (6). Despite age did not significantly affect take rate in our study participants aged 29 to 68 years ($p=0.84$), evidence suggests an association of improved graft take in younger patients and initiation of AFG at a younger age to be preferable (144). Apart from age being a potential risk factor for impaired graft take, the indication for reconstructive AFG in elderly patients should generally be considered with special care.

As the most prevalent addiction in society, nicotine abuse is known as a common troublemaker in surgery (145), which may affect graft revascularization and survival in addition to age (6). In general, nicotine abuse is known to have several harmful effects on cellular structure and circulatory components and is a frequently cited as a factor in many surgical complications, such as wound healing disorders, fat necrosis, underperfusion or flap ischemia (145). As a particular risk factor AFG, smoking may cause vasoconstriction leading to reduced blood flow

and oxygen saturation in subcutaneous tissue (146–148). To date, there is no clear evidence as to why cigarette smoking leads to a reduction in peripheral tissue perfusion, but the release of catecholamines during smoking is considered the most likely reason (146,147). Although it is generally believed that smoking may affect fat graft survival, there is limited evidence for this particular risk factor (145). Despite a lack of statistical significance attributed to the small number of cases, a trend towards a higher take rate in non-smokers ($p=0.07$, $r = -0.65$) was shown in our study collective, indicating smoking as a risk factor for impaired raft survival. Similar results were obtained in a clinical trial on the effects of nicotine abuse in facial AFG, as smokers were found to have a reduced take rate of approximately 40% (146). In this study cohort, two subjects were heavy smokers (smoked more than 30 per day) and the remaining 16 subjects were average smokers (smoked between 10 and 30 cigarettes per day) (146). In our study cohort, only one subject was a heavy smoker and two subjects were average smokers with a respective take rate of 39.55%, 31.89%, and 41.13% whereas the mean take rate of non-smokers was 50.67%.

A preclinical study in Sprague-Dawley type rats using the smoke exposure model by Gazzalle *et al.* (149) also reported a weight loss of grafted adipose tissue of 80% in rats, exposed to smoke throughout the procedure compared with 54% in the control group (145). Interestingly, immunohistochemical analysis furthermore revealed a significantly higher amount of pre-adipocytes as well as mature adipocytes in the control group, highlighting the deleterious effects of nicotine abuse at the cellular level (145). Further clinical and experimental studies are warranted to determine the extent of harmful effects of nicotine abuse in AFG (146).

There is also evidence that increased BMI affects adipocyte function and viability (96). A reduction in differentiation and proliferation capacity of ADSCs has been observed in adipose tissue of obese patients (BMI of >30 kg/m^2) (96).

However, studies have also not found a statistically significant association between increased BMI and impairment of adipocyte viability (96). We observed an approximate correlation of body weight and BMI in our study collective, suggesting a potential higher graft take in patients with a respective higher body weight. At this point, these results could also be due to a higher pre-existing thickness of the SAT at the recipient site, which may improve the take rate.

However, because of breast cancer and oncologic treatments, patients undergoing postmastectomy reconstruction may be underweight (150) and harvesting autologous fat can be

challenging especially if multiple sessions are required. Although usually contraindicated in underweight patients, results of a clinical trial on aesthetic breast augmentation with AFG by Cheng-Hung stated, that the same extent of augmentation can be achieved in underweight subjects (BMI <18.5) compared to normal-weight subjects (151).
As with overweight, it is recommended for underweight, to improve the patients nutrition and overall health before AFG to ensure adequate nutrition and oxygenation of the autologous fat grafts (79) and allow adequate volumes of autologous fat to be harvested. A balanced diet including the six basic nutrients protein, carbohydrates, fat, minerals, vitamins and water, as well as adequate nutrient intake are essential for the recovery after cancer treatment (150).
Micronutrients such as bromelain, magnesium, selenium, or vitamin B, which indicate better graft viability following surgery may also be used in patients seeking AFG with a low BMI (79).

A variety of other patient-related risk factors such as diabetes mellitus type 1 and 2 , previous chemo- or radiotherapy or menopausal status may affect cell functionality and viability (96). A better understanding of these variables is considered of utmost importance for deriving a theoretical framework to predict outcome in AFG and for identifying patient populations that would benefit from versatile techniques for autologous fat graft enrichment (96).

4.3 Adipocyte markers

To date, there have been few studies examining the expression of human adipocyte markers in lipoaspirates harvested from various sites using different harvesting methods and they are not fully comparable to the present study.

Preclinical evidence suggests, that the metabolic state of harvested and subsequently grafted adipose tissue may play a role in graft take (152). Analysis of adipocyte marker expression in our study collective revealed a strong indirect convergence of FABP4 and PLIN1 and take rate. In general, conclusions about the amount of viable adipocytes in lipoaspirates can be drawn via the expression of PLIN1 (153,154), and a lower expression is associated with higher lipolysis rates in humans (155). According to these findings, lower PLIN1 expression might be associated with a better outcome in AFG.

On the other hand, FABP4 has often been used as a marker for differentiated adipocytes, but evidence suggests that its expression is not restricted to mature adipocytes, but also detects a pool of undifferentiated progenitor cells associated with the vasculature of adipose tissue (156). The expression of this marker is related to adipocyte differentiation (157) and higher expression of markers associated with adipogenesis, such as FABP4, but also PPARG or CEBPA, might be associated with improved long-term outcomes (158). However, we did not observe a correlation between take rate and expression of PPARG or CEBPA in our study population. Despite the lack of statistical significance, the role of PPARG in adipogenesis and fat metabolism has been extensively investigated *in-vitro* (159) and based on these findings, we believe that this specific marker may play a crucial role in the initial and long-term survival of grafted autologous fat. Statistical significance may not have been achieved because of the small sample size, we nevertheless consider the measurement of these adipocyte markers to be a valuable approach for future human trials in larger cohorts.

Furthermore, regeneration after AFG may not be based on adipose tissue per se, but the presence and amount of stem cells ubiquitously distributed (152). Using SVF cells and particularly ADSCs may therefore soon become increasingly important in the search for optimal techniques for improved outcomes in AFG (6). While no connection of the take rate and the expression of macrophage (CD45), endothelial cell (CD31) and stem cell markers (CD34) in the SVF were observed, there is still a great demand for more in-depth studies to investigate the contribution of differentiated adipocytes and ADSCs to the graft take.

4.4 Limitations and outlook

This exploratory trial, which aimed to identify clinically relevant patient- and surgery-related parameters that affect the graft take, is limited in several ways. Because of the small study cohort, retrieved results may not be fully transferable and representative, which may also be the reason why certain significances were not reached but trends were revealed.

Although the trial was planned to reduce potential bias caused by different harvesting and grafting techniques of autologous fat, the individual techniques used may not be completely uniform in all study participants and a bias due to individual (surgeon-related) factors is to be considered a limiting factor.

Furthermore, the expression of several cell death markers was below the limit of detection, which is why indirect assumptions about cellular damage in harvested fat, for example by grading in SEM, can be made. In addition, no clear conclusion can be drawn about cellular damage and whether the viability of mature adipocytes or regenerative cell populations is of importance for improved graft take.

Since the study participance was limited to female patients, no clear statement can be made about the results in AFG in male patients.

Ultimately, there is a great need for strategies to optimize the technique of AFG, for which this explorative trial has provided highly relevant baseline data. We consider our results an important new aspect in the challenge of finding an optimal, minimally invasive technique in AFG. Definition of a universally applicable technique for a broad application spectrum of AFG and investigation of potential gender-related differences is only feasible through the implementation of future larger cohort studies with a sufficient long-term follow-up, which may be planned on the basis of these fundamental data.

5 Conclusion

The minimally invasive approach of AFG continues to gain popularity as an effective tool for soft-tissue augmentation particularly in reconstructive breast surgery. The current main goal is to overcome the low and unsatisfactory predictability of outcome due to the unpredictable graft survival and fat stability. Consequently, there is a high and growing demand for well-defined, large prospective studies that investigate the clinical relevance of potential risk factors for an impaired graft take. A variety of patient- and surgery-related parameters were found to be correlated or approximately correlated with a decreased graft take in this study cohort. We consider these findings to be an important new aspect in the challenge of determining a gold standard of this technique. The implementation of human, large-cohort studies examining these parameters in greater detail are crucial to support the development of a comprehensive theoretical model that supports the definition of a universal protocol for AFG for aesthetic and reconstructive purposes in clinical practice.

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