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The viability of the lipoaspirate after sieving into different particle size fractions: an experimental study

A thesis submitted in partial fulfilment of the requirements for the degree of Master of Medicine

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Abstract

Background: Lipofilling or autologous fat grafting with aspirated adipose tissue has emerged as a widely used surgical procedure for reconstructive and aesthetic purpose. One of the main indications is the enhancement of breast contour after a patient had a segmentectomy or after mastectomy followed by breast reconstructive surgery. The main drawback remains the unpredictability of the result due to resorption of the fat graft after injection.

Goal: We try to determine an ideal size of fat particles ready for injection in terms of cell viability and composition of the suctioned adipose tissue. Higher viability and a more favourable composition could help to prevent resorption of the fat graft and, consequently, decrease the need for secondary lipofilling sessions.

Methods: Lipoaspirates from 7 female human beings were gained by liposuction, centrifuged and divided according to the size of the fat particles by means of sieving. Samples were stained with Hoechst 33342 and propidium iodide and the cellular viability was analysed using an inverted microscope. Fluorescent histological sections with S100 staining were created to evaluate the composition with a fluorescence microscope.

Results: The mean cellular viability appeared to be 64.6 ± 30.5% in the lipoaspirate with particles smaller than 500 µm, 56.2 ± 23.3% in the lipoaspirate with particles between 500 and 1 000 µm, 50.2 ± 30.7% in the lipoaspirate with particles between 1 000 and 1 600 µm and 52.1 ± 23.0% in the lipoaspirate with particles larger than 1 600 µm ($p = 0.094$). The fraction of S100-positive cells, which were considered as mature adipocytes and preadipocytes, was 14.8 ± 5.9% in the sample with particles smaller than 1600 µm, whereas this percentage raised to 20.0 ± 6.3% in the sample containing fat particles larger than 1 600 µm ($p = 0.102$).

Conclusions: No significant differences were found in viability and composition of the different size classes. However, due to a lack of included patients, we can neither draw hard conclusions nor provide practical guidelines regarding the possible advantage of sieving the lipoaspirate prior to lipofilling. Our hypothesis remains an interesting research question that requires a larger study group.

Keywords: liposuction, lipofilling, fat grafting, fat transfer, autologous fat, viability, composition, fat particles, adipocytes, Hoechst 33342, propidium iodide, S100.

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1. Background

1.1 The application of lipofilling in reconstructive surgery

Liposuction has become one of the most common procedures in plastic surgery. Most of the time the goal is to remove excessive fat in order to improve the body contour. Liposuction may, however, be part of reconstructive procedures. In such cases, the aspirated fat can be transferred to various acceptor sites, e.g., to the thorax after a breast segmentectomy or after a mastectomy followed by breast reconstruction (figure 1). Several case reports[1] describe this fat grafting technique or lipofilling to the breasts for augmentation or correction of contour or volume defects due to medical conditions such as breast cancer, micromastia, tuberous breasts and Poland's syndrome. Multiple reports show that autologous fat grafting can enhance healing and improve scar quality in mature burn wounds[2-3], chronic ulcers[4] and skin areas affected by radiation therapy[5-6]. Lipofilling can also be used for soft-tissue augmentation in hand rejuvenation[7-8] or facial rejuvenation[9-13], when injected in volume lacking areas.

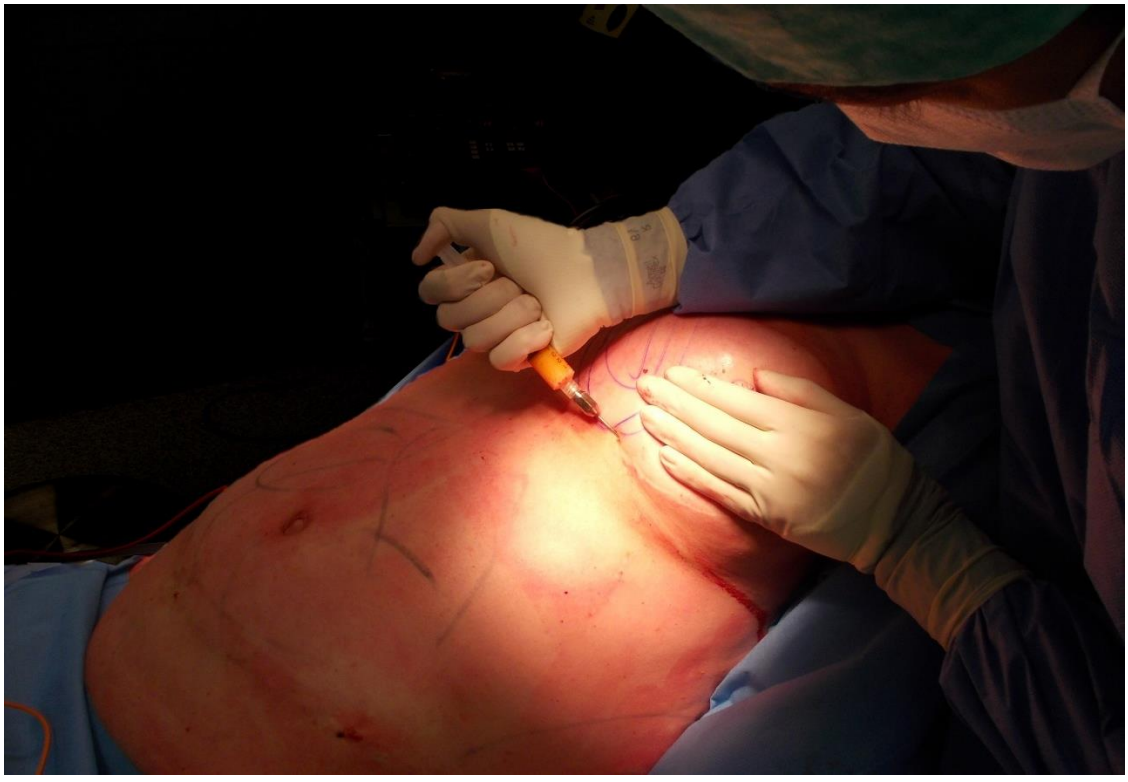

Figure 1. Injection of autologous fat (lipofilling) in a patient who previously underwent breast reconstructive surgery (deep inferior epigastric artery perforator flap or DIEP-flap) after a mastectomy for breast cancer. Permission to use this photograph was given by the patient.

1.2. The advantages of the lipofilling technique

The injected autologous fat resembles the characteristics of the subcutaneous tissue and the procedure of autologous fat grafting has many advantages. Major surgery is not required to obtain these lipoaspirates, nor for lipofilling which doesn't lead to large scars, especially when compared to myocutaneous flaps which are also used to reconstruct defects. The autologous fat, which is available in adequate quantities in most patients, is cheaper and causes less antigenic and allergic reactions than fillers and tissue substitutes such as hyaluronic acid. Other early postoperative complications are rare. Liponecrosis is the most common, followed by local infectious complications such as abscess formation and cellulitis, especially in irradiated breasts.[14-15]

1.3. Lipofilling intends to improve the quality of life in breast cancer patients

Last year Le Brun et al.[16] studied the quality of life of 42 patients in relation to the lipofilling they received in Nantes after they underwent a mastectomy for breast cancer, followed by breast reconstruction with prosthesis, a muscular flap or both. They took into account the aesthetic result, the secondary effects, the emotional impact and the information given by the surgeon or clinical staff prior to the operation. The results showed there was a significantly better appearance of the reconstructed breast and more harmony between the two breasts after lipofilling, according to the patients. 64.1% were satisfied with the evolution of the result, but 75.6% felt the need for a second or even more lipofilling sessions. In 29%, adhesions persist at the site of injection. The hesitation to wear a swimming suit and the apprehension of touching the reconstructed breast significantly decreased after the lipofilling procedure. These positive results are reflected by the sexual behaviour of the patient: 28% of patients regained normal sexual activity after the corrective fat grafting procedure, while only 9.5% were satisfied before lipofilling. Similarly, the decreased libido is slightly less marked after lipofilling (35.7%) than before (42.9%). In general Le Brun et al. conclude that lipofilling can improve the patients' quality of life from an aesthetic, affective and social point of view.

Schultz et al.[17] sent a comparable questionnaire to 43 patients after they underwent lipofilling for breast contour irregularities as a side effect of a mastectomy or breast-conserving surgery. Questions were asked regarding the consistency, the size, the shape, the

sensitivity and irregularities of the breast and the skin quality. The minimal time span between fat grafting and answering the questionnaire was six months. 67.4% of the surveyed patients answered positively on the question whether the irregularities had improved after lipofilling. 60.5% observed a better shape and consistency of the breast. A gradually disappearing effect of the lipofilling after a good initial result was mentioned by only 9.3% of the patients.

1.4. Safety of the lipofilling technique

1.4.1. Recent studies cannot prove an increased risk of breast cancer recurrence but further research is needed

Apart from isolated cases, such as the injection of autologous fat in the soft tissue of the nose during a rhinoplasty by Bruning[18] in 1911, we have to wait until the eighties for the first lipofilling procedures as we now know them.[19-20] This concept of treating defects by using the aspirated fat emerged after the introduction of liposuction.[21-23] In the nineties and the 21st century, lipofilling gained in popularity and was performed universally. Since indications broadened and the popularity of the autologous fat grafting increased, important questions regarding safety of lipofilling to the breast have to be answered. This is a logical result of the available epidemiological data concerning obesity as a risk factor for breast cancer[24] and biochemical experiments exploring the effects of soluble factors secreted by adipocytes. These so-called adipokines, such as adiponectine and leptin, can interact with remaining breast cancer cells in their “dormant” state after segmentectomy or even after mastectomy was performed. Based on the experiments, leptin seems to have a stimulating effect on breast cancer, whereas adiponectin can suppress tumour growth.[25]

Nevertheless, in 2007, the American Society of Plastic Surgeons (ASPS) formed the Fat Graft Task Force to conduct an assessment about safety and efficacy of lipofilling and to make recommendations for future research. Based on an evaluation of 187 scientific publications they could not confirm a potential increased risk of breast cancer recurrence or metastasis related to the autologous fat grafting procedure. However, the articles and reports gathered low numbers of patients and didn’t reach a high level of evidence due to the lack of a control group or a short period of follow-up.[1]

In 2011, Rietjens et al.[14] published a prospective study in which 158 patients who underwent 194 lipofilling procedures were followed during 18 months. 98% of the 158 cases were previously diagnosed with breast cancer and treated with breast conserving surgery or mastectomy with breast reconstruction. Only one local relapse was detected during these 18 months. Since the local relapse was recognised two weeks after the lipofilling procedure, it is doubtful that fat grafting provoked this relapse, and it is plausible that it was a misdiagnosed relapse at the time of the lipofilling. Although only this one case showed a relapse in 18 months, they emphasised that it is still unclear whether the grafting of adipose tissue can wake "dormant" tumour cells.

Because an important number of patients and a longer period of follow-up were needed to determine the risk of recurrence in breast cancer patients, Petit et al.[26] collected 321 patients who were operated for breast cancer between 1997 and 2008 and underwent lipofilling afterwards. Each patient was compared with two control patients having matching characteristics who also had surgery for breast cancer but who did not undergo a lipofilling procedure. They found no significant difference between the two groups in the incidence of local events (local relapse or locoregional relapse) after conservative breast surgery, nor after mastectomy. They also concluded patients with invasive breast cancer didn't have a higher risk for local events after lipofilling, compared with patients who didn't have lipofilling. A significant difference was however found when the analysis was limited to patients with ductal intraepithelial neoplasia or lobular intraepithelial neoplasia: the lipofilling group showed a higher risk of local events (4 events) compared with the control group (0 events). This statistical difference was questioned by the authors themselves in the article and could have been the result of a sampling error. A possible bias in this type of study design is that patients who were subjected to the lipofilling technique started "disease free" because they had a physical examination at the moment of the lipofilling, whereas patients from the control had their last check-up earlier.

In general, the authors of the mentioned studies consider lipofilling as a safe surgical procedure in the armamentarium of the plastic and reconstructive surgeon for patients who have breast cancer, but they stress the importance and the urgent need for further prospective studies with a large number of patients and a matched control group, a detailed definition of the cancer status and a long-term follow-up.

1.4.2. The influence of lipofilling on breast imaging

The Fat Graft Task Force emphasised the fat grafting technique can impede the breast examination and that there might be a potential risk of interference with radiological detection of breast malignancies. Once again, no proof in the literature was found that strongly suggests this interference, and when imaging is inconclusive, biopsies can be performed for diagnostic certainty.[1] In the previously mentioned study by Rietjens et al.[14], it was shown that there was little alteration in mammograms after lipofilling. 5.2% of the patients who had conservative surgery showed minor alterations which were considered as benign images. Petit et al.[15] published in 2011 the results of a retrospective multicenter study in which they included 513 patients who underwent 646 lipofilling procedures after conservative breast surgery or mastectomy. They concluded that lipofilling did not affect radiological follow-up and that standard imaging technologies (ultrasound, mammography and MRI) can identify the fat grafts, microcalcifications and suspicious lesions. It seems by all means useful to us to inform the radiologist whether the patient underwent lipofilling sessions in the breast tissue.

1.5. The resorption of the fat graft remains the main drawback

As in every surgical intervention, the success of autologous fat grafting depends on many determinants. The past couple of years, research projects have concentrated on the best techniques to carry out the harvesting of the fat, i.e. the liposuction, followed by the processing of the lipoaspirate and the lipofilling procedure. This is because, to date, the main drawback of this lipofilling technique remains the unpredictability of the outcome. Mainly due to resorption, not the entire injected material will survive, with a reduction in volume as result. This can increase the need for more grafting procedures after the initial lipofilling, as seen in the study by Le Brun et al.[16] discussed in section 1.3, or can lead to overcorrection when trying to prevent these secondary procedures. This reduction of volume can vary, from 10% one week after surgery to 70% or more in the 20th week after surgery, as proven by Choi et al. by using 3D imaging.[27]

A study on 20 patients who underwent breast reconstruction and secondary lipofilling, conducted by Cigna et al.[28], shows that the appreciation of the cosmetic outcome may reflect this resorption of the grafted fat over time. An assessment of the aesthetic satisfaction was

made by means of evaluation forms, filled out by both the patient and an independent plastic surgeon. This was done preoperatively and repeated one month and six months after the lipofilling procedure. The results showed that, one month postoperatively, patients considered their breast as more cosmetic in comparison with the preoperative situation (the average score evolved from 5.2 to 7.9 on a visual analogue scale). This value decreased to 7.2 six months postoperatively. The score given by the plastic surgeon showed the same tendency (from 4.9 preoperatively to 7.6 after one month and 7.1 after six months).

1.6. In search of the highest viable fat sample

Various parameters in the process from liposuction to lipofilling have been investigated over the years in order to reduce resorption and improve long term results. The cell survival theory[29-30] states that fat graft survival volume corresponds to the number of viable cells at the time of lipofilling. Therefore, most of the research regarding this subject aims to determine the ideal parameters which lead to maximum cell viability in the fat graft prior to injection. To reproduce the findings of our literature review, the surgical procedure from the beginning to the end is divided into 4 parts: donor site selection, harvesting, processing and injection.

1.6.1. Donor site selection

One of the studied parameters is the donor site selection. Ullmann et al.[31] performed an in vivo experiment in which they examined the long-term survival of human fat grafts in nude mice. The donor of the fatty tissue was a woman who had liposuction from the abdomen, the thighs and the breasts which led to three samples of lipoaspirates from different donor sites in one and the same patient. 45 nude mice were divided into three groups, according to the three donor sites. The scalp of each mouse was injected subcutaneously with 1 cc of fat from one region. 16 weeks after this injection, the grafts were explanted and their weight and volume were measured. Histological sections with hematoxylin-eosin staining were made to evaluate tissue organization, quality of the nucleated fat cell, vascularisation, cyst or vacuole formation, fibrosis, inflammation and necrosis. The results showed no significantly differing characteristics between the lipoaspirates from the three donor sites. Recently, these results were confirmed by Li et al.[32] who harvested fat tissue from the flanks, upper abdomen, lower abdomen, lateral thighs and inner thighs, and also injected it subcutaneously into nude mice.

After 12 weeks, the explanted fat grafts did not differ in weight, volume or histological parameters.

This similarity of the different donor sites was previously described by Rohrich et al.[33] who compared the viability of adipocytes harvested from the abdomen, the flanks, the thighs and the knee regions of 5 patients. To analyse viability, a quantitative in vitro colorimetric assay of cell proliferation was performed, which could not show any significant difference between the four body regions.

Since the characteristics of the aspirated fat from different commonly used donor sites do not seem to differ, no guidelines can be proposed concerning the ideal donor site. Therefore it is preferred to harvest in an easily accessible and safe region of the body, taking into account the wish of the patient as well.

1.6.2. Harvesting of the fat graft

Erdim et al.[34] compared the adipocyte viability in lipoaspirates obtained by liposuction cannulas with different diameters. It is important to harvest fat that can be injected through a small needle when performing lipofilling, but the suction cannula has to be large enough to maintain a certain level of fat tissue architecture. They saw that the use of liposuction cannulas with a diameter of 6 mm provided a significantly higher viability of the adipocytes than the cannulas with a diameter of 2 mm or 4 mm. Preference for suction cannulas with larger diameters was confirmed by Kirkham et al.[35] They analysed weight and histology of fat grafts in the sixth week after fat transplantation in nude mice, after these lipoaspirates were obtained in adult women by means of suction cannulas with a diameter of 3 and 5 mm. At six weeks, they explanted the fat grafts and histological analysis showed more intact nucleated adipocytes, less infiltrate and less fibrosis in the fat grafts which were previously collected through the 5-mm cannula. Additionally, a difference was found in the graft weight with more retained adipose tissue in the 5-mm group.

In a recent review of studies related to fat grafting research, Pu[36] states that syringe aspiration should be considered as a technique of choice for harvesting of fat grafts. The advantage of syringe aspiration over conventional liposuction with a negative pressure machine is that the former is a less traumatic method, albeit slower. Shiffman et al.[37]

examined histological sections of lipoaspirates which were harvested with different suction forces. They saw fat cell damage was more present in the lipoaspirates collected by a vacuum pressure of 1 atm (approximately 10-15 %), compared to lower vacuum pressures (2% or less fat cell damage). Crawford et al.[38] performed a Trypan blue staining on lipoaspirates obtained with syringe aspiration and conventional liposuction. A greater number of viable fat cells were found in hand-suctioned specimens relative to standard liposuction.

1.6.3. Processing of the fat graft

Many techniques have been described for the processing of the lipoaspirate as well. The processing consists of the removal of blood, aspiration fluid and the oily layer which results from ruptured adipocytes. However, which is the best technique, and even whether or not processing is necessary remain controversial. Currently, 4 methods are mainly used in the clinical practice: decantation by gravity, washing, filtration and centrifugation.

Until 2008 it was not clear whether centrifugation had any impact on the viability of lipoaspirates but it was plausible that centrifugation would have a harmful effect due to mechanical injury. Xie et al.[40] compared the viability in human lipoaspirates after centrifugation for 2 minutes and with four different centrifugal forces between 1 000 and 4 000 rpm. It was shown that centrifugation had a detrimental effect on the viability and they discovered a linear relationship between increasing centrifugal force and reduced viability. Especially when rotation speed exceeded 4 000 rpm, they saw a significant drop in the cell viability and they observed significantly distorted and fractures adipocytes on histological analysis.

In the previously mentioned article from Rohrich et al.[33], in which a viability assay did not reveal any differences between the four donor sites, a second criterion was investigated. They performed centrifugation at 500 g for 2 minutes at 25 degrees Celsius on samples from each of the four sites. Centrifuged and non-centrifuged lipoaspirates appeared to be equally viable.

Figure 2. Example of freshly aspirated fat, before centrifugation.

Figure 3. The same tube with aspirated fat, after centrifugation. Four fractions can be identified: the upper oily fraction, the lipid layer, the serosanguinous layer and the pellet.

Condé-Green et al.[41-42] studied the effect of centrifugation on the composition of the lipoaspirates, by comparing it with fat samples that decanted slowly under the action of gravity. They saw the decanted adipose tissue contained more intact nucleated adipocytes and mesenchymal cells, which are important for the angiogenic and adipogenic effect of the fat graft. Less mesenchymal cells and more altered adipocytes were found in the lipid layer of the centrifuged lipoaspirates. This lipid layer is one of the four fractions in the lipoaspirate after centrifugation (figure 2 and 3) and is used for injection, whereas the upper oily fraction and the serosanguinous fraction are of no use according to most plastic surgeons. In the lowest layer of the centrifuged lipoaspirate which is usually discarded, the pellet, they found however the greatest concentration of endothelial cells and mesenchymal cells. Condé-Green et al. concluded that, however centrifugation is a quick method to separate the required adipocytes from other substances such as blood, lipids and other components, it leads to a higher number of damaged cells and the separation of mesenchymal cells, which are needed for vascularisation of the fat graft and which are more resistant to trauma. They also stated that the pellet does not have to be discarded after centrifugation, but injected together with the lipid layer, in order to prevent graft resorption.

1.6.4. Injection of the fat graft or lipofilling

Erdim et al.[34] not only compared the influence of the size of the liposuction cannula, but they also analysed how the size of the injection needles affects the numbers of live adipocytes in the fat grafts. They injected lipoaspirates through injection needles of 14, 16 and 20 gauge. The viability of the adipocytes was similar to each other in the three groups but compared to fresh lipoaspirates which were not injected, the count of viable adipocytes was significantly lower.

Choi et al.[27] also studied the effect of the injected volume in the breast. 123 breasts were divided in three groups based on the volume of fat injected: the first group receiving an average volume of 151 cc, the second group receiving a smaller volume with an average of 51 cc. The third group received an intermediate volume with an average of 93 cc. In the long term (the latest observation was after 140 days) it was found that the retention of transplanted adipose tissue was significantly higher in the group with the largest volume injections.

The same patients were divided by Choi et al.[27] according to possible prior radiotherapy on the acceptor site: 28 irradiated breasts were compared to 95 non-irradiated breasts. After 140 days of observation, no statistical difference was found.

1.7. Viability analysis

Several options are available to determine the viability of fat cells. Manual cell counting on a haemocytometer after staining of the cytoplasm with trypan blue was a frequently used method. However some drawbacks limit the quality of this method, such as its inter-observer variability and the lack of cytoplasm in mature adipocytes.[43]

Lee et al.[44] tried a protocol utilizing an automated cell counter. They used two viability stains: trypan blue and CellTiter Blue. Manual cell counting of the samples stained with trypan blue showed a number of live adipocytes between 2 750 000 and 19 200 000 per ml. Counts with the automated cell counter ranged from 3 230 000 to 4 290 000 cells per ml, which is significantly different from the manual cell counting. The automated cell counter can additionally measure the cell size and a count of the cells between 40 and 150 micrometer,

which is the size range of adipocytes, yielded 1 040 000 to 1 420 000 live cells per ml. Automated cell counting of the samples stained with CellTiter Blue yielded 993 000 to 1 340 000 viable cells per ml. They concluded that using the automated cell counter does not lead to the high inter-observer variability, seen in manual counts, and that it can count cells in a certain size range. Therefore, this is a more reliable method to determine the live adipocyte population than manual cell counting. To overcome the problems related to counting adipocytes, such as the fragility of adipocytes and their tendency to float, they also prepared samples which contained carboxymethyl cellulose and the tissue fixative formalin. Carboxymethyl cellulose was found to decrease the sampling variability by keeping the adipocytes evenly distributed in a homogenous sample, whereas formalin avoided the fall in cell count over four hours by stabilizing the adipocytes.

In 2007 Suga et al.[45] compared different methods in order to assess cell viability and cell numbers in freshly aspirated fat tissue. After processing by digestion with collagenase and centrifugation (at 430 g for 5 minutes) was done, they stained samples from the adipocyte layer, the watery layer and the bottom layer (pellet) of the lipoaspirates with Hoechst 33342 and propidium iodide to distinguish viable cells from dead cells. Hoechst 33342 has the ability to stain the nuclei of viable and dead cells, whereas propidium iodide specifically stains the nuclei of dead cells. The adipocyte layer contained $4.4 \pm 0.66 \times 10^5$ viable adipocytes, $2.5 \pm 0.28 \times 10^5$ dead adipocytes, $1.5 \pm 0.23 \times 10^5$ viable non-adipocytes and $7.5 \pm 0.72 \times 10^5$ dead non-adipocytes. The watery fraction did not contain any cells. The bottom layer contained $10.0 \pm 2.8 \times 10^5$ viable non-adipocytes and $2.3 \pm 0.34 \times 10^5$ dead non-adipocytes. No adipocytes were found in the bottom layer. Multicolour flow cytometry revealed that 37% of the non-adipocytes in the bottom layer were adipose-derived stem cells, another 37% consisted of blood-derived cells, 15% were endothelial cells and the remaining 11% were categorised as other cells.

Xie et al.[40] introduced in 2008 the glucose transport test to assess the viability of the lipoaspirates obtained from the lower abdomen of five young women. Given that adipocytes can take up glucose, the transported glucose into adipocytes is a marker for their metabolism level and therefore their viability. They compared their results with a MTT assay, which is a tetrazolium salt assay, and there appeared to be a good correlation between the results from both methods. The glucose transport test does not measure the viability of single adipocytes but measures the overall viability of the adipose tissue.

Medina et al.[46] performed a study to predict long-term survival of fat grafts in 128 nude mice. During the first ten days after lipofilling they explanted fat lobules and examined apoptosis, weight, histology and DNA content of the grafts. They compared these observations with the grafts they explanted six weeks after the initial grafting procedure. This procedure allows assessing early and late performance of the fat grafting. It was shown that the apoptosis rate after ten days reflects the absorption rate and hence the outcome of the lipofilling. Even though the graft undergoes apoptosis during the first ten days, the DNA content and weight of the graft initially remains stable, which can be explained by the avascular nature of the graft and the inability to remove dead cells. When the blood supply to the graft and its drainage develops, the absorption will occur. This is evident by the decrease in weight, DNA content and histological changes. They concluded that by measuring the apoptosis rate after the first ten days, an estimation can be made regarding the long-term performance of the lipofilling procedure. A remarkable incidental finding was that the weight of the graft decreased by about 20 to 25% on the first day after lipofilling, while the weight remained unchanged over the next ten days. The authors believed this initial weight loss is caused by dehydration of the grafts.

Boschert et al.[47] discovered in 2002 that after centrifugation of a fresh lipoaspirate, the highest cell viability can be found in the bottom part of the adipocyte layer. It contains 2.5 times more viable cells than the most superficial layer, just beneath the oily layer. This can be explained by a higher number of intact cells at the bottom, while a higher number of disrupted cells are present at the top because of their lower density. Therefore the authors recommended using the adipocytes from the lower fatty layer. However, in our view, the selective use of this lower fatty layer is difficult to carry out practically.

Miyazaki et al.[48] discovered that when collecting lipoaspirates, several cells that are closely adherent to mature adipocytes can generate fibroblastic cell populations with the potential of multiple differentiation along mesenchymal lineage to produce adipocytes, osteoblasts and chondrocytes. They saw that frequency of adipogenic differentiation was higher in the ceiling cell population (the upper layer after centrifugation) than in the bottom cell population.

1.8. Cryopreservation

A possible solution in addressing the problem of resorption after lipofilling is storage of the remaining adipose aspirates after the initial liposuction. Studies have been done to determine whether cryopreservation is a valid option to store adipocytes. Theoretically, if resorption occurs, the previously aspirated adipose tissue could be thawed and injected. This could be done on an outpatient basis and under local anaesthesia. The main advantage of this method would be that it eliminates the need for more liposuction procedures under general anaesthesia and, consequently, that it reduces hospitalisation time, costs and operative or anaesthetic risks.

In 2004 Pu et al.[49] conducted a study to determine whether lipoaspirates could be a valid source of processed lipoaspirate cells after cryopreservation at -196 degrees Celsius. They counted the number of processed lipoaspirate cells after cryopreservation (with cryoprotective agents dimethyl sulfoxide and trehalose), processing and a 2-week culture and compared it to freshly aspirated adipose tissue after processing and a 2-week culture but without preservation. In the frozen and thawed tissue, they counted a number of cells equal to 90% of the yielded number of cells in the fresh adipose aspirates. The length of time until these processed lipoaspirate cells from the cryopreservation group became adherent to the culture plate after initial culture was also recorded. This revealed to be a longer time span compared to fresh cells (48 to 72 hours in the cryopreservation group, 24 hours in the control group). Despite this latency of cell growth after cryopreservation, they concluded that cryopreserved lipoaspirates can still be a good source of processed lipoaspirate cells.

In 2010 Pu et al.[50] published their findings about an in vitro study in which they compared the adipocyte viability immediately after the harvest of the fat from the lower abdomen with the viability after cryopreservation at -196 degrees Celsius. The lipoaspirate of each patient was mixed with cryoprotective agents (dimethyl sulfoxide and trehalose) before storage. Evaluation with the trypan blue vital staining did not show a statistically significant difference between these two groups in terms of adipocyte viability. By performing a glycerol-3-phosphatase dehydrogenase assay, which measures the activity of an adipocyte specific enzyme but requires enzyme digestion and multiple centrifugations, they found that the activity of this enzyme appeared to be significantly lower in the samples that underwent freezing and thawing. This indicates a less optimal cellular function of the adipose tissue.[51]

Histological analysis showed no differences between the two groups. The authors concluded by stating that the frozen fat grafts have a decent viability, but, due to their lower level of glycerol-3-phosphatase dehydrogenase activity, that they might not survive well after being transplanted.

Different temperatures for cryopreservation have been tested by Li et al.[52] They have stored human lipoaspirates at -20, -80 and -196 degrees Celsius. Viability tests by using a MTT assay were done after 2 and 7 days of cryopreservation. Although a downward trend could be seen, there was no significant difference between the viability on day 2 and day 7 after harvesting and cryopreservation, nor were there significant differences in cell viability among the cells at the three temperatures. Additionally they studied the influence of hydroxyethyl starch as a cryoprotective agent by adding it to the samples before freezing it. Again, there were no significant differences in viability between the three temperatures or between the time spans from freezing to thawing (2 days versus 7 days). 2 and 4 weeks after cryopreservation, the adipose tissue was injected in nude mice, so these fat grafts could be harvested again for histological analysis, 3 months later. This showed less viable adipose tissue in the 4 week group but, once again, no significant differences were found among the samples preserved at various temperatures with or without the cryoprotective agent. In brief, Li et al. summarize their study by suggesting that cryopreservation of the lipoaspirates is a clinically practical method for secondary lipofilling sessions. They state that an expensive and large facility to create liquid nitrogen temperatures (-196 degrees Celsius) does not yield a significantly better viability than commercial freezers up to -80 degrees Celsius. This is in contrast with the findings by Son et al.[53] who conclude that fat banking at -70 degrees Celsius or warmer is inadequate to maintain viability. They saw a rapid drop in adipocyte viability after storage at -15 and -70 degrees Celsius. After 1 day of storage, they observed that viability was reduced to 12-13% in both temperature groups, while this number amounted to 80% in freshly aspirated tissue, and the viability decreased gradually during the following 8 weeks (after 8 weeks, only 5% was viable). However, they did not add any cryoprotective agent, which is needed to prevent intracellular ice formation and osmotic stress to maintain optimal viability when cryopreserving adipose tissue.[43,54]

Several recent studies in the cryopreservation of adipose tissue show promising results but emphasize the importance of an adequate freezing and thawing protocol at the right temperature and with proper cryoprotective agents. If in vivo experiments can confirm the

usefulness and benefits of lipofilling procedures by means of cryopreserved lipoaspirates, it will become a valid solution to the issue of resorption of the fat graft. Nevertheless, we believe that further research is needed to improve the viability of the injected fat graft, which could render secondary fat grafting unnecessary.

2. Hypothesis

2.1. The concept of sharp needle intradermal fat grafting

In 2012 Zelter et al.[55] reported about their 3-year experience with the SNIF-technique (Sharp Needle Intradermal Fat grafting) as an alternative to classic intradermal fillers in the treatment of facial wrinkles. 250 patients treated with this autologous fat grafting technique were evaluated by clinical examination and photographs. 96 of them have been followed for at least 1 year. The clinical outcome was positive and stable with results lasting longer than resorbable dermal fillers and a low rate of complications. The SNIF technique uses a small harvesting cannula (with a 2 to 3 mm diameter and 1 mm holes with sharp edges) allowing the harvest of fat particles which are smaller than the fat particles aspirated by classic liposuction. Furthermore, a sterile nylon cloth with 0.5 mm mesh size was used to rinse the lipoaspirates with a saline solution and, consequently, fat particles larger than 500 micrometer were ready for intradermal injection by means of a 23-gauge needle.

The underlying idea of the SNIF-technique and what is considered the key to its success, is based on the concept of inverse proportionality between the radius of the injected fat particles and the total surface of the fat particles (figure 4). Injecting smaller fat particles leads to a larger total contact surface, which is available to capillaries in the receptor area, essential for nutrition and respiration of the newly grafted fat. It is also believed that this large area of contact can integrate the fat better so that it feels more natural and smooth and that it can prevent migration of the fat graft, which is of great importance when injecting it in facial volume lacking areas or wrinkles.[9] A possible additional reason for the success of the SNIF-technique can be the composition of the injected fat. After liposuction, the fat particles larger than 0.5 mm were separated from the smaller ones by means of a nylon cloth and, finally, they injected the fat graft with a 23-gauge needle (which has an outer diameter of 0.64 mm and an inner diameter of 0.34 mm). They basically select a fat sample that contains particles larger than 0.5 mm and smaller than a certain size due to the narrow harvesting cannula and small injection needle. This maximum size, however, is difficult to estimate since the flexible fat particles are aspirated with suction force and injected with pushing force respectively.

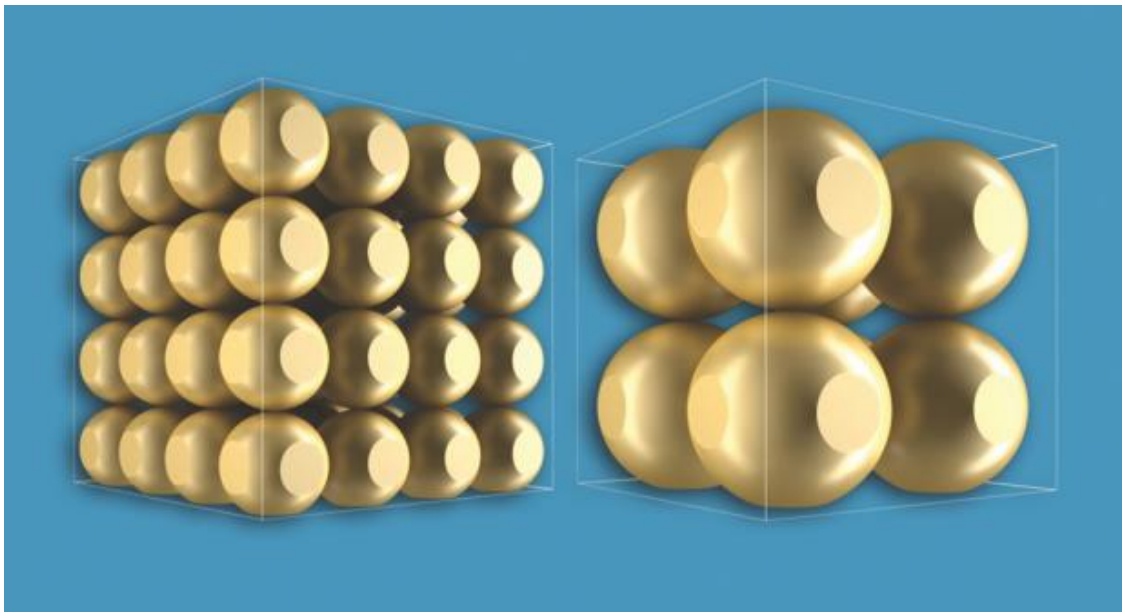

Figure 4. Concept of inverse proportionality in fat grafting. The injection of fat particles with a shorter radius leads to a larger total contact surface, compared to the injection of the same volume of larger fat particles. Image copied from *Sharp-needle intradermal fat grafting (SNIF)* from Zeltzer et al.[55] Permission to copy this image was given by the author.

2.2. The viability and composition of different size classes of fat samples

It is interesting to look closely at this concept of selecting fat samples based on the size of the fat particles and other components in the lipoaspirate. A precise separation of freshly aspirated fat tissue in a laboratory setting could help us investigate whether a higher viability or a more favourable composition can be found in a particular size class. This may lead to a quick and uncomplicated additional step in the process of autologous fat grafting surgery, after the harvesting and prior to lipofilling. Specifically, one would select a sample with more advantageous characteristics for lipofilling and, theoretically, a better postoperative result can be expected, i.e. a more predictable result that is less affected by resorption and will require less additional lipofilling sessions.

It can be expected that the overall viability in smaller fat particles is better than in large fat particles. This is mainly as a result of the presence of cells other than adipocytes. The freshly aspirated adipose tissue is composed of heterogeneous cell populations including adipose-derived stem cells, erythrocytes, leucocytes, vascular cells such as endothelial (progenitor) cells, smooth muscle cells and pericytes, and other cells.[56] When dividing the aspirated fat tissue according to size, adipose-derived stem cells, preadipocytes and other cells may be present in varying degrees due to their different diameters. Since these cells have physical

characteristics that differ from those of mature adipocytes, they might as well influence the quality of the injected fat and the results in the long-term after a lipofilling procedure. While mature adipocytes tend to fail to survive if vessels from the recipient site don't restore the vascularity in the fat graft within the first four days, preadipocytes are less dependent on this nutrient and oxygen supply.[57] It is also believed that preadipocytes are more prone to survive the process of transplantation because of their smaller size and lower intracellular content of lipid.[9,30,57] Furthermore, preadipocytes still have a self-renewing capacity and the capacity to differentiate into mature adipocytes after lipofilling takes place, which can be of importance in the prevention of graft resorption.

In 2005 van Harmelen et al.[58] stated that adipocytes represent approximately two-thirds of the total cell number in the adipose tissue and, due to their large cell size, make up more than 90% of the tissue volume. However, the advantageous physical characteristics of preadipocytes and adipose-derived stem cells, their survival and the high percentage of adult stem cells in fatty tissue[9,59], make them an interesting research focus in the domain of fat grafting. Therefore, we perform our laboratory research on the complete lipoaspirate, without discarding the upper oily layer, the lower aqueous layer or the pellet, and we concentrate not merely on the adipocytes but include all cell types in our viability assay.

Due to their characteristics and the inverse proportionality between radius and total contact surface which leads to a better vascularisation and integration of the fat graft, we expect a clinically more stable result when injecting relatively small fat samples that contain enough preadipocytes. However, the smaller the fat sample, the lower the concentration of mature adipocytes given their large diameter and their tendency to adhere to other adipocytes in a fat particle. We believe a balance should be found, i.e. a sample that contains enough immature adipocytes but is also rich in viable adipocytes. Therefore, histological sections are made to determine the composition of the different fat samples. A distinction will be made between adipocytes and other cells. We expect to find a greater portion of adipocytes in the total cell number in the larger fat samples.

2.3. Additional studied parameters

In 2003, van Harmelen et al.[60] studied the influence of body mass index (BMI) and age on the differentiation capacity and total number of adipocytes and preadipocytes in dissected (not

aspirated) breast tissue. They discovered there seems to be a constant ratio between the number of adipocytes and the number of preadipocytes, which is not significantly different in obese patients compared to patients with lower BMI and which is the same in different age groups. In patients with higher BMI, the number of adipocytes and preadipocytes per body increases, but due to their increasing cell size, the number of adipocytes and preadipocytes per gram adipose tissue was lower. Increasing BMI also correlates with lower differentiation capacity of the preadipocytes. No significant correlations were found between the patient's age and the number of adipocytes, the number of preadipocytes, their ratio and the differentiation capacity of preadipocytes. We will also take into account the effect of the BMI and age on the viability in the different fat samples. Other parameters, such as chemotherapy and radiotherapy, will be studied as well.

3. Goal

Despite the growing interest in fat grafting and improved scientific understanding, the composition of the lipoaspirate is still not clear. More specifically, the exact composition and the ratio of cell types (fat cells, adipose-derived stem cells, fibrocytes, erythrocytes,...), and their viability remain uncertain. The method of aspiration of adipose tissue, their processing "on the bench" in the operating theatre and the insertion varies from surgeon to surgeon because scientific knowledge is lacking.

The goal of this project is to understand the scientific basis of this technique, which already has its clinical application, and, if possible, to develop an innovative application. More specifically, the purpose of the research project is the analysis of the composition of the lipoaspirate, which we obtained performing liposuction, and the viability of the cells it contains, with a special interest regarding the size of the fat particles. Since mature adipocytes, stem cells and other cells differ in size, an interesting parameter that has not been studied yet might be the classification of the lipoaspirate content according to the size of the fat particles. In general, a better understanding of the scientific basis of the existing techniques will lead to better clinical practice and possibly the discovery of innovative applications.

Since lipofilling is already a common procedure in many departments of plastic and reconstructive surgery and the incidence of patients who are diagnosed with breast cancer and possibly have to undergo breast reconstructive surgery are very high, the relevance of research in this domain is important. The varying resorption of the fat graft and the unpredictability of the outcome remain the most important drawbacks of this procedure. Optimisation of the technique can lead to less resorption, better long term results and, consequently, a decreased need for secondary lipofilling procedures with less risks and costs associated to surgery and hospitalisation.

4. Methods

4.1. Patient selection

7 patients undergoing elective liposuction at the Department of Plastic and Reconstructive Surgery at the Brussels University Hospital between October 2012 and April 2013 were recruited for this prospective study. A limitation was that patients could be included only when the general investigator (M.G.) was present during the intervention. Of the patients, all of them were Caucasian female, with ages ranged from 38 to 79 years and a mean age of 55 years. The mean body mass index was 27.2 kg/m² (range 20.3-44.1 kg/m²). Exclusion criteria for this study were haematological abnormalities, connective tissue diseases and lipodystrophy. None of the patients were smokers.

4.2. Donor site selection

Lipoaspirates were harvested from the lower abdomen, inner thighs or trunk because most studies show no difference in viability of fat grafts from these different regions.[31,33]

4.3. Harvesting of the lipoaspirate

The procedure is performed under general anaesthesia in dorsal decubitus in the main surgical theatre. Following in-hospital protocol, infiltration of donor sites was performed with a mixture of 500 to 1 000 ml of sodium chloride 0.9%, 20 ml of Xylocaine® (lidocaine) 1% with 1 : 200 000 adrenaline, and 20 ml of Naropin® (ropivacaïne HCl) 0.75% using an infiltration cannula after we incised the skin with a surgical scalpel number 11. Subsequently the harvesting was carried out using a liposuction cannula with a 4 mm inner diameter and a conventional liposuction machine (Aspirator 2 by Euromi®). The suction pressure to perform liposuction was -0.9 bar (corresponds to approximately -13 psi or -675 mmHg). All donor site incisions were closed with surgical sutures. Surgical dressings and compression garments were applied at the liposuction site. Compression of the lipofilling site is avoided: patients are asked not to wear a brassiere for one month.

4.4. Processing of the lipoaspirate

4.4.1. Centrifugation of the lipoaspirate

The obtained lipoaspirate (figure 2) consists not only of fat tissue but also of a liquid fraction, which is aspirated along with the adipocytes. This liquid portion contains peripheral blood, ruptured cell material and the injected solution for local anaesthesia and vasoconstriction. These components are easier to distinguish after centrifugation in 10 cc tubes following Coleman's protocol (for 3 minutes at 3 000 rpm, figure 5). On the tubing horizontal lines are drawn indicating the four fractions: the upper oily level which is the least dense and which is composed of free fatty acids released after adipocyte rupture, the lipid layer containing adipocytes, the watery layer containing water, blood and any aqueous element, and the pellet on the bottom (figure 3). We measured the ratios between these 4 layers. The tubes were transferred immediately and at room temperature to the laboratory for further investigation.

Figure 5. A conventional centrifugation machine (Aspirator 2 by Euromi®) will centrifuge the 10 cc tubes with the freshly aspirated fat for 3 minutes at 3 000 rpm (Coleman's protocol).

4.4.2. Sieving of the lipoaspirate into 4 size classes

By means of polyamide sieves with square meshes of 500, 1 000 and 1 600 micrometer (figure 6), we divided the fat grafts into four groups according to the size of the components in the lipoaspirates: a sample containing the components smaller than 500 micrometer, a sample with components between 500 and 1 000 micrometer, a sample with components between 1 000 and 1 600 micrometer and finally a sample with components larger than 1 600 micrometer (figure 7).

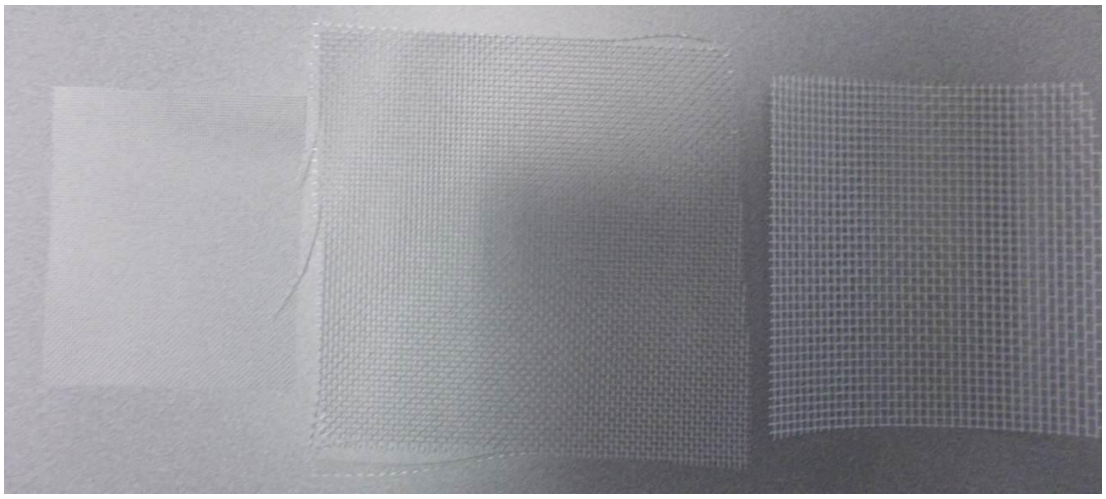

Figure 6. Examples of the polyamide sieves with square meshes of different sizes. Left: 500 µm. Center: 1 000 µm. Right: 1 600 µm.

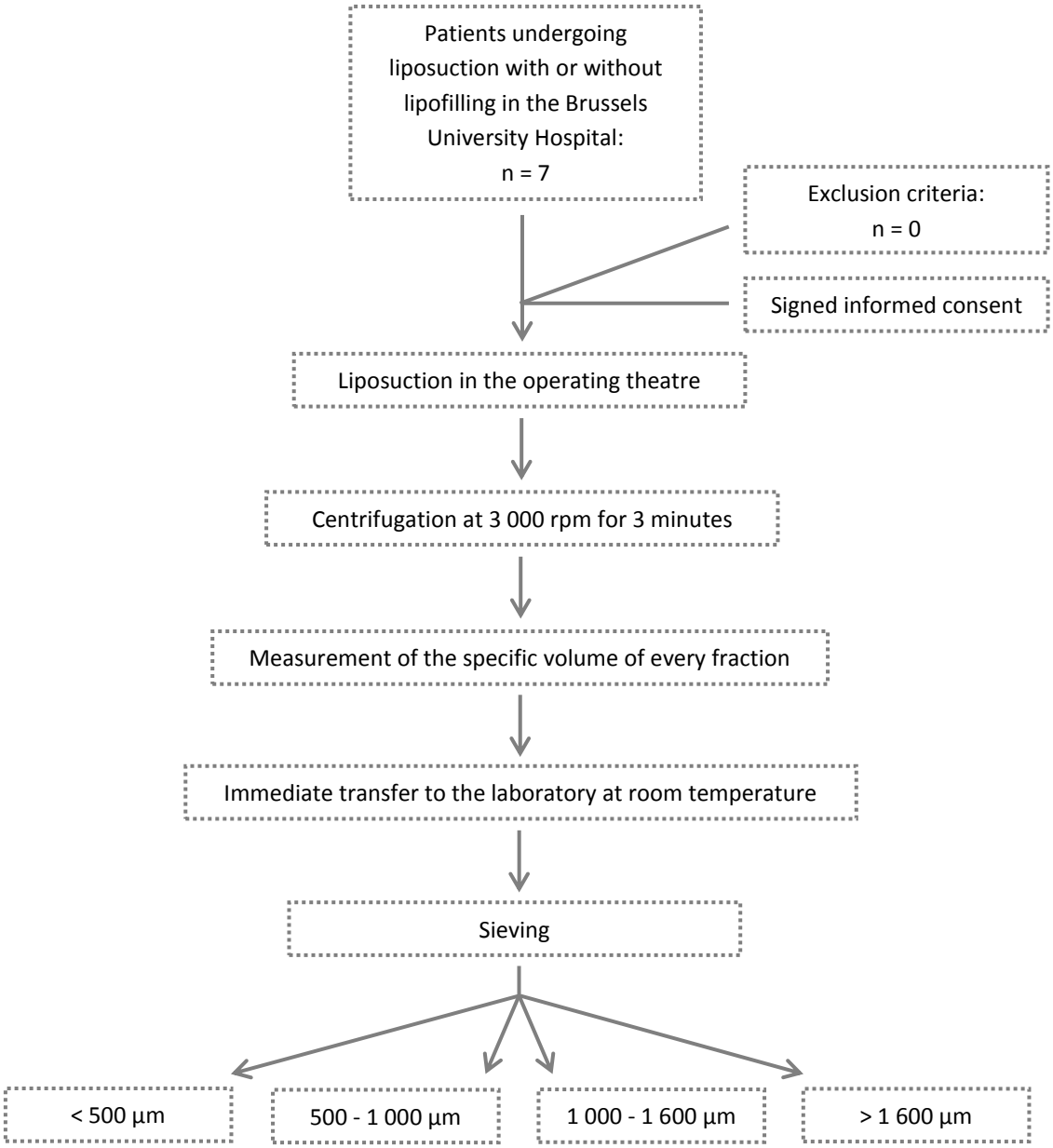

Figure 7. Concise schematic representation of the research protocol: from the selection of patients to sieving of the harvested material into different groups.

4.5. Viability analysis

Of each sample we aspirated cells and brought them into a multi-well plate (figure 8). We added 1 ml Hanks' balanced salt solution (HBSS), 15 µl Hoechst 33342 and 15 µl propidium iodide to the four samples to distinguish viable cells from dead cells. Since Hoechst 33342 has the ability to stain the nuclei of both viable and dead cells, whereas propidium iodide specifically stains the nuclei of dead cells, we defined cells stained only with Hoechst 33342

as viable cells, and cells stained with propidium iodide were counted as dead cells. Erythrocytes were not included in the count because they lack a cell nucleus.

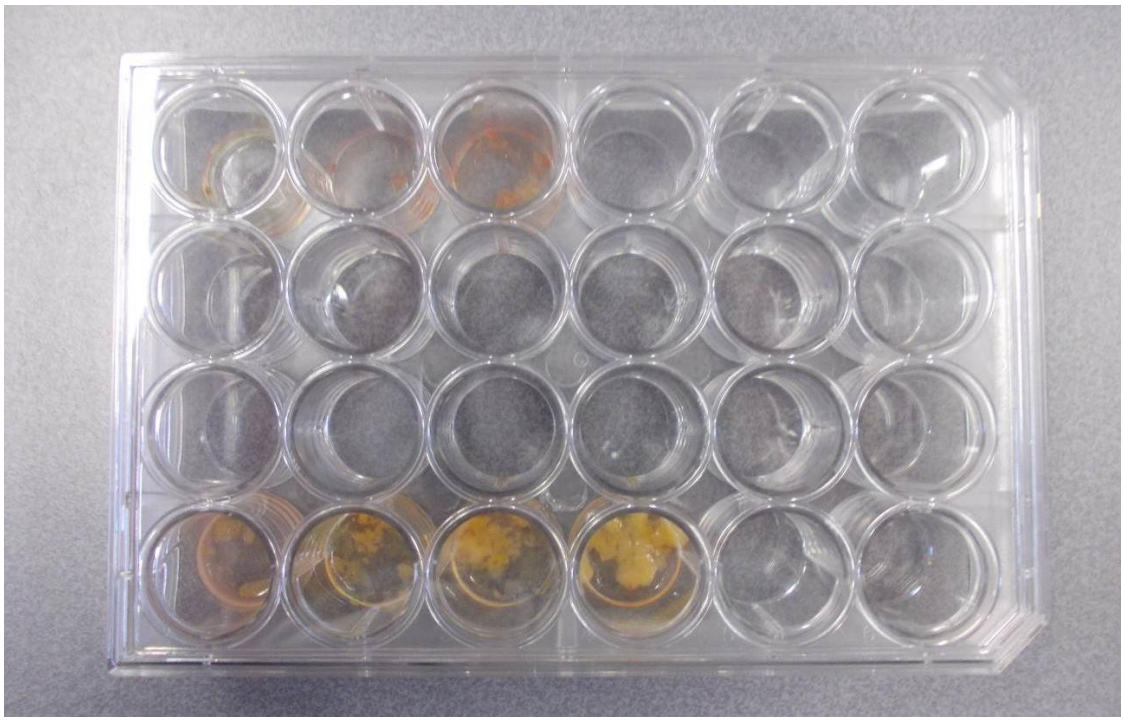

Figure 8. Example of a multi-well plate with 4 groups of aspirated, centrifuged and sieved fat in the bottom row. From left to right: a sample with particles smaller than 500 µm, a sample with particles between 500 and 1 000 µm, a sample with particles between 1 000 and 1 600 µm and a sample with particles larger than 1 600 µm.

This protocol is in analogy with the study by Suga et al.[45] in which different methods to assess cell viability and cell numbers in freshly aspirated fat tissue were compared. After preliminary testing, we decided to not process our samples any further by digestion with collagenase and secondary centrifugation prior to staining with Hoechst 33342 and propidium iodide. During preliminary testing we saw digestion with collagenase led to a considerable destruction of viable cells. Because our aim was to analyse samples which resemble as closely as possible the fat grafts that can be injected in patients, this step was rejected from the protocol.

We performed a count of 1 000 nuclei in each sample with distinction of nuclei that were stained with only propidium iodide and nuclei that were stained by both propidium iodide and Hoechst 33342. We used an inverted fluorescence microscope type Axiovert 135M with a 20x objective by Carl Zeiss (Jena, Germany; figure 9) for this viability analysis.

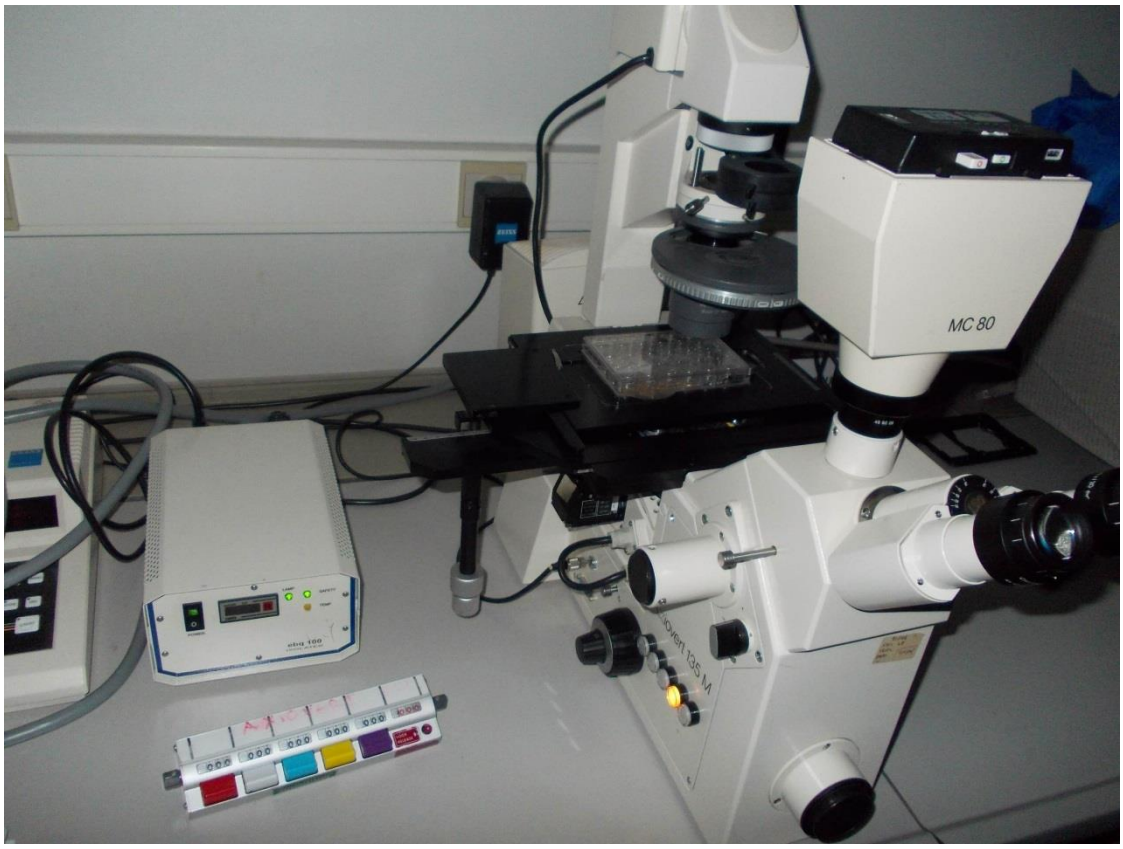

Figure 9. Counting viable and non-viable cells with an inverted fluorescence microscope (Axiovert 135M with a 20x objective; Carl Zeiss, Jena, Germany).

4.6. Composition analysis

Fluorescent histological sections with S100 staining were made for the evaluation and numbering of different cell types.

4.6.1. Preparation of histological sections

4.6.1.1. Chemical fixation with formaldehyde

After sieving of the lipoaspirates was done and samples were taken for the viability analysis, we conserved the remaining lipoaspirate larger than 1 600 µm in a 4% buffered formaldehyde solution for 72 to 96 hours at room temperature to prevent tissue degradation and preserve cells by cross-linking proteins. The 3 groups consisting of the smaller fat particles were put together in a mixed group. This new distribution of the lipoaspirate had to be done because of the difficulties we discovered during the preliminary tests. We observed that fat particles shrunk and decreased in number during the different dehydration and

clearing steps. Also there was a possible loss of fat particles when removing the clearing agent from the samples because of their translucent appearance after clearance. Eventually we were not able to prepare these small amounts of fat specimens for histological sections. It was not always possible to acquire a larger volume of aspirated fat for research to overcome this problem, because the study was performed in a hospital setting and medical care of the patient took precedence. In some slender patients requiring lipofilling, additional fat samples for investigation were not available. Consequently, a new classification was set, consisting of a group of fat particles smaller than 1 600 µm and a group of fat particles larger than 1 600 µm. The former group was also conserved in a 4% buffered formaldehyde solution for 72 to 96 hours at room temperature.

4.6.1.2. Dehydration protocol

During pilot tests for this project, it was difficult to prepare the lipoaspirates for histological sections due to their liquid state. Eventually we established a modified dehydration and clearing process which allowed us to remove water and prepare histological sections.

This dehydration process is carried out by transferring the tissue through solutions of increasing ethanol concentration:

1. ethanol 70% for 2 hours
2. removal of the ethanol 70%
3. ethanol 70% for 2 hours
4. removal of the ethanol 70%
5. ethanol 90% for 1 hour
6. removal of the ethanol 90%
7. ethanol 90% for 1 hour
8. removal of the ethanol 90%
9. ethanol 100% for 1 hour
10. removal of the ethanol 100%
11. ethanol 100% for 1 hour
12. removal of the ethanol 100%

This process takes place at room temperature.

4.6.1.3. Clearing protocol

For the following step, the dehydrated samples were transferred to glass jars. This next step consists of removal of the dehydrating substance from the tissue and is called “clearance” because of the translucent appearance of the tissue after the dehydrating agent has been removed. This is done with a clearing agent that serves as a transition medium between two immiscible compounds, ethanol and paraffin. There are many clearing agents in use. We chose to use toluene, formerly known as toluol. Toluene is an agent that clears properly and fairly rapidly, that does not overharden the tissue like xylene tends to do, is safer than benzene (which is a known carcinogen) and that is miscible with ethanol as well as with embedding paraffin.[61-62]

The clearing process consists of the following steps:

1. toluene for 45 minutes
2. removal of the toluene
3. toluene for 45 minutes
4. removal of the toluene

This process takes place at room temperature as well.

4.6.1.4. Infiltration

We transferred the dehydrated and cleared samples into metal moulds and impregnated them with paraffin. These moulds were stored overnight in the paraffin oven at 60 degrees Celsius.

4.6.1.5. Embedding in paraffin

The next day, we aspirated the used paraffin and began the embedding of the samples to form a paraffin block around the samples.

The embedding was performed at room temperature following these steps:

1. the metal mould containing the fat sample is placed on a cold plate for approximately 5 seconds

2. transfer to hot plate
3. filling of the mould with paraffin
4. a plastic cassette covers the mould and is labelled with the patient number and the size of the fat particles
5. additional filling with paraffin in order to fix the plastic cassette
6. transfer to cold plate for approximately 1 hour (figure 10)

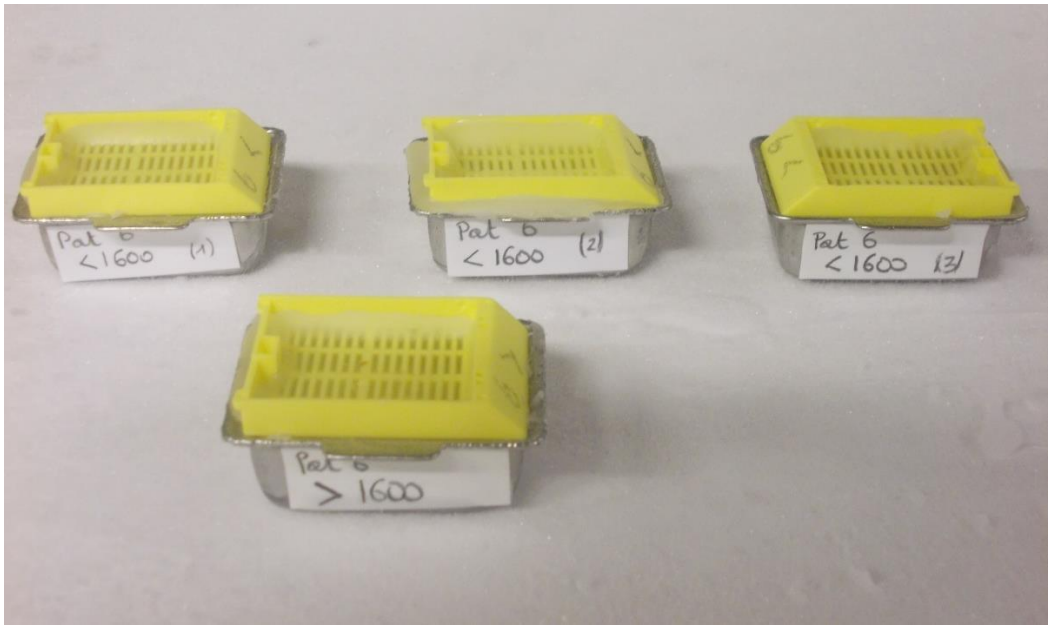

Figure 10. The metal moulds, containing the fat samples of different sizes and covered with the plastic cassette, on the cold plate.

4.6.1.6. Microtome sectioning

After hardening of the paraffin, the paraffin blocks can be cut in the Microm HM 450 sliding microtome by Thermo Scientific (Waltham, Massachusetts, USA; figure 11) at a thickness varying from 4 to 6 µm. Sections are placed on a Superfrost glass slide and dried on a warm plate at 40 degrees Celsius.

Figure 11. Microm HM 450 sliding microtome by Thermo Scientific (Waltham, Massachusetts, USA).

4.6.2. Fluorescent antibody stained sections

We chose to use polyclonal rabbit anti-S100 antibodies to detect adipocytes (Dako, Glostrup, Denmark).[63] This antibody reacts with the S100 equivalent protein in humans, more specifically it cross-reacts strongly with human S100B and weakly with S100A1 and S100A6. S100B is most abundant in glial cells of the central and peripheral nervous system, in melanocytes, chondrocytes and adipocytes.[64-66] Polyclonal rabbit anti-S100 antibodies are used in the identification of S100-positive neoplasms since they label cells expressing S100 in Langerhans' cell histiocytosis, schwannoma, malignant melanoma and chondroblastoma.[67-70]

The sections were prepared for analysis with fluorescence microscopy following these steps:

1. bake at 56 degrees Celsius for 1 hour
2. immerse slides in toluene bath 1 for 5 minutes
3. immerse slides in toluene bath 2 for 5 minutes
4. immerse slides in isopropylalcohol 100% bath 1 for 2 minutes
5. immerse slides in isopropylalcohol 100% bath 2 for 2 minutes
6. immerse slides in isopropylalcohol 90% bath for 2 minutes

7. immerse slides in isopropylalcohol 70% bath for 2 minutes
8. rinse with running tap water for 5 minutes
9. rinse with distilled water for 5 minutes
10. treat with a citrate buffer in a water bath at 99 degrees Celsius for 20 minutes
11. cooling down 20 minutes at room temperature
12. rinse with phosphate buffered saline (PBS) for 5 minutes
13. dry carefully around section and incubate for 30 minutes with normal goat serum (PBS containing 10% normal goat serum and 0.8% bovine serum albumin)
14. remove excess fluid on the section and dry the edges
15. incubate overnight with the primary antibodies: polyclonal rabbit anti-S100 antibodies (dilution 1/200; Dako, Glostrup, Denmark) at 4 degrees Celsius in a humidity chamber
16. rinse with PBS for 5 minutes and repeat 2 times
17. dry carefully around the section and incubate for 60 minutes at room temperature with the secondary anti-rabbit antibodies conjugated with Alexa Fluor 555 dye (dilution 1/500; Molecular Probes, Invitrogen, Eugene, Oregon, USA) which is spectrally similar to but more photostable than Cy3 dye
18. rinse with PBS for 5 minutes in the dark and repeat 2 times
19. mount sections with DAPI mounting medium (8 drops of Dako mounting medium and 1 drop of Vectashield mounting medium with DAPI)

4.6.3. Composition analysis

For the analysis of the composition, we used a fluorescence microscope type Axioplan 2 by Carl Zeiss (Jena, Germany) with a 20x objective, an ORCA-R2 camera by Hamamatsu (Japan) and the SmartCapture software by Digital Scientific (Cambridge, United Kingdom).

Cells expressing S100 were determined as mature adipocytes or preadipocytes, since S100 is present in preadipocytes[71-72] and its concentration rises during differentiation into mature adipocytes.[73-74] We were able to identify the morphological characteristics of mature adipocytes, consisting of a narrow rim of cytoplasm and a eccentrically placed nucleus.

Cells which were negative for S100-staining were considered as fibroblasts, endothelial cells, adipose-derived stem cells, erythrocytes, leucocytes, smooth muscle cells and pericytes.[41,42,56,72]

4.7. Statistical analysis

Categorical data are summarised as number of patients and percentages and continuous data as means (with standard deviation, SD) and medians. Groups of interest are compared by non-parametric tests. Friedman's test (related-samples two-way analysis of variance Friedman's by ranks test) is used when comparing 3 groups or more. When comparing two groups we use a related-samples Wilcoxon signed rank test. Correlations are assessed by Spearman's rho non-parametric correlation coefficient. A p-value lower than 0.05 is considered significant. Computational procedures were performed using Excel 2003 (Microsoft® Office Excel 2003) and IBM® SPSS® Statistics, version 20 (IBM Corporation, 2011).

4.8. Safety precautions

A risk associated with the manipulation of the lipoaspirates is the contact with blood and consequently the transmission of infectious diseases such as hepatitis and HIV. Precautionary measures were taken such as wearing gloves and hand hygiene. Gloves were also worn during the process of preparing the histological sections. All manipulations of toluene were done in the fume hood.

5. Results

5.1. Viability of the lipoaspirate in the different size classes

By means of cell staining with propidium iodide and Hoechst 33342, we distinguished viable cells from dead cells in the lipoaspirate. We performed a count of 1 000 nucleated cells in each of the four size categories from every patient. On average, the smallest lipoaspirate material, consisting of particles smaller than 500 µm, showed a cellular viability of 64.6 ± 30.5%. The mean viability in the lipoaspirate material with particles between 500 and 1 000 µm was 56.2 ± 23.3%. A mean viability of 50.2 ± 30.7% was found in the samples with a particle size between 1 000 and 1 600 µm. In the lipoaspirate material consisting of particles larger than 1 600 µm, the mean viability was 52.1 ± 23.0% (table 1). The related-samples Friedman's two-way analysis of variance by ranks test was used to determine if a statistically significant difference could be found between these four groups. A p-value of 0.094 indicates the absence of any statistical significance.

		Lipoaspirate containing particles smaller than 500 µm	Lipoaspirate containing particles between 500 µm and 1 000 µm	Lipoaspirate containing particles between 1 000 µm and 1 600 µm	Lipoaspirate containing particles larger than 1 600 µm
Viable cells: positive Hoechst 33342 staining and negative propidium iodide staining	mean ± SD minimum maximum	646 ± 305 336 962	562 ± 233 233 794	502 ± 307 162 890	521 ± 230 181 803
Dead cells: positive Hoechst 33342 staining and positive propidium iodide staining	mean ± SD minimum maximum	354 ± 305 38 664	438 ± 233 206 767	498 ± 307 110 838	479 ± 230 197 818
Cellular viability	mean ± SD minimum maximum	64.6 ± 30.5% 33.6% 96.2%	56.2 ± 23.3% 23.3% 79.4%	50.2 ± 30.7% 16.2% 89.0%	52.1 ± 23.0% 18.1% 80.3%
p-value	0.094				

Table 1. Viability of the lipoaspirate after sieving into different size classes. Nuclei stained with both Hoechst 33342 and propidium iodide are considered as dead cells, whereas nuclei that are only stained with Hoechst 33342 are counted as viable cells. Mean values and standard deviation are shown as well as the minimum and maximum value for each size class.

A box plot (figure 12) displays the differences in median viability between the four different size classes, together with its dispersion and skewness.

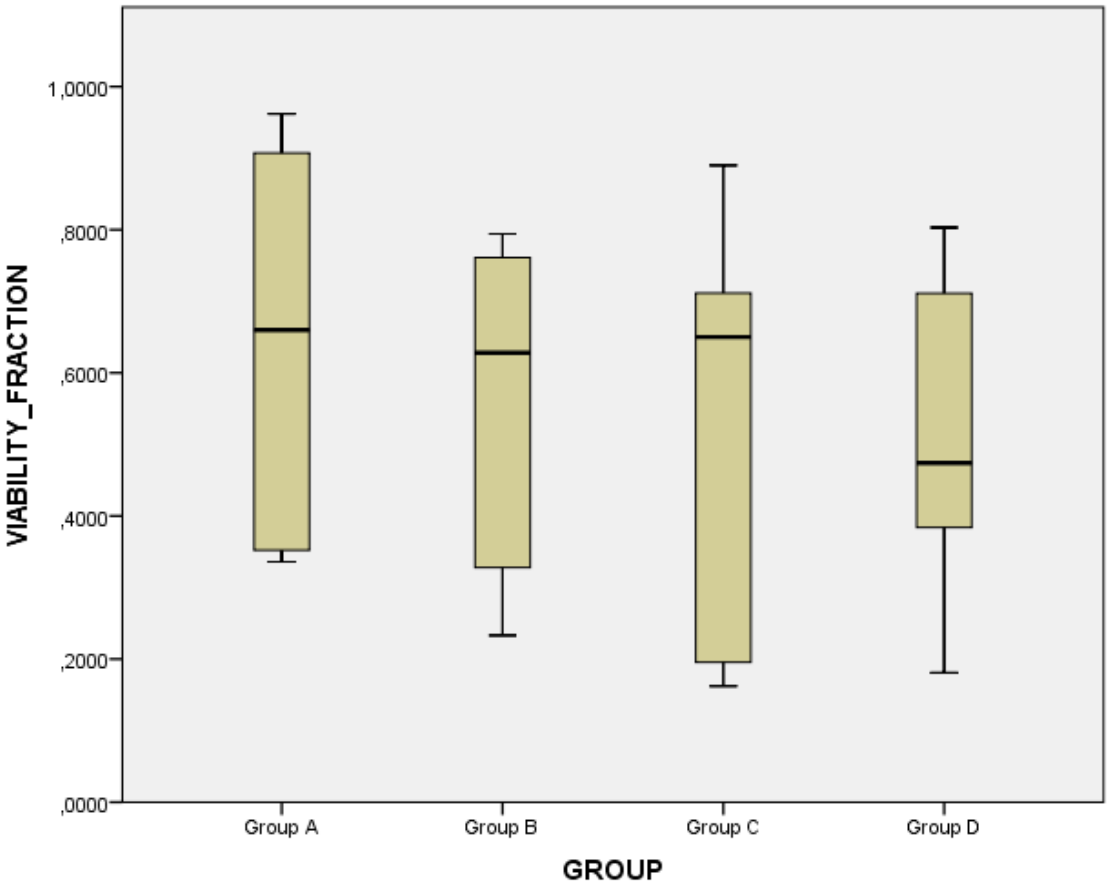

Figure 12. A box plot displays the median cellular viability and the spread of the results in the different size classes. The black horizontal line in the box indicates the median value. The central rectangle demarcates the first quartile (25th percentile) and third quartile (75th percentile) and contains the interquartile range. The vertical lines (whiskers) indicate the variability outside the first and third quartiles and extend to the minimum and maximum data values. Group A: fat particles smaller than 500 µm. Group B: fat particles between 500 and 1 000 µm. Group C: fat particles between 1 000 and 1 600 µm. Group D: fat particles larger than 1 600 µm. 0,0000: 0% viability. 1,0000: 100% viability.

5.2. Composition of the lipoaspirate in the different size classes

Fluorescent antibody stained histological sections were analysed to determine possible differences in composition between large and small fat samples (figure 13). In the samples containing fat particles smaller than 1 600 µm, we measured a fraction of 14.8 ± 5.9% S100-positive cells, which were considered as mature adipocytes and preadipocytes. This percentage of S100-positive cells raised to 20.0 ± 6.3% in the lipoaspirates containing fat particles larger than 1 600 µm (table 2). The related-samples Wilcoxon signed rank test was

used to test for statistical significance. The calculated p-value of 0.102 indicates no statistical significance.

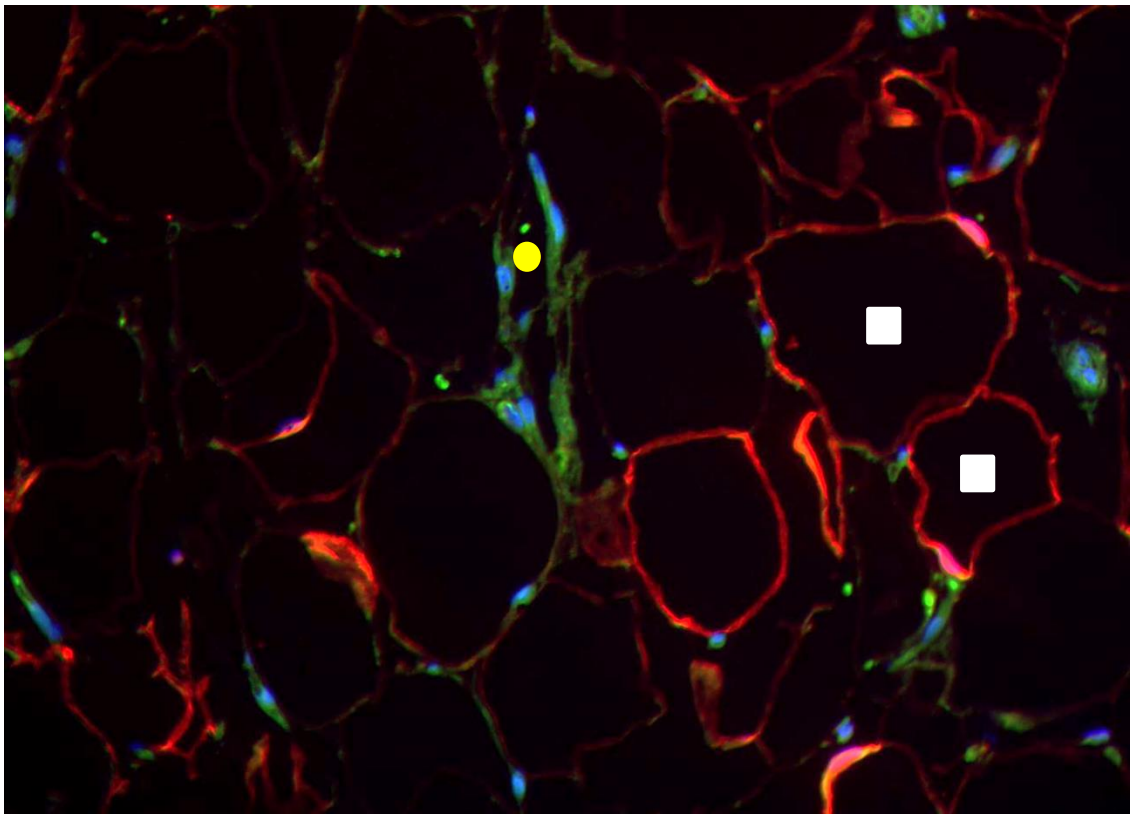

Figure 13. Histological section of adipose tissue gained by liposuction. The red Alexa Fluor 555 dye marks the anti-S100 antibodies and detects cytoplasm of adipocytes. The blue stain is DAPI which is a fluorescent non-specific nuclear marker. The white squares indicate examples of adipocytes with a clearly recognizable nucleus. The yellow circle is surrounded by vascular endothelial cells. The little green doughnut shaped cells are erythrocytes.

		Lipoaspirate containing particles smaller than 1 600 µm	Lipoaspirate containing particles larger than 1 600 µm
S100-positive cells	mean ± SD	56 ± 40	86 ± 18
	minimum	13	53
	maximum	92	113
S100-negative cells	mean ± SD	282 ± 122	379 ± 158
	minimum	142	184
	maximum	366	657
Fraction of S100-positive cells	mean ± SD	14.8 ± 5.9%	20.0 ± 6.3%
	minimum	8.4%	10.5%
	maximum	20.1%	31.9%
p-value	0.102		

Table 2. Composition of the lipoaspirates after sieving into different size classes. Mean values and standard deviation are shown as well as the minimum and maximum value for each size class.

5.3. The oily fraction as a marker of the viability of the lipoaspirate

We investigated whether there was a correlation between the fraction of the oily component in the centrifuged sample (table 3) and the viability of the lipoaspirate. To determine the correlation coefficient, we performed a Spearman's rho correlation analysis. A correlation coefficient of -0.08 indicates the absence of a linear relationship (figure 14).

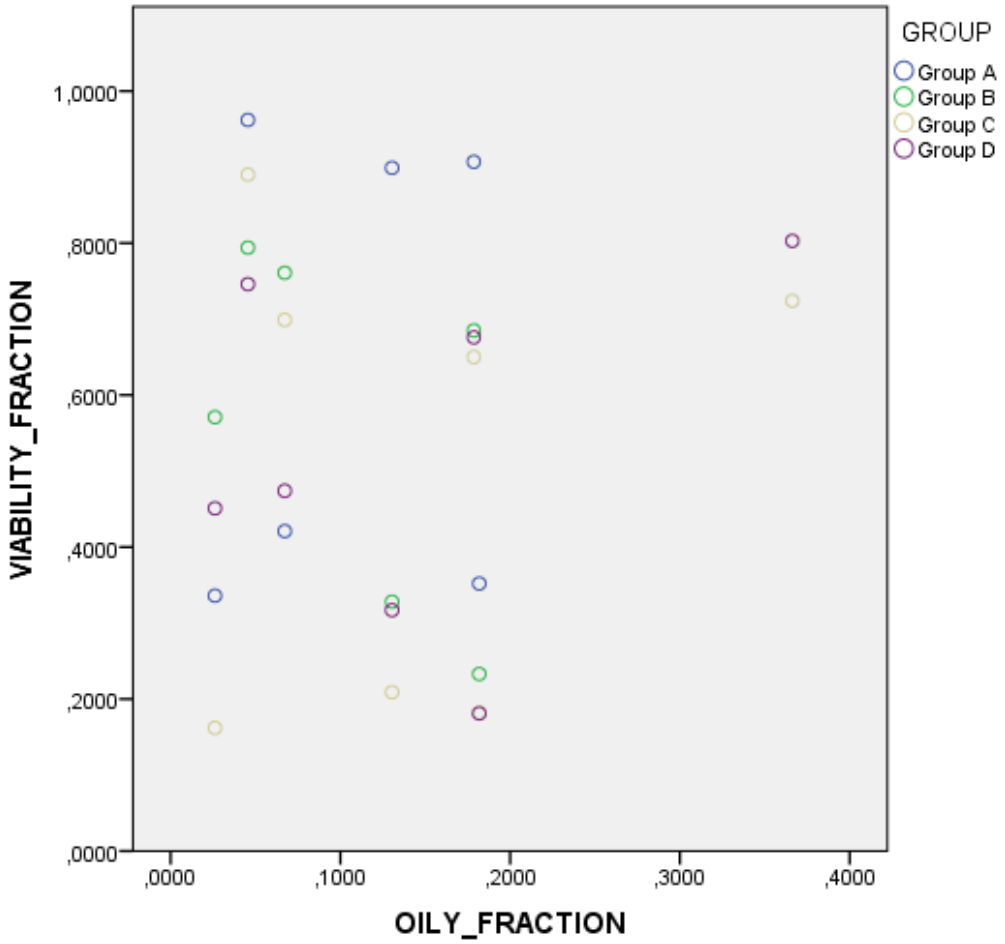

Figure 14. A scatter plot shows the relationship between the oily fraction in the fresh centrifuged lipoaspirate and the viability of the different size classes in the lipoaspirate. Group A: fat particles smaller than 500 µm. Group B: fat particles between 500 and 1 000 µm. Group C: fat particles between 1 000 and 1 600 µm. Group D: fat particles larger than 1 600 µm. 0,0000: 0% viability. 1,0000: 100% viability.

		Oily layer	Lipid layer	Serosangui-nous layer	Pellet layer
Fraction of the component to the total volume of the lipoaspirate in the tube	mean ± SD	14.2 ± 11.7%	54.1 ± 12.0%	28.4 ± 16.8%	3.3 ± 0.9%
	minimum	2.6%	40.2%	4.2%	2.1%
	maximum	36.6%	71.3%	52%	4.6%

Table 3. Mean fractions of the different layers in the centrifuged lipoaspirates.

5.4. The influence of the patient's age on the viability of the lipoaspirate

To determine whether the patient's age has an impact on the viability of the lipoaspirate, a Spearman's rho correlation analysis was done. The patients' ages ranged from 38 to 79 years and the mean age was 55 years. We discovered a correlation coefficient of 0.12 indicating a very weak relationship between age and viability of the lipoaspirate (figure 15).

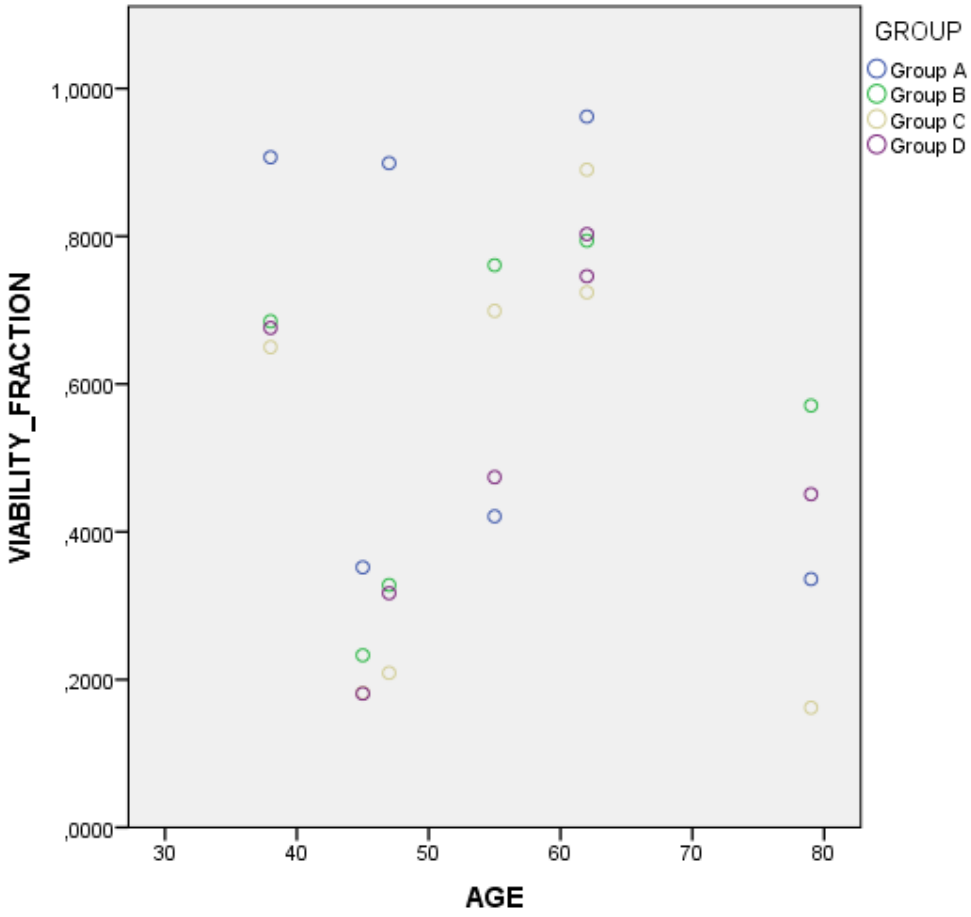

Figure 15. A scatter plot showing the relationship between the age of the patient and the viability of the different size classes in the lipoaspirate. Group A: fat particles smaller than 500 µm. Group B: fat particles between 500 and 1 000 µm. Group C: fat particles between 1 000 and 1 600 µm. Group D: fat particles larger than 1 600 µm. 0,0000: 0% viability. 1,0000: 100% viability.

5.5. The influence of the patient's BMI on the viability of the lipoaspirate

We performed a Spearman's rho correlation analysis to determine the correlation coefficient between the patient's BMI and the viability of the lipoaspirate. The mean body mass index was 27.2 kg/m² (range 20.3-44.1 kg/m²). A correlation coefficient of -0.08 was found which is too low to be meaningful and indicates the absence of a linear relationship in the investigated patients (figure 16).

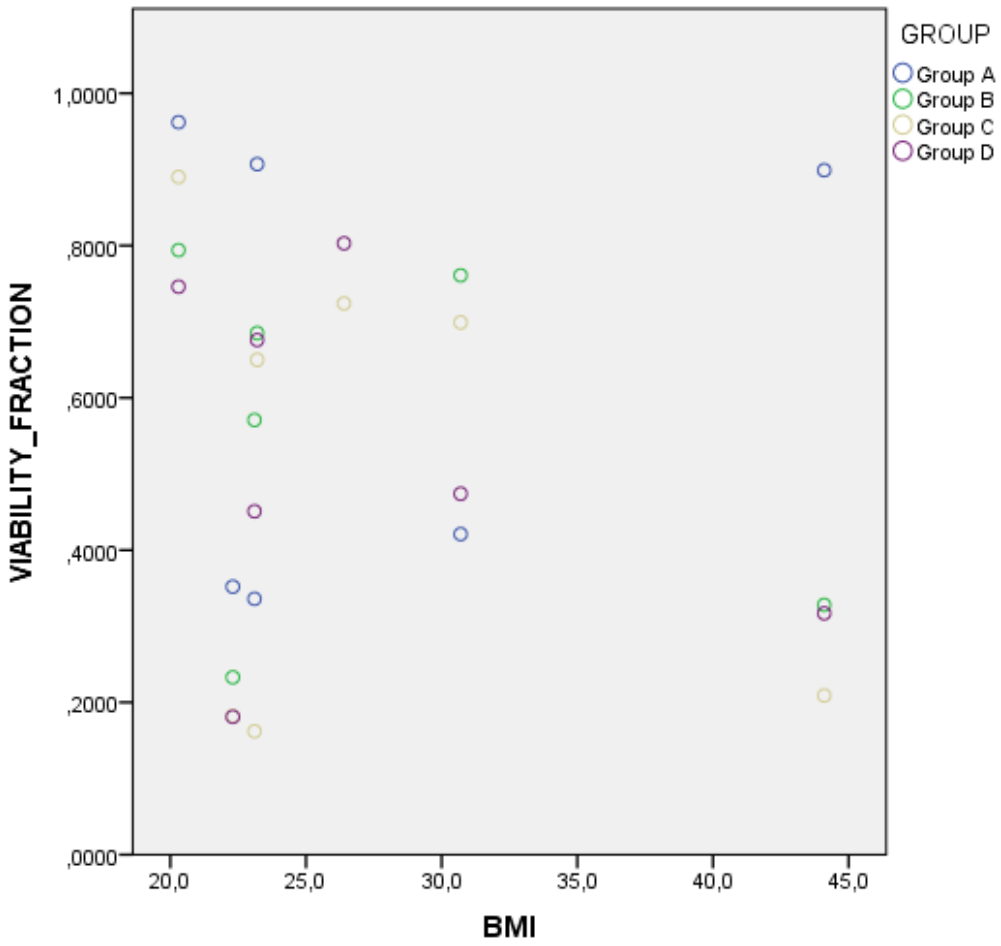

Figure 16. A scatter plot showing the relationship between the BMI of the patient and the viability of the different size classes in the lipoaspirate. Group A: fat particles smaller than 500 µm. Group B: fat particles between 500 and 1 000 µm. Group C: fat particles between 1 000 and 1 600 µm. Group D: fat particles larger than 1 600 µm. 0,0000: 0% viability. 1,0000: 100% viability.

5.6. The influence of chemotherapy on the viability of the lipoaspirate

We studied the influence of the patients' medical history on the viability of the lipoaspirates. 3 patients out of the studied 7 patients underwent chemotherapeutic treatment for breast cancer. To determine whether there is a relationship between chemotherapy and the viability of the lipoaspirates, a Spearman's rho correlation analysis was done. A weak correlation was found with a correlation coefficient of 0.17 (figure 17). The p-value is 0.41 and indicates no statistical significance.

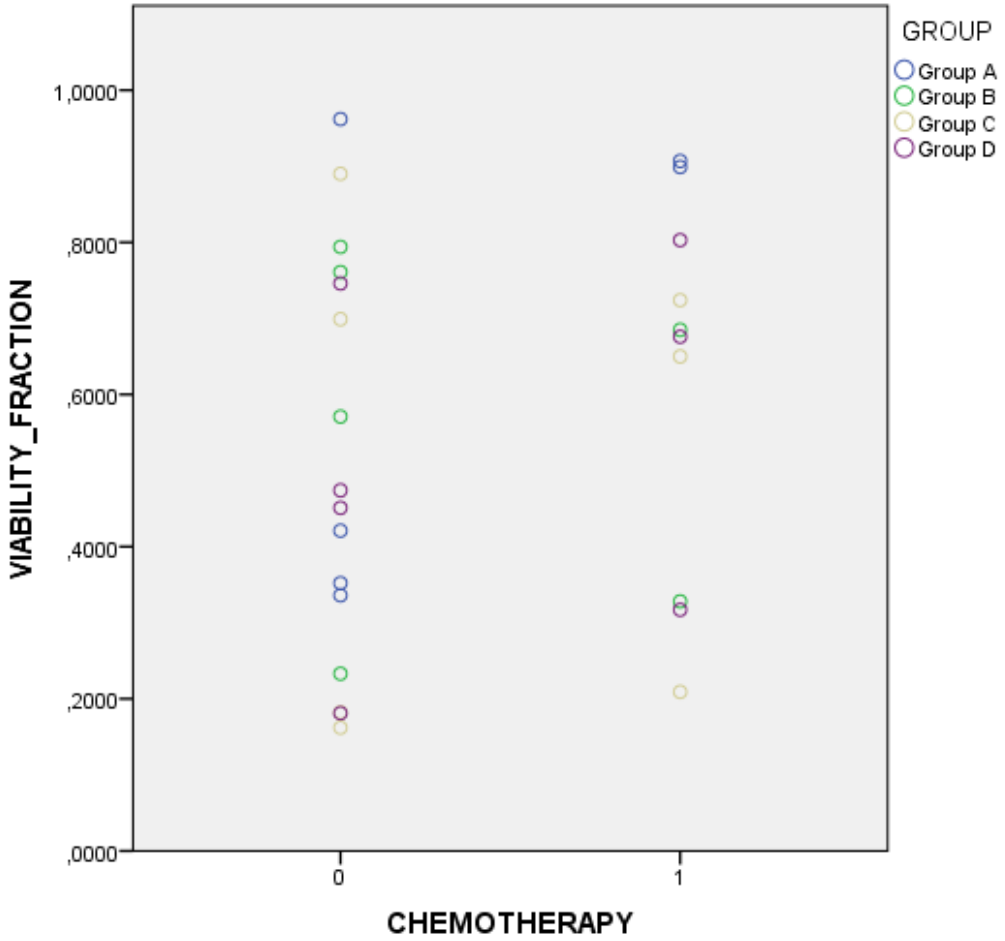

Figure 17. A scatter plot showing the relationship between the presence of chemotherapy in the patient's medical history and the viability of the different size classes in the lipoaspirate. 0: no chemotherapy in the medical history. 1: chemotherapy in the medical history. Group A: fat particles smaller than 500 µm. Group B: fat particles between 500 and 1 000 µm. Group C: fat particles between 1 000 and 1 600 µm. Group D: fat particles larger than 1 600 µm. 0,0000: 0% viability. 1,0000: 100% viability.

5.7. The influence of radiotherapy on the viability of the lipoaspirate

Another studied parameter is the absence or presence of radiation therapy in the patients' medical history. 4 patients in the study group had radiotherapy for breast cancer. We performed a Spearman's rho correlation analysis to investigate whether there is a relationship between radiotherapy and the viability of the lipoaspirates. A very low correlation was found with a correlation coefficient of -0.14 (figure 18).

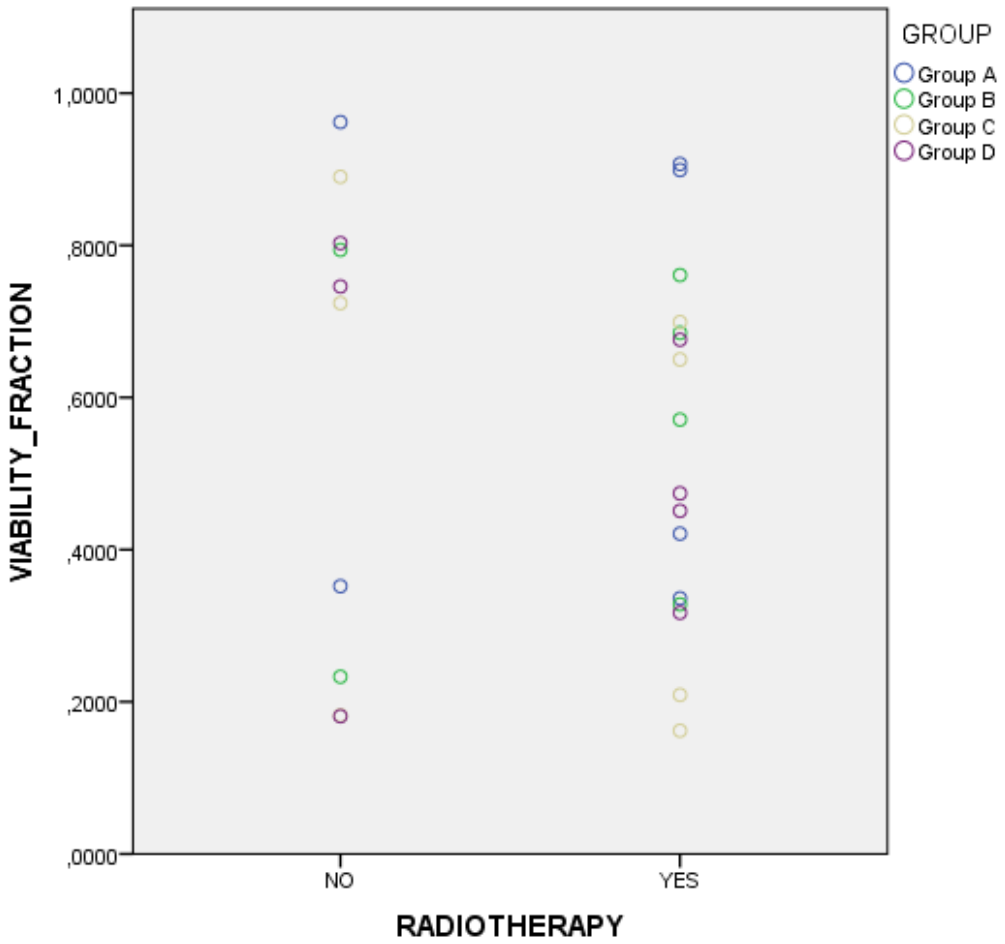

Figure 18. A scatter plot showing the relationship between the presence of radiotherapy in the patient's medical history and the viability of the different size classes in the lipoaspirate. No: no radiotherapy in the medical history. Yes: radiotherapy in the medical history. Group A: fat particles smaller than 500 µm. Group B: fat particles between 500 and 1 000 µm. Group C: fat particles between 1 000 and 1 600 µm. Group D: fat particles larger than 1 600 µm. 0,0000: 0% viability. 1,0000: 100% viability.

6. Discussion

Lipofilling is a surgical technique with the aim of augmenting or restoring volume and contour with autologous adipose tissue that has been harvested with liposuction. An important indication is the injection of the lipoaspirate in the breast after a patient underwent breast-conserving surgery or a mastectomy followed by breast reconstructive surgery in the treatment of breast cancer. Lipofilling is an elegant procedure which can be done on an outpatient basis and can avoid major surgery such as a myocutaneous flap or the use of an implant. The grafted fat is relatively resistant to infection and the healing generally proceeds without complications.[14-15] Nevertheless, touch-up procedures can be necessary in order to achieve an optimal result. It is difficult to predict the right volume to inject which is required to obtain a symmetric appearance of the breasts. This is caused by the variable rate of resorption of the grafted material which leads to a decreasing volume in the postoperative period.[1] Based on the cell survival theory[29-30], numerous research projects concentrate on the viability of the lipoaspirate and how it can be optimised prior to insertion. We investigated the impact of the sieving of the lipoaspirate on the viability and composition. By sieving the adipose tissue in four different groups based on the size of the fat particles, we searched for a difference in viability in a particular group.

Statistical analysis of the data shows that no significant differences have been detected in the viability of the size classes. Furthermore, the high standard deviation of the mean viability indicates that the data are spread out over a large range of values. However, when comparing the mean viability of the different groups, the highest value could be found in the smallest group consisting of particles with a diameter of 500 µm or lower (table 1). The box plot (figure 12) demonstrates that the median cellular viability was the lowest in the group with particles larger than 1 600 µm. This information indicates a possible trend of declining viability as the size of the fat particles increases. This would be consistent with our hypothesis.

Our analysis of the fluorescent antibody stained histological sections of the lipoaspirates with either particle diameters above or particle diameters below 1 600 µm does not allow us to draw hard conclusions concerning the different composition of the two groups. The proportion of S100-positive cells which were considered as mature adipocytes and preadipocytes was higher in the largest group, as expected in our hypothesis based on the

normal diameter of adipocytes, but, once again, no statistically significant difference was noted (table 2).

We compared our results with those of Suga et al.[45], who also used the Hoechst 33342 and propidium iodide nuclear staining. They stained samples from the adipocyte layer, the serosanguinous layer and the pellet, and made a distinction between viable adipocytes, dead adipocytes, viable other cells and dead other cells. We have copied their results, which were reported as absolute values, in table 4. In 1 g of aspirated adipose tissue, they measured a general cellular viability of 56.4% (we divided the number of viable adipocytes and other cells by the total number of adipocytes and other cells) which is very similar to our overall viability. The viability was lower in the adipocyte layer (37.1%) than in the bottom layer (81.3%). This corresponds to our finding: the sample with the smallest particles, which has the highest viability in our study, contains more of the components of the pellet because these cells have a smaller diameter and will pass through narrow sieve openings.[44] We can calculate from the same table that adipocytes represent 24.5% of the total number of cells in this 1 g of aspirated fat tissue. This percentage exceeds our result. A possible explanation for this difference may be that they processed the lipoaspirate by digestion with collagenase for 30 minutes, whereas we left this step out of the protocol because we attempted to analyse tissue which matches the material that can be injected in patients.

	Viable adipocytes	Dead adipocytes	Total number of adipocytes	Viable other cells	Dead other cells	Total number of other cells
Adipocyte layer	4.4 ± 0.66	2.5 ± 0.28	6.9	1.5 ± 0.23	7.5 ± 0.72	9.0
Serosanguinous layer	0.0	0.0	0.0	0.0	0.0	0.0
Pellet	0.0	0.0	0.0	10.0 ± 2.8	2.3 ± 0.34	12.3
	4.4 ± 0.66	2.5 ± 0.28	6.9	11.5	9.8	21.3

Table 4. Composition and viability of the different layers in 1 g of aspirated adipose tissue as published in *Numerical measurement of viable and nonviable adipocytes and other cellular components in aspirated fat tissue* by Suga et al.[45] Mean values and standard deviation are shown (x10^5).

The fraction of the oily component in a freshly aspirated and centrifuged sample (table 3) did not appear to be a predictor of the viability of the lipoaspirate. Because this upper layer consists of free fatty acids due to rupture of the adipocytes, we suspected a lower viability in samples having a considerable amount of this oily component. As seen in the scatter plot (figure 14), no pattern can be retained.

The impact of the patient's characteristics and medical history on the viability of the adipose tissue was determined. Statistical analysis did not reveal any correlation between the sample viability and the patient's age or BMI. Neither the presence of chemotherapy in the patient's medical history nor radiotherapy seemed to exert an influence on the viability (figures 15-18).

Based on the outcome of our experimental study and the absence of statistically significant results, no hard conclusions can be drawn and we cannot recommend the introduction of an additional processing step prior to the actual lipofilling. However, given the observed trend of decreasing viability in samples with increasing particle diameter, a subsequent study with a larger patient group might reveal a statistically stronger relationship. This brings us to the weakness of this experimental study, i.e. small patient sample size. This is due to the time-consuming process of making histological sections and the limited time available during this project. With a paired t-test we determined the required sample size to detect statistically significant differences in the viability of the different lipoaspirate samples. It indicates that a number of 26 patients are needed for the conventional 80% power assuming a 5% significance level. For 90% power, a sample size of 34 patients is required.[75-76]

To our knowledge, this is the first time that the quality of the fat graft has been tested following sieving into different samples. Therefore it remains interesting to carry out further research with an extended study group. An additional test would be a viability analysis of the lipoaspirate after it has been sieved and passed through a conventional lipofilling needle (usually 18 to 20 gauge). This value would reflect the real viability of the graft at the moment of transfer to the acceptor site and is clinically more relevant. If a higher viability can be determined in one of the lipoaspirate samples after sieving and passing through a needle, then this particular size class can later be selected and injected during the lipofilling procedure. This would theoretically lead to a more stable postoperative outcome with less resorption of the fat graft as stated by the cell survival theory.[29-30] Additional lipofilling sessions would have to be performed less frequently which leads to a higher patient satisfaction, fewer expenses, less operative risks and no repeated trauma of the donor site.

To better understand the determining factors in the mechanism of resorption and survival of the grafted fat, it is important to conduct research on the exact composition of the lipoaspirates. Since preadipocytes and adipose-derived stem cells are smaller and have a less

delicate structure than mature adipocytes, they are considered as equally important in the fat grafting process.[9,30,57] Moreover, preadipocytes and adipose-derived stem cells have a proliferating and differentiating capacity and are less dependent on the vascularisation of the receptor site, whereas mature adipocytes rely on the quality of the nutrient and oxygen supply in the treated region.[57] An accurate determination of the amount of different cell types in the various size classes of the lipoaspirate after sieving remains an interesting point of research. In our histological analysis, no further differentiation was made between mature adipocytes, preadipocytes and other cells.

In order to investigate our theoretical beliefs, an experimental study could be performed on rats or mice. The human lipoaspirate can be sieved, according to the method described in this experiment, and analysed for viability and exact composition, and subsequently, a given volume can be injected in the immunocompromised animal to assess the clinical outcome. Postoperative evaluation of the graft by means of a three-dimensional imaging technique or by histological analysis after explanting of the graft, can help us determine the practical usefulness of the sieving as an additional step in the lipofilling procedure.

7. Conclusion

Lipofilling is an excellent surgical option in the treatment of morphological defects after breast-conserving or breast reconstructive surgery. The primary obstacle remains the resorption of the fat graft which sometimes necessitates additional lipofilling procedures. We investigated whether a difference could be found in the cellular viability and composition of the fresh lipoaspirate after we sieved it into different size groups. However a trend could be observed, statistical analysis did not reveal any significant differences, which can be partly attributed to the small study group. In conclusion, a greater number of patients are needed to fully investigate our hypothesis, and, for the time being, we cannot provide guidelines regarding the possible beneficial use of the injection of autologous fat selected by sieving.

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