Name of department:	Department of Plastic Surgery and Burns Treatments, Rigshospitalet
PhD program:	Surgical Sciences
Author:	Mikkel Herly, MD
Title and subtitle:	Fat grafting in humans: An assessment of the resorption dynamics of fat grafts and evaluation of methods to measure fat graft retention in the breast
Topic description:	Studies concerning the resorption dynamics of fat grafts and an investigation into the correct method for measuring fat graft retention in the breast. The studies support a randomized clinical trial protocol which is also included in the thesis
Supervisor:	Professor Christian von Buchwald, MD, DMSc
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Preface

This PhD thesis is comprised of research that I have conducted at the Department of Plastic Surgery and Burns Treatments, Rigshospitalet from 2014 to 2020. In this period, I have developed a protocol for a phase III Randomized Clinical Trial of using adipose derived-stem cells for breast reconstruction with fat grafting. The trial has been approved by the Danish Medicines Agency and the National Committee on Health Research Ethics and it is included as study 5 in this thesis. The studies presented in this thesis were made in part as a preparation for this RCT.

Furthermore I have used a large portion of my time to help my supervisor and colleague, Peter Vester-Glowinski, MD, PhD to conduct a randomised controlled trial of fat grafting enriched with ex-vivo expanded adipose-derived stem cells for breast augmentation that we wanted to finish before I initiated the trial concerning breast reconstructions. Peter Vester-Glowinski was the trial PI on this study and therefore, this work is not directly included in this thesis. However, it has had important implications for the future perspectives of my research and therefore, I discuss the results of this trial in the perspectives section.

My long-term goal is to find a way to use adipose-derived stem cells to improve fat grafting for breast reconstruction after mastectomy. The studies included in this thesis are all necessary investigations for the design of a new randomised controlled trial of breast reconstruction with fat grafting and adipose-derived stem cells.

The studies described in this thesis were funded by the Danish Cancer Society, the Department of Plastic Surgery and Burns Treatments, Rigshospitalet and the Research Fund of Rigshospitalet.

Mikkel Herly, July, 2020

Acknowledgments
I would like to thank Christian von Buchwald for offering supervision of my efforts to achieve a PhD. Furthermore, I greatly appreciate Professor Buchwald personally and look forward to hopefully collaborate with him on many future research projects.

Peter Vester-Glowinski is my closest colleague and a great friend. I worked with him on his PhD projects as a medical student and this experience has shaped my research career in a very positive way. Our talents and shortcomings complement each other in a great way. I hope we will be able to continue our work together and that the research group we are building will make many exciting discoveries in the future.

Dr. Mathias Ørholt has worked with me on all my past and current research projects as a medical student alongside his studies. Now he has finished medical school and he is working to start his PhD. He is very intelligent, and he has a lot more aptitude for many things including statistical analysis than I have. I have the greatest respect for him, and I hope I will have the opportunity to work with him going forward.

Dr. Mathilde Hemmingsen has helped me on the studies of methods to measure fat graft volume retention in the breast. She started as a medical student and has now graduated as a medical doctor and started her PhD. Mathilde is a very talented researcher and I look forward to work with her on her many new research projects in the future.

Andreas Larsen has been helping me with a lot of my research including the meta-analysis that is included in this thesis. Andreas is a very intelligent and talented medical student with incredible work ethics. He also has a talent for organizing a research group and early on he took charge of the research projects. I am sure he has a very bright future ahead of him and I am grateful for the opportunity to work with him.

Krzysztof Drzewiecki has been my supervisor since I started my research in plastic surgery in 2014. Prof. Drzewiecki is a great mentor and sparring partner for research questions, and he has also helped me with career advice.

Jens Jørgen Elberg has helped to recruit volunteers from his private clinic, Amalieklinikken, for the MRI studies. Dr. Elberg is a great mentor for me in research and even more so in plastic surgery. His approach of thinking about the root cause of surgical problems when choosing the surgical treatment, is something that I will use for the rest of my career. I am very grateful for all I have learned from him.

Finally, I wish to thank my wonderful wife Cecilie for her support of my research. She is my closest ally and I always consult her with problems, and she has been listening patiently. Cecilie

is great at putting things into perspective and to call me out when I obsess over small obstacles. I am very lucky.

Mikkel Herly
July, 2020

Table of contents

List of studies

Study 1 – Published paper in Appendix 1
Quantifying Long-Term Retention of Excised Fat Grafts: A Longitudinal, Retrospective Cohort Study of 108 Patients Followed for up to 8.4 Years[1]

Authors: Mikkel Herly, Mathias Ørholt, Peter V Glovinski, Christian B Pipper, Helle Broholm, Lars Poulsgaard, Kåre Fugleholm, Carsten Thomsen, Krzysztof T Drzewiecki

Study 2 – Published paper in Appendix 2
Efficacy of breast reconstruction with fat grafting: A systematic review and meta-analysis[2]

Authors: Mikkel Herly, Mathias Ørholt, Andreas Larsen, Christian B Pipper, Rikke Bredgaard, Christina S Gramkow, Adam J Katz, Krzysztof T Drzewiecki, Peter V Vester-Glowinski

Study 3 – Published paper in Appendix 3
The current gold standard breast volumetry technique seems to overestimate fat graft volume retention in the breast: A validation study[3]

Authors: Mikkel Herly, Felix Christoph Müller, Mathias Ørholt, Joachim Hansen, Sophie Sværke, Mathilde N Hemmingsen, Bo S Rasmussen, Jens J Elberg, Krzysztof T Drzewiecki, Peter V Vester-Glowinski

Study 4 – Manuscript in Appendix 4
Lipovol: Validated Software for Measuring Fat Graft Volume Retention in the Breast with MRI

Authors: Mikkel Herly, Mathias Ørholt, Felix Christoph Müller, Mathilde Hemmingsen, Joachim Hansen, Andreas Larsen, Bo Sonnich Rasmussen, Jens Jørgen Elberg, Christian von Buchwald, Krzysztof T. Drzewiecki, Peter V. Vester-Glowinski

Study 5
PROJECT PROTOCOL: Fat Grafting with Ex-Vivo Expanded Adipose-Derived Stem Cells for Breast Reconstruction Following Mastectomy, EudraCT: 2016-005186-31

Authors: Mikkel Herly, Krzysztof T Drzewiecki, Anne Fischer-Nielsen, Niels Kromann, Lea Munthe Fog, Christina Stunk Gramkow, Rikke Bredgaard, Tove Tvedskov, Peter V Vester-Glowinski

Abbreviations

ASC: Adipose-Derived Stromal Cell (and Adipose-Derived Stem Cell)
BCS: Breast Conserving Surgery
CD…: Cluster of Designation (Surface marker system)
CT: Computed tomography
EudraCT: European Union Drug Regulating Authorities Clinical Trials Database
GCP: Good Clinical Practice unit (international unit for monitoring drug trials)
GMP: Good Manufacturing Practice
HBSS: Hank's Buffered Saline Solution
HCG: Human Chorionic Gonadotropin
HMT-3522 T4-2: Breast cancer cell line for in vitro studies
MCF-7: Breast cancer cell line for in vitro studies
MSC: Mesenchymal Stem Cell
MRI: Magnetic resonance imaging
P0, P1, etc: Cell passage denomination, P0 being unpassaged cells
POCR: Point of complete reconstruction
PC: Platelet Concentrates
pHPL: pooled Human Platelet Lysate
RCT: randomised controlled trial
SVF: Stromal Vascular Fraction

Danish summary of the PhD thesis/Dansk resume af PhD-afhandlingen

Fedttransplantation i sin nuværende form er en relativt ny teknik til at tilføre blødt væv til dele af kroppen, hvor man ønsker mere volumen, f.eks. i forbindelse med en rekonstruktion af brystet. Den består i at man suger fedt fra andre steder på kroppen end der hvor man mangler væv. Derefter sprøjtes fedtet ind i små kanaler og i så mange planer som muligt på recipientstedet. Dette gøres, for at fedtcellerne fra transplantatet er så tæt på en ny blodforsyning som muligt på det nye sted på kroppen, hvor man gerne vil have fedtet til at overleve. Denne teknik er især lovende til brystrekonstruktion, hvor de nuværende teknikker, der består af frie lapper og brystimplantater, har nogle negative sideeffekter for patienten i form af donorstedsmorbiditet efter høst af den frie lap og kapseldannelse omkring brystimplantater.
Fedttransplantation har dog den ulempe, at en stor del af transplantatet forsvinder efter operationen. Dette er formentlig fordi ikke alle de tilførte celler får en ny blodforsyning i tide, hvorefter de dør og bliver resorberet af kroppen.[4–6] Man forventer at omkring halvdelen af transplantatet forsvinder efter en fedttransplantationsoperation.[7] Af denne grund er mange forskere ivrige efter at udvikle nye teknikker til at forbedre fedttransplantaters overlevelse. En af de mest lovende teknikker er at tilsætte patientens egne fedtderiverede stamceller (ASC'er) til transplantatet, som menes at kunne øge bevarelsen af fedttransplantatets volumen. Et studie fra 2013 af Kølle et al. fra min forskningsgruppe har vist at fedttransplantater tilført patientens egne stamceller kunne øge overlevelsen af transplantatet signifikant.[8] Disse resultater satte vi os for at undersøge om vi kunne videreføre til brug i brystet. Studiet gav anledning til at to yderligere studier blev planlagt: Første studie skulle undersøge ASC-beriget fedttransplantation til en brystforstørrelse (EudraCT 2014-000510-59) og et andet som skulle undersøge ASC-beriget fedttransplantation til at rekonstruere et bryst efter en mastektomi (EudraCT 2016-005186-31). Førstnævnte studie var planlagt at Peter Glowinski skulle stå i spidsen for og jeg fik muligheden for at være med i dette studie, som medicinstuderende fra begyndelsen af studiet. Jeg fik senere muligheden for at stå i spidsen for det sidste studie, som skal undersøge ASC-beriget fedttransplantation til brystrekonstruktion hos kvinder der har undergået mastektomi.

Der var dog flere ting, som fortsat var ukendt om dynamikken i fedttransplantaters resorption, der gjorde det svært at undersøge, hvordan man kan forbedre den. Der manglede viden om hvor lang tid resorptionsprocessen varer, og hvor meget af fedtet der resterer, efter at resorptionen er tilendebragt. Derudover var der brug for en valideret metode til at måle retentionen af fedttransplantater, så man kunne sammenligne resultater på tværs af forskningsgrupper.

I denne PhD afhandling vil jeg præsentere fire studier, der undersøger fedttransplantaters resorption og teknikker til at måle det. De fire studier er bundet sammen af, at de alle

beskæftiger sig med fedtretention på forskellige niveauer, gående fra undersøgelse af målemetoder til at beregne fedtretention, over en basal undersøgelse af fedttransplantaters udvikling over år og til en mere klinisk relevant undersøgelse af, hvor mange fedttransplantationsseancer, der skal til for at rekonstruere et bryst efter en mastektomi. Hvert af de første fire studier har selvstændige forskningsspørgsmål, som adresseres i artiklerne. Derudover er de fire studier vigtige forarbejder til det randomiserede kliniske studie, som skal undersøge om ASC-berigelse kan forbedre volumenbevarelsen af fedttransplantater til at rekonstruere et bryst efter en mastektomi (studie 5).
Spørgsmålene og hvordan de individuelle studier understøtter det randomiserede kliniske studie (studie 5) kan beskrives ved de forskningsspørgsmål som skulle besvares:

Spørgsmål 1: Hvor lang tid går der fra en fedttransplantation til fedtresorptionsprocessen er tilendebragt? Dette spørgsmål skulle besvares for at vælge den korrekte opfølgning af patienterne i det randomiserede kliniske studie (studie 5).

I studie 1 undersøges fedttransplantater i kraniet hos patienter, som har fået fjernet en tumor udgået fra de schwannske celler omkring n. vestibularis. Disse patienter var fulgt med CT- og MR-skanninger for at undersøge for recidiv med en gennemsnitlig opfølgning på 2,7 år og op til 8,4 år. Skanningerne blev brugt til at undersøge fedttransplantatets retention over tid og til at finde ud af, hvornår fedttransplantaternes volumen var stabilt. Dette kunne bruges til at beslutte opfølgningsperioden i fremtidige prospektiver studier af fedttransplantater, herunder det randomiserede kliniske studie (studie 5).

Spørgsmål 2: Hvor mange fedttransplantationsbehandlinger skal der normalt til for at rekonstruere et bryst efter en mastektomi? Dette spørgsmål var vigtigt for designet af det randomiserede kliniske studie (studie 5), da vi gerne ville undersøge om stamcelleberigelsen kunne mindske antallet af behandlinger til at rekonstruere et bryst.

I studie 2 er litteraturen gennemgået systematisk for tidligere studier af brystrekonstruktion med fedttransplantation. Dernæst har vi brugt meta-analytiske metoder til at estimere, hvor mange fedttransplantationsbehandlinger der normalt skal bruges til at rekonstruere et bryst efter hel eller delvis kirurgisk fjernelse af brystet pga. kræft eller en familiær disposition til kræft. Denne viden kunne bruges til at lave en power-beregning til det kliniske studie om brystrekonstruktion med stamcelleberiget fedt.

Spørgsmål 3: Hvordan måles volumenoverlevelsen af et fedttransplantat i brystet? Volumenoverlevelsen af fedttransplanter i brystet er det primære endemål i det randomiserede kliniske studie (studie 5), som denne PhD afhandlings arbejder lægger sig op ad. Derfor var der brug for at sikre, at vi havde en valideret målemetode til at måle det korrekt.

I studie 3 undersøgte vi målenøjagtigheden og præcisionen af den mest brugte MRI-baserede metode til at måle brystvolumen og volumenretention af fedttransplantater i brystet. Metoden

blev valideret for dens evne til at måle ændringer i brystvolumen hos kvinder, der undergik en brystforstørrende operation med implantater eller fedttransplantation. I studie 4 præsenterer jeg vores eget bud på en metode til at måle fedtretention i brystet med MR-skanninger. Metoden valideres ud fra samme principper som i studie 3.

I studie 5 præsenteres et resume af protokollen for det randomiserede kliniske studie af stamcelleberiget fedttransplantation til brystrekonstruktion efter mastektomi, som blev understøttet af de fire første studier.

Resultater

Studie 1 viste overordnet set, at opfølgningstiden efter fedttransplantation bør være længere end først antaget. Vores model over fedttransplantaternes volumenretention over tid viste at fedttransplantaternes volumen var først stabilt efter 2,2 år i kraniet. Dette kan selvfølgeligt være anderledes for fedttransplantater i brystet, som injiceres med Coleman-teknik[6], men resultatet gjorde alligevel, at vi tilføjede en opfølgning efter to år til protokollen for det randomiserede kliniske studie, som er beskrevet i studie 5.

I studie 2 kunne vi bruge publicerede studier til at estimere hvor mange behandlinger der typisk bruges for at rekonstruere et bryst efter en mastektomi. Vores undersøgelse viste, at der bruges ca. tre behandlinger for at rekonstruere et bryst efter en hudbevarende mastektomi. Det mest overraskende ved dette studie var, at der ikke ser ud til at skulle bruges flere behandlinger til at rekonstruere et bryst efter en radikal mastektomi end efter en hudbevarende mastektomi. Til gengæld viste studiet at der skal bruges signifikant flere behandlinger på at rekonstruere et bestrålet bryst end et ikke-bestrålet bryst.

Studie 3 og studie 4 viste vores arbejde med at finde en metode til at måle fedtretention i det kliniske studie med udgangspunkt i MR-skanninger. Resultatet af de to studier er vores nye metode, som giver ret konsistente resultater på tværs af observatører og forskellige patienter. Den nye metode (Lipovol) er blevet brugt til at måle fedtretention i et netop færdiggjort klinisk studie og vi har planlagt at den også skal bruges i det randomiserede kliniske studie, som beskrives i studie 5.

<u>Foreløbige resultater fra det randomiserede studie af ASC-beriget fedttransplantation til brystforstørrelse</u>

Det andet studie der skulle undersøge ASC-beriget fedttransplantation til en brystforstørrelse er tilendebragt. Resultaterne er præsenteret ved kongres[9] og manuskriptet afventer indsendelse til et fagfællebedømt tidsskrift. Desværre viste studiet ingen effekt af ASC-berigelsen. Brysterne som blev behandlet med ASC-beriget fedt havde ikke en højere bevarelse af transplantaternes volumen end de normale fedttransplantater (54,0% vs. 55,9%). Resultaterne fra dette studie har haft stor betydning for mine planer om at igangsætte det næste kliniske studie om ASC-beriget

fedttransplantation til brystrekonstruktion. For at undersøge diskrepansen mellem det første studie som blev udført af Kølle et al. (Lancet 2013)[8] og det efterfølgende studie af Glowinski et al.[9] har jeg lavet en forskningsplan for studier som skal undersøge forskellene mellem det første studie, der blev publiceret i 2013 og det ovenfor beskrevne studie. Disse studier skal undersøge isoleringen af SVF, ekspansion af ASC'er og cellernes egenskaber, for at finde frem til om der er vigtige forskelle mellem cellerne i de to studier, som er afgørende for at de kan understøtte fedttransplantaters overlevelse. Identifikation af sådanne forskelle ville muliggøre opstart af det sidste kliniske studie om ASC-beriget fedttransplantation til brystrekonstruktion, som er beskrevet i studie 5.

Konklusion

Denne PhD afhandling består af fem arbejder som undersøger dynamikken i fedttransplantaters resorption og undersøger metoder til at måle fedttransplantaters retentionsrate i brystet. Det sidste arbejde i PhD'en er en videnskabelig protokol til et randomiseret klinisk studie der skal undersøge om ASC'er kan forbedre volumenbevarelsen af fedttransplantater til rekonstruktion af brystet. De første fire studier har alle haft en rolle i designet af protokollen.

De foreløbige resultater fra det andet randomiserede kliniske studie af ASC-beriget fedttransplantation til brystforstørrelse fra vores gruppe[9] viste ingen effekt af ASC-berigelsen. Dette resultat giver anledning til en række nye studier, som skal undersøge årsagen til den manglende effekt af ASC-beriget fedt til brystet i forhold til den signifikante effekt, som blev observeret i studiet på overarmene af raske forsøgspersoner i 2013[8]. Jeg har lavet en foreløbig forskningsplan for studier, som skal muliggøre at vi kan starte det sidste randomiserede studie af ASC-beriget fedttransplantation til brystrekonstruktion, så vi forhåbentligt kan tilbyde vores patienter en bedre behandling i fremtiden.

Summary of the PhD thesis

Fat grafting is an increasingly popular technique for the correction of soft tissue defects and now has an important role in plastic surgery. The procedure consists of harvesting fat from one part of the body where fat is abundant and transplanting that fat to another part of the body where more soft tissue volume is needed. The fat is harvested as a block of tissue and placed via an incision or it is harvested as lipoaspirate and injected in a fan like pattern in multiple channels and planes with a blunt cannula. Fat grafting is used for both reconstructive and cosmetic purposes. The advantages of fat grafting are a low risk of donor site morbidity, the absence of foreign material, and a natural feel. The major drawback of the fat grafting technique is that some of the fat cells are lost to resorption, which result in a loss of volume. For this reason, multiple sessions of fat grafting are often necessary to achieve the desired result, which poses a challenge when planning the surgery.
The resorption of the fat graft is thought to be due to a lack of oxygenation and nutritional support of the transplanted fat cells.[4–6] It is expected that approximately half of the transplanted fat is resorbed after surgery.[7] Therefore, many researchers are eager to design interventions that may improve the volume retention of fat grafts. One of the most promising techniques is to add the patient's own adipose-derived stem/stromal cells (ASCs) to the fat graft. A study performed by Kølle et al.[8] from our group, which was published in 2013, showed that fat grafts enriched with expanded adipose-derived stem cells can significantly increase the volume retention of bolus injected fat grafts. This discovery led to the planning of two additional RCT's that would be used to translate the first proof-of-concept study to clinical practice. The first study would investigate ASC-enriched fat grafting for breast augmentation (EudraCT 2014-000510-59) and the other ASC-enriched fat grafting for breast reconstruction (EudraCT 2016-005186-31). The first of these follow-up-studies has been conducted with Peter Glowinski as the principal investigator and I had the opportunity to be a part of the trial group from the beginning as a medical student. subsequently, I got the opportunity to be the principal investigator of the final trial that would investigate ASC-enriched fat grafting for breast reconstruction in women who had undergone a mastectomy.

However, there were still many aspects of non-enriched fat grafting that had yet to be studied, to ensure the highest possible quality of the RCTs. We learned that there was a lack of studies investigating the length and extent of the normal resorption process. Furthermore, there was a need for validated and reproducible techniques for measuring the fat graft retention rate in the breast, which could be used to compare results between research groups.

In this PhD thesis, I present four studies that investigate the resorption dynamics of fat grafts and methods for measuring the volume retention of fat grafts. The four studies share a common foundation; they all investigate fat graft retention. The four studies investigate fat graft retention at different levels: two studies are concerned with methods for measuring fat graft volume

retention in the breast, one study is concerned with the basic resorption dynamics of excised fat grafts over time and finally, one study is concerned with the very clinically relevant question of how many fat grafting treatment sessions are needed to complete a breast reconstruction. Furthermore, all four studies are important supporting studies for a randomized clinical trial (RCT) that I plan to conduct as the principal investigator. The RCT is summarized in study 5.

The first four studies presented in this thesis build upon individual research questions that are addressed in manuscripts and summaries of this thesis. However, the studies also have roles to play in relation to the planning of the randomized clinical trial (study 5) that will be addressed exclusively in this thesis. The research questions and their relation to the trial protocol are described below:

Question 1: What is the time to volumetric steady state of an excised fat graft, and what is the retention rate at steady state? The answer to these questions would help us choose an appropriate follow-up time to measure fat graft volume retention in the randomized clinical trial (study 5).

In study 1, I present a retrospective analysis of patients who have undergone surgery to remove a vestibular schwannoma. The patients were reconstructed with an excised fat graft and followed by CT and MRI for many years after the surgery to identify relapse of the schwannoma. These images were used to measure fat graft volume retention over time and to identify the time to steady-state of the fat graft's volume retention in a statistical model. We used the analysis as a guide for the length of the clinical follow-up in the RCT of ASC-enriched fat grafting for breast reconstruction after mastectomy.

Question 2: How many treatment sessions are needed to complete a breast reconstruction after a skin-sparing mastectomy? This question is important for the RCT (study 5) in which we sought to investigate whether a potentially increased fat graft volume retention from the ASC-enrichment could decrease the number of fat grafting treatment sessions needed to complete a breast reconstruction.

In study 2 we conducted a systematic review of the literature on breast reconstructions performed with fat grafting as the only treatment modality. The studies of this review were in turn used to estimate the number of fat grafting sessions needed to complete a breast reconstruction following a skin-sparing mastectomy, a modified radical mastectomy and breast conserving surgery. These estimates were used in the design of the RCT of ASC-enriched fat grafting for breast reconstruction, which had "number of treatment sessions needed to complete the reconstruction" as a secondary endpoint.

Question 3: What is the most accurate and objective method for measuring fat graft volume retention in the breast? The RCT (study 5) relies on a measurement of fat graft volume

retention in the breast as the primary endpoint, and therefore it is necessary to ensure that the method for measuring this endpoint is accurate and precise.

Study 3 is a validation study of the technique that we believe to be the most commonly used method for measuring fat graft volume retention in the breast based on MRI scans. To the best of our knowledge, this technique has not been previously validated for its ability to measure changes in breast volume, although this is prerequisite for measuring fat graft volume retention. Study 4 is a presentation of a new method for measuring fat graft volume retention in the breast for use in future studies. The method was validated according to the same principles used in study 3 and it will be used to measure fat graft volume retention in the RCT, which is described in study 5.

In study 5, the trial protocol for the RCT (EudraCT 2016-005186-31) is summarized. The knowledge acquired from the first four PhD studies were used to design the trial.

Results

Study 1 was used to model the resorption process of excised fat grafts placed in the mastoid cavity. The model estimated that it takes approximately 2.2 years for the fat grafts to reach volumetric steady state. This tells us that the resorption process has the potential to go on for a very long time, although we do not yet know whether the resorption process of injected fat grafts in the breast is equally long. However, the results of this study led us to change the follow-up period of the primary endpoint in the randomized clinical trial (study 5) to two years, and we generally plan for a longer follow-up periods in future studies of fat grafting.

Study 2 provided an estimate of the number of treatments typically needed to reconstruct a breast after a mastectomy. Our analysis relied on meta-analytical methods of the literature and estimated that approximately three treatment sessions are necessary to completely reconstruct a breast that has not been treated with radiotherapy after a skin-sparing mastectomy. This estimate was used in the planning of the randomized clinical trial (study 5) by giving us an estimate of the background population. The study found that significantly more fat grafting treatment sessions were necessary to reconstruct an irradiated breast than a non-irradiated breast. Surprisingly, we also found that the number of treatment sessions to completely reconstruct the breast was not dependent on whether the patient had been treated with a modified radical mastectomy or a skin-sparing mastectomy.

Study 3 and 4 comprise our efforts to find an accurate and precise method for measuring the volume retention of fat grafts in the breast based on MRI scans. We found that the most commonly used method for measuring fat graft retention in the breast overestimated increases in breast volume. We also presented our new method for measuring fat graft volume retention

in the breast and showed that the method was able to measure changes in breast volume with high accuracy and reproducibility across observers. The new method which we call Lipovol will be used in future studies in our group and we plan to use it in the randomized controlled trial of breast reconstruction with ASC-enriched fat grafting that I describe in study 5. Furthermore, we hope that other researchers will find the method useful in their studies. The method will be freely available to other researchers upon request.

Preliminary results from the randomized controlled trial of ASC-enriched fat grafting for breast augmentation (EudraCT 2014-000510-59)

The second RCT of our group to investigate ASC-enriched fat grafting in women undergoing a breast augmentation has been finalized. The results have been presented[9], and the scientific manuscript is in the final stages before being submitted to a peer-reviewed journal. Unfortunately, the trial did not show any effect of the ASC-enrichment. The breasts treated with ASC-enriched fat grafting did not show a significantly higher fat graft volume retention than the breasts treated with normal fat grafting (54.0% vs. 55.9%). These results have important implications for my plans to initiate the next randomized controlled trial of ASC-enriched fat grafting for breast reconstruction. This recent development has prompted me to outline a research plan to investigate the differences between the first trial that was published in 2013[8], which showed a significant effect; and the second trial that did not. These studies will investigate in detail how to isolate and expand ASCs and ensure that the cells maintain the properties that make them able to improve fat graft volume retention.

Conclusion

This PhD thesis consists of five studies. The thesis is generally concerned with the resorption dynamics of fat grafts and methods to measure fat graft volume retention in the breast. The final study of the PhD thesis is a protocol for a randomized controlled trial that will investigate ASC-enriched fat grafting for breast reconstruction. The first four studies all played a role in the design of the trial protocol.

The preliminary results of the second RCT investigating ASC-enriched fat grafting for breast augmentations recently showed no effect of the ASC-enrichment. This outcome has prompted us to conduct a series of studies that will investigate the discrepancy between the first study that found a significant effect of the ASC-enrichment in the upper arms of healthy individuals and the second RCT that failed to reproduce this effect in the breast. The research plan will be used to optimize the isolation and expansion of ASCs which hopefully, will enable us to improve breast reconstructions with fat grafting so that we can offer a better treatment to our patients in the future.

Background

Fat transplantation was first performed by Neuber in 1893 for correcting a scar-induced depression in the face with fat harvested from the arm.[7] The breast was first used as a recipient site for fat transplantation in 1895 by Czerny, who transferred a lipoma into the breast tissue to fill out a volume defect after breast surgery.[10] The fat grafting technique was refined by the contributions of several surgeons, with some of the most prominent being Holländer[11] and Lexer.[12] Höllander revolutionized the fat grafting technique by heating the fat graft until it was fluid and was thereby able to inject the graft into tissue defects.[11] Lexer took on the procedure of fat injection and described a wide range of situations in which this technique could be utilized.[12] These situations included breast asymmetry, scar corrections, Dupytren contractures, lipodystrophies, traumas and other minor contour deformities.[12] Lexer was also one of the first surgeons to describe the main drawback of fat grafting as a soft tissue filler. He assessed that approximately 60% percent of the fat graft was resorbed in the period after transplantation.

Fat grafting in its current form is relatively new and was made popular among plastic surgeons by Dr. Sydney Coleman, who introduced the structural fat grafting technique and its application to the breast and face.[6,13,14] The process is described by Coleman to include three steps: harvesting of the fat, processing of the fat and injection of the fat. The harvesting is commonly performed via liposuction from the abdomen, hips, thighs or buttocks with a blunt cannula after infiltration of either Ringer's lactate or lidocaine with or without epinephrine. Afterwards, the fat is processed by centrifugation or sedimentation to eliminate excessive water, blood, irrigation fluid and oil from the ruptured fat cells. The processing ensures that components that will be rapidly resorbed are removed from the fat graft and is thought to increase the final fat graft retention rate. Lastly, the fat is transferred to small syringes and injected through small incisions in the skin with a blunt cannula. The fat is injected in a fan-like pattern in multiple planes by releasing small droplets of fat while retracting the syringe. The multiplane pattern and "pearls-on-a-string" approach secures a maximal surface area of the fat and thereby facilitates oxygenation of the fat cells.[4]

After the introduction of structural fat grafting by Coleman, the popularity of fat grafting increased because it was regarded as the ideal soft tissue filler. Many surgeons such as Illouz[15], Delay[16], Bircoll[17] and Rigotti[18] have contributed to the popularization of fat grafting and have highlighted the advantages of low donor site morbidity, the absence of foreign material and a natural feel. Although the technique of fat grafting has evolved throughout many decades, the main drawback of fat grafting persists, specifically, that a large portion of the graft is lost to resorption after surgery.[15,16] Fat graft resorption complicates planning of the reconstruction and necessitates multiple procedures if a large volume reconstruction is required. Therefore, strategies to improve the procedure focus on increasing the retention rate of the fat graft.

Strategies to improve fat graft volume retention

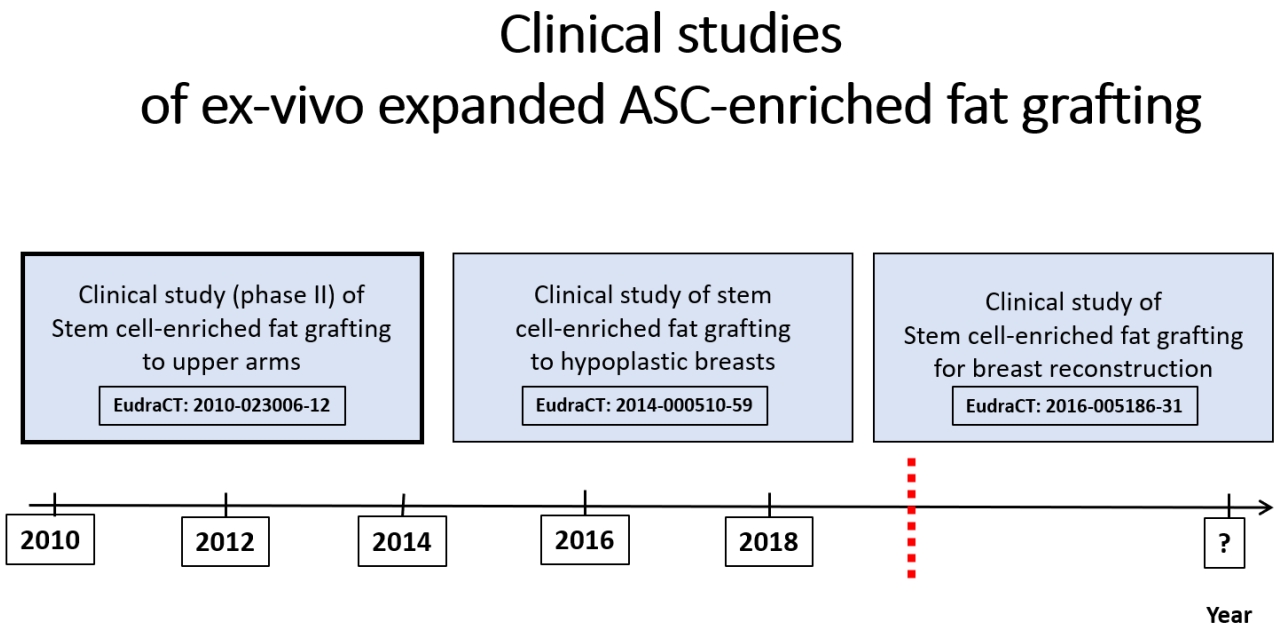
Several methods have been proposed to improve the volume retention of fat grafts.[19–24] They can generally be divided into techniques for improving the transplant and techniques for improving the recipient site. In 2010, Dr. Ueberreiter described a novel method for harvesting fat cells, called BEAULI.[19] This method differed from the traditional manual harvesting methods because it use a water-jet to free fat cells from the fat tissue (Water-jet assisted lipotransfer, WAL) simultaneously with the liposuction. The method is believed to increase the survival of fat cells due to the reduced mechanical stress during harvesting. After harvesting, the fat is separated from the irrigation fluid via centrifugation, and then, the fat graft is ready for injection. Another approach for improving fat graft retention that focus on the recipient site is to pre-expand the breast with an external bra-like device called BRAVA. The BRAVA device creates a vacuum over the breasts.[20–23] Preclinical studies have shown that the use of external expansion provides less tension in the breast after the fat grafting, and induce a neoangiogenic response in the recipient bed.[25] This in turn would support cell survival due to increased nutritional support for the transplanted fat cells. However, subsequent reports that many patients do not tolerate the externally applied BRAVA device, and as an alternative Dr. Stilleart proposed the use of an internal tissue expander for breast reconstruction.[24] The method, which is called “Intratissular expander-mediated fat grafting” is similar to the BRAVA treatment in many ways. It is performed by placing a tissue expander in the breast and successively deflating it while replacing the volume by injecting fat into the surrounding tissue. The procedure is thought to help prepare the breast for the increase in volume so that the fat graft can be placed with less pressure. Furthermore, the capsule that forms around the expander implant is believed to increase blood supply and prevent the fat from pooling in the cavity formed by the mastectomy. However, both BRAVA and intra-tissular expansion are time consuming and not always tolerable by the patient. Moreover, intratissular expansion involves foreign bodies and thus may lead to a whole range of other complications. In addition, both procedures are costly.

Another promising idea to improve fat graft volume retention is to add adipose-derived stromal cells, which are also called adipose-derived stromal cells (ASCs), to the fat graft.[26,27] ASCs are a type of mesenchymal stem cell that resides in adipose tissue and can differentiate into adipocytes, chondrocytes and osteocytes, among other cell types, predominately from the mesodermal germ layer. The addition of these cells to a fat graft has been hypothesized to increase volume retention of fat grafts either by the ASCs differentiating into new adipocytes or by the secretion of paracrine factors that stimulate adipocyte survival and proliferation in the recipient site.[28] Multiple studies have investigated the enrichment of fat grafts with ASCs or stromal vascular fraction (SVF).[29–33] SVF is a mixed cell population that include ASCs that can be obtained from fat tissue. Studies by Gentile, Tissiani and Yoshimura have demonstrated a significant increase in fat graft volume retention in the breast through addition of non-expanded SVF to fat grafts.[29–31] Tissiani reported fat graft retention up to 79% after SVF

enrichment.[30] However, studies by Peltoniemi[32] and Chiu[33] have not been able to reproduce the beneficial effect of SVF on fat graft volume retention. These conflicting results should be viewed considering the potential risk of bias in the conducted studies that are non-randomized, not blinded, and in some cases without control groups. This highlights the need for future studies with randomization, blinding and objective volumetric measurements.

Studies of ASC-enriched fat grafting in our research group

Randomized controlled trials of ASC-enriched fat grafting require a multidisciplinary team effort to enrol patients, perform surgeries, isolate and expand ASCs in a GMP-grade facility and evaluate the graft retention rate with a validated measurement technique in the follow-up period. Therefore, to put the work of this PhD thesis into the context of the activities of my current research group and its history, I think it is relevant to briefly describe the work of my research group. The group planned to conduct three randomised controlled trials of ASC-enriched fat grafting which are illustrated in the figure below.



Long-term research plan to investigate ASC-enriched fat grafting in humans

The first RCT investigated the efficacy of ex-vivo expanded ASC-enriched fat grafting (EudraCT 2010-023006-12).[8] The trial was finalized before I joined the research group. The trial which was conducted by Stig-Frederik Kølle as principal investigator and Krzysztof Drzewiecki as Sponsor was a proof-of-concept trial that investigated ASC-enrichment of fat grafts to enhance fat graft volume retention. Ten voluntary participants were included to undergo liposuction to obtain ASCs, which were then culture-expanded ex vivo for 14 days. After the culturing period,

the expanded ASCs were added to an autologous fat graft. The ASC-enriched fat graft was injected as a bolus in the upper arm of the participants, and in the same procedure, the participants received a bolus of non-enriched fat grafting to the contralateral upper arm. The bolus technique was chosen to optimize visualization of the graft on MRI examinations for measurement of the fat graft retention rate. The study showed a highly significant effect of the ASC-enrichment with a fat graft retention of 80.9% in the enriched grafts and 16.3% in the contralateral control grafts that had not been enriched with ASCs.[8] However, the findings needed to be translated into a clinically relevant setting. Therefore, two additional trials were planned. A second RCT (EudraCT 2014-000510-59) was planned to investigate ASC-enriched fat grafting for cosmetic breast augmentations in 10 participants using the structural fat grafting technique with Peter Vester-Glowinski as the principal investigator. The purpose of this study was to validate the effect of ex-vivo expanded ASCs in a more relevant recipient site with a homogenous patient population and with the use of the structural fat grafting technique. However, one session of fat grafting is often sufficient for a cosmetic breast augmentation and the impact of the ASC-enrichment of fat grafts is therefore limited in this patient population.[34] Thus, we also designed a third RCT, which is described in a detailed summary in study 5 of this thesis. The purpose of the third RCT (EudraCT 2016-005186-31) is to investigate ASC-enriched fat grafting in patients undergoing breast reconstruction after a mastectomy. This patient population is more challenging because the recipient bed is smaller, which reduces the blood supply to the transplanted adipocytes and thereby graft survival. Therefore, many sessions are often needed, ranging from 2-7.[2] This makes reconstruction with fat grafting a long surgical treatment course for patients who often have already undergone extensive treatment before initiating the breast reconstruction treatment. The hypothesis is that ASC-enrichment may increase graft survival and as a result minimize the number of sessions needed for breast reconstruction, which would reduce the surgical morbidity and discomfort in this patient group. If successful, ASC-enriched fat grafting may become a minimally invasive alternative to free flaps and breast implants for patients who chose to undergo breast reconstruction after surgical treatment of breast cancer.

Necessary studies for designing an RCT of ASC-enriched fat grafting for breast reconstruction following a skin-sparing mastectomy (study 5)

During the planning of the RCT (study 5) we encountered several problems that had not been sufficiently addressed in the literature which interrupted the design process of the trial.

Time to steady state of the fat graft

No consensus exists on the exact amount of fat that is resorbed or on when the fat graft reaches a steady state in which no further resorption occurs. It is commonly stated in the literature that the fat graft resorption process ends 6 to 12 months after surgery.[16,19,35]

However, this is based on unproven assumptions because the studies rarely follow the patients for more than one year. To the best of our knowledge, only two studies have followed the fat graft resorption process for more than one year, and these studies both indicated that the resorption process continued for more than a year.[36,37] However, the fat graft volume retention was estimated clinically (i.e., with no objective volumetric method), and the point where the fat grafts reached a steady state was not determined. Because no studies were able to point towards an exact time to steady state, it was not possible to determine the best follow-up period for the RCT (study 5). Therefore, we conducted study 1, which provided a unique opportunity to map the exact resorption process of a fat graft. We analyzed a sequence of MRI scans from patients with vestibular schwannoma who underwent a mastoidectomy with transfer of an en-bloc excised fat graft for closure of the dura mater. Furthermore, this patient population underwent an extensive radiological control program. This provided us with a mean follow-up time of up to 8.4 years and thus enough time to evaluate the time to a steady state of the fat grafts. Such analysis had not been described in the literature previously.

Number of sessions needed for complete breast reconstruction

As background for the RCT (study 5), we also needed to know how many fat grafting sessions are typically needed to undergo a breast reconstruction after a skin-sparing mastectomy. We were also interested in investigating the effect of radiotherapy on the number of sessions needed for complete reconstruction. No consensus exists in the literature on the mean number of sessions for complete reconstruction, with a wide range of 2 to 7 sessions for complete breast reconstruction.[38–41] Therefore, we conducted a systematic review and meta-analysis to investigate the number of sessions needed to complete a breast reconstruction. We stratified patients into 5 groups based on the type of breast surgery the patient had undergone prior to the breast reconstruction. The stratifications were made based on whether the patient had undergone a skin-sparing mastectomy, non-skin-sparing mastectomy or breast conserving surgery. These three groups were further subdivided by whether the patients received radiotherapy.

Measurement of fat graft volume retention

The primary endpoint of the RCT (study 5) was the fat graft volume retention in each breast after one year. Many techniques have been suggested to measure fat graft volume retention, with the most commonly used methods being MRI[42–44] and 3D-photography.[45–47] The current gold standard for measuring changes in breast volume is arguably MRI, as proposed by Dr. Herold et al.[43] In this method, an MRI scan is performed before the fat grafting procedure and a second scan is performed post-operatively. Next, the borders of the entire breast are delineated manually, and the volume difference is compared to the injected fat graft volume. However, we hypothesized that the method was associated with a systematic measurement error and that using the method risked overestimation of changes in breast volume.[48] We

believed that this was mainly due to lax tissue surrounding the breast being drawn into the augmented breast. Because the lax tissue is included in the post-operative scan and not in the preoperative scan, there is a risk of systematic overestimation. In study 3 we validated the above-mentioned MRI technique for measuring changes in breast volume in 28 patients. Our results would have implications for studies that have used the method for both estimating fat graft volume retention[22,23,49] and studies that investigated interventions to improve the fat graft volume retention.[32,50] These investigation led us to develop Lipovol, a new method for measuring fat graft volume retention in the breast (study 4). The method differed from the gold standard by using fixed osseous markers to ensure that the same block of tissue was analysed on both the pre-operative and post-operative scan. Thereby, we considered the lax tissue surrounding the breast, and systematic overestimation was avoided. The method was validated by measuring the breast volume in 14 patients before fat grafting and 3 hours after the procedure. The change in breast volume measured with MRI was then compared with the injected volume. All measurements were performed by four independent and blinded observers.

Study 1 - ***Quantifying Long-Term Retention of Excised Fat Grafts: A Longitudinal, Retrospective Cohort Study of 108 Patients Followed for up to 8.4 Years***

Authors: Mikkel Herly, Mathias Ørholt, Peter V Glovinski, Christian B Pipper, Helle Broholm, Lars Poulsgaard, Kåre Fugleholm, Carsten Thomsen, Krzysztof T Drzewiecki

Study objectives

To investigate the long-term retention of excised fat grafts in the craniofacial area and to calculate the time-to-steady-state volume retention for the grafts.

Study objectives in relation to the PhD thesis

When studying the volume retention of fat grafts, it is important to know whether the resorption process has ended at the time of the measurement (i.e., that the graft volume is in steady state). To compare retention rates of fat grafts between studies, assessments must be performed at the same time in the resorption process, and preferably after the resorption process is finalized. In this study, I calculated the time to volumetric steady state of excised fat grafts in the mastoid cavity. To the best of my knowledge, this is the only study that has demonstrated the steady state of fat grafts with a volumetric analysis. Although the type of fat graft that was examined in this study is very different from a structural fat graft in the breast, the study population presented a unique opportunity to track fat grafts over an extended period of time, which is necessary to find the time to steady state. The results of this study were considered when we decided on the follow-up period of the primary endpoint in the RCT that is described in study 5 because because no previous studies have investigated the time to steady state of structural fat grafts in the breast.

Project summary

<u>Background</u>

The main drawback of fat grafting is that a part of the graft is lost to post-operative resorption. It is essential for the plastic surgeon to have an idea of how much of the graft will stay in place after the resorption period. Furthermore, it is very important to know the length of the resorption period to plan any additional fat grafting procedures. The latter is poorly understood and to the best knowledge of this author, prior to the publication of this study in 2017, no studies had shown the plateau phase of fat graft resorption (i.e., steady state).

<u>Methods</u>

In this study, we investigated existing radiological images of patients who had undergone ablative surgery for vestibular schwannoma and volume restoration with an excised fat graft from the abdomen. These patients were followed up with magnetic resonance imaging (MRI) or computed

tomography (CT) scans on a regular basis to detect any recurrence of the schwannoma. The fat graft was delineated on all image slices that contained the fat graft. These areas were then multiplied by the slice thickness and summed to calculate the total volume of the graft. Figure 2 in Appendix 1 illustrates the delineation of a fat graft. We only included patients who had undergone a post-operative scan within 14 days after surgery. The post-operative scan was used to calculate the fat graft volume at baseline and was set as 100% graft volume. The fat graft volume retention was calculated by dividing the fat graft volume calculated on the follow-up scans with the baseline volume to estimate a retention rate. All retention rates that were calculated based on follow-up scans were plotted against the number of days after surgery. The fat graft retention over time was modeled with a four-parameter log logistic dose-response curve. Additionally, we planned a priori to analyze the effect of sex and baseline fat graft volume with a cut-off volume above or below 16 mL on fat graft volume retention. Steady state fat graft volume retention was defined as a resorption rate of less than 5% of the baseline fat graft volume per year. For histological evaluation, biopsies were taken of the fat grafts in patients who underwent revision surgery during the study period.

Results

Between 2006 and 2015, 208 patients underwent ablative surgery for vestibular schwannoma; 108 of these patients were included in the study. The average observational period was 2.7 years, (range 17 days – 8.4 years). The baseline fat graft volume was 18.1 mL on average (95% CI, 17.2 mL – 18 mL). Volumetric steady state was estimated to be 806 days after surgery, at which time the average fat graft retention rate was 50.5% (95% CI, 46.4% - 54.7%). Patient characteristics and study variables can be seen in Table 1 in Appendix 1, and a model of the fat graft retention trajectory can be seen in Figure 4 in Appendix 1.

The fat graft retention rate was significantly higher in male patients than in female patients ($p < 0.001$). The fat grafts in male patients reached volumetric steady state after 714 days, at which time the average volume retention was 57.7% (95% CI, 51.4% - 63.2%), whereas the fat grafts in female patients achieved steady state after 874 days, at which time the average fat graft volume retention was estimated to be 44.5% (95% CI, 39.5% - 50.1%). A model of the graft retention rate over time in female patients vs. male patients can be seen in Figure 4 of Appendix 1. We did not find any statistically significant difference in fat graft volume retention between the low-baseline volume group (average baseline volume = 13.6 mL) and the high-baseline volume group (average baseline volume = 20.1 mL).

Five patients who had revision surgery in 2016 consented to have biopsies taken of their fat grafts. The specimens were obtained 22 days, 1.3 years, 3.3 years, 4.4 years and 4.6 years after the primary surgery. In the four grafts obtained more than 1 year after surgery, we found mature fat tissue with limited amounts of fibrosis. In the graft obtained after 22 days, we found focal necrosis with surrounding macrophages. Histological images of two selected grafts can be seen in Figure 6 of Appendix 1.

Discussion of the results and their relation to previous research

To the best of our knowledge, this study was the first to investigate the resorption dynamics of fat grafts via imaging analysis with a follow-up of more than one year. Furthermore, to the best of our knowledge, this study was also the first to estimate time to volumetric steady state of fat grafts. The time to steady state of 2.2 years after surgery was longer than what we had anticipated. Several previous studies state that the time to steady state fat graft volume retention is approximately 6 months[31,51] because that is how long it takes for the adipocytes to succumb to the hypoxia, and for the body to clear the dead cells from the recipient site. However, to the best of my knowledge of the literature, this has not been confirmed by a volumetric analysis. Our study show that it can take significantly longer than 6 months for fat grafts to reach steady state volume retention, which demonstrate the need for investigations into this area with other types of fat grafting and in other recipient sites.

Another interesting finding in our study was that we did not find statistically significantly different fat graft retention rates between the high baseline fat grafts and the low baseline fat grafts. We had hypothesized that excised fat grafts with large volumes would have a higher necrosis rate because a larger part of the graft would be too distant from the nearest blood supply to survive on diffusion alone. In fact, a previous study by Capaneda et al. suggests that fat more than 1.5 mm from the nearest blood supply will not survive.[52] This study has been cited multiple times as a reference to the scientific foundation for the structural fat grafting technique. In this study, we investigated fat grafts that had an average distance from the graft center to the nearest blood supply of 9.6 mm. If all fat tissue more than 1.5 mm from nearest blood supply necrotized, we would have expected lower retention rates or more scar tissue formation in our study. On the contrary, we found a retention rate of more than 50%, which is similar to what Neuber found by a clinical estimate in 1893[7]. Furthermore, the histological and radiological analyses showed that the majority of the fat grafts consisted of mature fat tissue, even years after the transplantation of the excised fat graft.

Interestingly, Bourne et al.[5] showed that fat grafting injected with the structural fat grafting technique, as described by Coleman[6], leads to pooling of fat deposits along tissue planes in the recipient site. Consequently, for a large portion of fat grafts, the distance to the nearest blood supply may be substantially greater than what is theoretically expected of the structural fat grafting technique.

Limitations

Our study has limitations that should be addressed. Being a retrospective study, we were limited by the clinical information that could be obtained from the included patients. For instance, we were very interested to investigate the effect of weight changes on the fat graft volume retention over time. Unfortunately, weight was not available from the time of the MRI examinations and thus, we were prevented from performing that analysis. Furthermore, the exclusion of patients and scans was a potential source of selection bias. However, the reasons for exclusion were all related to technical problems such as individual scans not including the entire fat graft. We did not

identify any scans or patients who were excluded based on the volume retention of the fat graft and therefore, I do not expect that selection bias has played a role in the results of the study.

In conclusion, our study highlights the need for further research into the most fundamental parts of fat grafting. The findings illustrate the need for broader investigations into the time to steady-state fat graft volume in other recipient sites and with the structural fat grafting technique.

Perspectives for additional research

The calculation of time to steady-state fat graft volume retention and the model of the resorption trajectory were estimated for excised fat grafts in the craniofacial area. Today, fat grafting is predominantly performed using the structural fat grafting technique[13] in which liposuctioned fat is injected in multiple channels and planes. We are currently investigating the resorption dynamics of fat grafts injected with the Coleman technique[6] in the breast in a prospective study. In this study, we include women who undergo a breast augmentation with fat grafting. We use the Lipovol software (which is described in study 4) to measure the fat graft volume retention in the breasts. The patients are followed annually for three years with MRI scans. The prospective study is headed by Dr. Mathias Ørholt with me as a supervisor. The study will be used to model fat graft volume retention over time and to estimate the time to steady state of structural fat grafts in the breast. We believe that the findings can be used to decide the follow-up time required in future studies but until then we will use the findings from Study 1 as a guide for follow-up of fat graft volume retention.

Study 2 - *Efficacy of breast reconstruction with fat grafting: A systematic review and meta-analysis*

Study objectives

To investigate the efficacy of breast reconstruction using only fat grafting in a systematic review of the literature. Specifically, we sought to use meta-analytical methods to find the number of fat grafting sessions needed to complete a breast reconstruction.

Study objectives in relation to the PhD thesis

This study contributes to the PhD objectives by providing background information on the number of fat grafting treatments needed to complete a breast reconstruction based on studies reported in the literature. This study supports the design of the RCT described in study 5 by investigating the number of fat grafting treatments that is typically needed to complete a breast reconstruction after a skin-sparing mastectomy. The reason we want to improve the fat graft volume retention in the RCT is to improve the treatment protocol for women in need of breast reconstruction after breast cancer. However, an improvement in the fat graft retention rate is less significant for the patient if the fat graft volume retention does not translate into fewer treatment sessions needed to complete the reconstruction. Therefore, we included 'the number of treatments needed to complete a breast reconstruction' as an endpoint in the RCT which is described in study 5. If cell-enrichment results in fewer treatment sessions needed to complete a breast reconstruction, it would provide a great benefit for patients.

Project summary

Background

Multiple procedures are required to perform a breast reconstruction after mastectomy with fat grafting alone. Previous reviews concerned with breast reconstruction with fat grafting have reported on patient satisfaction[53] and the oncological safety of the procedure[54,55], but to the best of our knowledge, no reviews have focused on the number of treatments needed to complete a breast reconstruction. On this background, we decided to conduct a systematic review of the literature on breast reconstruction with fat grafting and to use meta-analytical methods to estimate the number of treatments needed to complete a breast reconstruction. Additionally, we calculated the average total fat graft volume needed to complete the reconstruction, which can be used as a guide to assess whether a given patient has sufficient fat available for liposuction that can be used for fat grafting.

Methods

The systematic meta-analysis and review was performed on observational studies. Therefore, it was conducted in accordance with MOOSE guidelines.[56] The selection of studies was conducted by

three observers in parallel, and all discrepancies were discussed until a consensus was reached. A search of the literature was performed July 2017 using the PubMed, EBSCO, EMBASE and Cochrane databases with the following search terms: (breast reconstruction OR mastectomy OR lumpectomy OR quadrantectomy OR BCS) AND (fat graft∗ OR lipofilling OR fat transfer OR lipotransfer OR lipografts OR fat transplantation OR lipomodeling).
We included studies based on consecutive, unselected patients who had undergone breast reconstruction with fat grafting as the only treatment modality after breast cancer or a genetic predisposition to breast cancer. The included studies were required to specify that the patients had completed their breast reconstruction and to include a minimum follow-up time of six months. We excluded studies in which the patients received a fixed amount of fat grafting sessions. When we encountered multiple manuscripts from the same authors with overlapping patient populations, we only included the manuscript with the highest number of patients or the most recently published manuscript. We contacted the corresponding authors of manuscripts when we were unsure about whether they fulfilled the inclusion and exclusion criteria of the review.

A meta-analysis of the patients in the included manuscripts was performed to estimate the number of treatment sessions needed to complete breast reconstruction based on the following five treatment categories: irradiated modified radical mastectomy, non-irradiated modified radical mastectomy, irradiated skin-sparing mastectomy, non-irradiated skin-sparing mastectomy and breast-conserving surgery.
The included manuscripts were assessed according to the Methodological Index for Non-Randomized Studies (MINORS) for potential bias. We modeled the number of treatments needed to complete a breast reconstruction based on treatment category using a shifted hypergeometric distribution of the study-specific probability of finishing the reconstruction in the next treatment. This was performed individually for the five treatment categories (irradiated modified radical mastectomy, non-irradiated modified radical mastectomy irradiated skin-sparing mastectomy, non-irradiated skin-sparing mastectomy and breast-conserving surgery). All other outcomes were reported with simple descriptive statistics. A Bonferroni correction was applied to adjust for multiple testing. The complication rates were compared with chi-squared tests.[2]

Results

The search identified 1556 studies. The screening for title and abstract provided 202 studies eligible for full text screening. Thirteen studies were included after full text screening. We then contacted the authors of 57 studies that may fulfil the inclusion criteria after some clarifications from the authors. This added an extra 8 studies to the review and meta-analysis, resulting in a total of 21 studies. The main reason for exclusion was a lack of stratification of the patients into the treatment categories (e.g., the number of treatment sessions were provided as an average from a mixed patient population who had undergone cancer treatment ranging from breast-

conserving surgery to irradiated radical mastectomy). See Figure 1 in Appendix 2 for a flowchart of the screening process.
The 21 studies originated from 11 countries and comprised a total of 1011 breast reconstructions in 834 patients (78.8% unilateral and 21.2% bilateral breast reconstruction). The mean follow-up time was 43.8 months. All the included studies were observational, two of them were prospective, 10 were retrospective, and nine were case reports/case series. The average MINORS score of the studies was 9.85 out of 16. Table 1 in Appendix 2 summarizes the characteristics of the included studies.

The number of fat grafting sessions needed to complete the breast reconstruction was modeled based on a total of 1011 breast reconstructions. The estimated number of treatments needed to complete the breast reconstruction was 2.84 (95% CI 2.69–2.98) for the non-irradiated modified radical mastectomy group, 4.27 (95% CI 3.27–5.28) for the irradiated modified radical mastectomy group, 2.93 (95% CI 2.25–3.61) for the non-irradiated skin-sparing mastectomy group, 4.66 (95% CI 3.12–6.19) for the irradiated skin-sparing mastectomy group, and 1.72 (95% CI 1.32–2.12) for the breast-conserving surgery group. The number of treatments needed to complete a breast reconstruction was significantly higher in the irradiated skin-sparing mastectomy group and the modified-radical mastectomy group than in the non-irradiated groups. Conversely, the skin-sparing mastectomy groups were not significantly different from the modified radical mastectomy groups in terms of number of fat grafting sessions needed to complete a breast reconstruction when stratifying for irradiation status.
The total injected fat graft volume, which was the sum of the injected fat graft volume of all fat grafting sessions used to reconstruct the breast, was 744.8 mL in the non-irradiated modified radical mastectomy group, 740.8 mL in the irradiated modified radical mastectomy group, 657.7 mL in the non-irradiated skin-sparing mastectomy group, 626.2 mL in the irradiated skin-sparing mastectomy group, and 230.5 mL in the breast-conserving surgery group. The total complication rates in the five groups were compared, and the irradiated mastectomy groups presented significantly more complications overall than the non-irradiated groups, with $p < 0.001$ for both the skin-sparing mastectomy groups and the modified radical mastectomy groups. See Table 3, Appendix 2 for specification of the complications in the five groups.

Discussion of the results and their relation to previous research

In this systematic review and meta-analysis, we estimated the number of fat grafting treatment sessions needed to complete a breast reconstruction based on the resection type and the use or non-use of radiotherapy. Unexpectedly, we did not find a significantly higher number of treatment sessions needed to reconstruct the breast after a modified radical mastectomy than after a skin-sparing mastectomy. We had hypothesized that the more constricted skin envelope would make more fat grafting sessions necessary to complete the reconstruction. However, this analysis relied only on the number of treatment sessions, and thus, it is possible that other relevant outcomes differ between the two groups. A previous retrospective study by Bailey et al. found that patients

undergoing breast reconstruction after a skin-sparing mastectomy were significantly more satisfied with their reconstructed breast than patients who had undergone reconstruction after a non-nipple-sparing mastectomy.
Our analysis found that a significantly higher number of fat grafting sessions was needed to complete a breast reconstruction with fat grafting in irradiated breasts than in non-irradiated breasts after both modified radical mastectomy and skin-sparing mastectomy. This association is supported by previous observations[57,58] and seems plausible from a clinical perspective. This meta-analysis suggests that 1-2 additional fat grafting treatment sessions are needed to reconstruct an irradiated breast after a mastectomy than are needed for non-irradiated breasts.

The strength of the meta-analysis lies in the unambiguous nature of the primary endpoint (number of treatment sessions needed to complete the reconstruction) and the large sample size distributed across multiple surgeons, surgical centers and countries. However, there are several limitations that should be considered. The analysis was conducted with 'breast' as the independent sampling unit, which has the consequence that studies with large patient populations dominate the analysis. This could have the unintended consequence that surgeons with the most experience would skew the number of treatment sessions needed to complete a breast reconstruction lower because they are more experienced. However, the funnel plot shown in Figure 4, Appendix 2 shows that the average results were evenly distributed between small studies and large studies, apart from two case studies that had especially high numbers of treatment sessions. These two case studies[39,59] were descriptions of especially challenging cases, but the two studies are not expected to have had a significant impact on the results of our analysis because of the overall sample size of more than 1000 breast reconstructions. Another limitation is that we chose to include studies with a minimum follow-up time of six months. We know from a previous study that the fat graft resorption process can continue for up to 2.2 years[1], but if we had decided to only include studies with a follow-up period of more than 2 years we would have to exclude many of the studies in this analysis. However, many of the very large studies that we included in the meta-analysis had a follow-up time of more than 24 months.[58,60,61] We decided on a six month cut-off limit as the best compromise to ensure a wide range of studies in the analysis while minimizing the risk of included patients undergoing subsequent fat grafting sessions after the follow-up period.

<u>Perspectives for additional research</u>
A comparison of the fat graft volume retention in the five treatment categories was not attempted in this meta-analysis because of heterogeneity in the measurement techniques used to calculate fat graft volume retention. Presently, there is no consensus regarding how fat graft volume retention is measured, and the techniques being used are generally not subjected to validation studies that compare measurements of fat graft volume retention between techniques and against known volume changes in the breast. This is the main reason for inclusion of the next two studies in this thesis. These studies investigated the accuracy of the current gold standard

technique for measuring fat graft volume retention in the breast and presented a new measurement technique of our own design.

Study 3 - *The current gold standard breast volumetry technique seems to overestimate fat graft volume retention in the breast: A validation study*

Study objectives

To investigate the most commonly used breast volumetry techniques based on MRI scans for their ability to measure changes in breast volume. The ability of each technique to measure changes in breast volume is crucial for measuring fat graft volume retention in the breast.

Study objectives in relation to the PhD thesis

The primary endpoint of the randomized controlled trial of fat grafting with adipose-derived stromal cells (ASCs) for breast reconstruction is fat graft volume retention, which would be the third clinical trial investigating ASC-enriched fat grafting conducted by our group. Fat graft volume retention was the primary endpoint in the first study of these cells conducted by our group[8] and was the primary endpoint of our second trial of fat grafting with ASCs for breast augmentation in healthy women.[9] When we conducted our second trial, which has been presented in part[9], we searched the literature for a validated technique for measuring fat graft volume retention in the breast. The most commonly used reference point for new techniques was a breast volumetric technique designed by Herold et al.[42,43,62] However, while this technique has been validated for its reproducibility when measuring breast volume and the volume of an implant by direct delineation of the implant on an MRI scan, this technique has not previously been validated for its ability to measure a change in breast volume between two scans of the same patient. Therefore, we decided to investigate this ability of this technique to measure changes in breast volume to validate the technique for measuring fat graft volume retention as the primary endpoint in our randomized clinical trials.

Project summary

<u>Background</u>

Several techniques for measuring breast volume have been proposed and validated.[43,47,63–68] Many of these techniques have subsequently been used to measure fat graft volume retention in the breast. We propose that techniques for measuring fat graft volume retention should be validated by assessing the ability of the technique to measure changes in breast volume and preferably an increase in breast volume. The reason for this suggestion is that accurate measurement of a change in breast volume is necessary for measuring fat graft volume retention. The volume of a fat graft in the breast cannot be delineated directly on an MRI scan because the fat graft is dispersed in multiple tunnels and planes in the breast to maximize blood supply to the graft. This is also the case for 3D photography that is based on the breast topography and is not able to visualize the inside of the breast. Therefore, the measurement of the volume retention of a fat graft is typically

calculated by measuring the volume of the breast before surgery and after surgery when the resorption process has ended. The increase in breast volume from the first scan to the second scan is interpreted as the residual volume of the fat graft, which can then be compared with the initially injected volume to calculate the retention rate. To summarize, the way fat graft volume retention in the breast is calculated with current methods hinges on the assumption that the difference in breast volume from the preoperative scan to the follow-up scan is equal to the residual fat graft volume. However, to the best of our knowledge, this is not how previous MRI techniques have been validated. Therefore, we conducted a validation study of one of the most commonly used techniques[43,44,62] for calculating fat graft volume retention in the breast by measuring changes in breast volume after breast augmentations and comparing the measured change in breast volume with the volume of the augmentation.

Methods

We included women undergoing breast augmentation with breast implants or with fat grafting in a private clinic, Amalieklinikken, Copenhagen and the Dept. of Plastic Surgery and Burns treatments, Rigshospitalet. One surgeon (JJE) performed all the breast augmentations in the study. We excluded women with known breast diseases, pregnancy or contraindications to MRI examination, such as implanted metal or claustrophobia. The patients who underwent implant-based breast augmentation were scanned within 10 days before the surgery and 4-7 months after the surgery. The two MRI examinations were performed at the same time in the woman's menstrual cycle. The patients undergoing breast augmentation with fat grafting were scanned the day before surgery and again within three hours after the surgery, at which time the edema would not yet have fully developed and fat graft resorption would not have occurred.

The MRI examinations were performed on a 3-tesla MRI unit, except for two women who were examined on a 1.5-tesla MRI unit. The volume assessments were performed independently by three readers (JH, SS, MNH) who did not know the true volumes of the implants/fat grafts. The breast delineations were performed according to Herold et al.[43,44,62]: the skin was used as the superficial border, the internal costal surface was used as the deep border, the center of the sternum was used as the medial border, and the point where the thickness of the breast becomes equal to the thickness of the skin was used as the lateral, superior and inferior borders. We contacted the corresponding author[43] of the papers to verify the steps, and he suggested that an equal number of slices should be used in the preoperative MRI examinations and the post-operative MRI examinations. To the best of our knowledge, this additional step has not been described previously in the literature. The accuracy of the technique was assessed by measurement of the error between the measured increase in breast volume and the true volume of the breast augmentation (i.e., volume of the implant or volume of the injected fat). The precision of the technique was assessed in milliliters by the inter- and intra-reader variation and as a percentage of the true volume of the breast augmentation. Previous studies have validated their MRI-based measurement technique by its ability to measure the size of a breast implant by direct delineation of the implant. We do not think this test is a genuine assessment of the ability of a

technique to measure a change in breast volume because the fat graft cannot be delineated. However, to assess the accuracy of the MRI scans and the observers' ability to delineate correctly, we also measured the implant volumes by direct delineation of the implant. This was performed after the measurements of changes in breast volume as to not unblind the observers.
The sample size power calculation was estimated with the Clopper-Pearson exact method which required 53 breast augmentations for the validation study to achieve a 90% confidence interval width of less than 10%. Based on this, we decided to include 28 patients for a total of 56 breasts with, 14 of the patients undergoing breast augmentations with implants and 14 of the patients undergoing breast augmentation with fat grafting.

Results

Twenty-eight women undergoing bilateral breast augmentation were included. Fourteen of these women underwent breast augmentation with implants, and the other 14 women underwent breast augmentation with fat grafting. The patient characteristics are shown in Table 1, Appendix 3. The mean measurement error across the three readers was 178.4 mL (95% CI 156–201 mL) when measuring the women who received breast implants and 153.4 mL (95% CI 138.4–168.5 mL) for the women who received fat grafting. These measurement errors correspond to percentage errors of 50.8% (95% CI 45.3% −56.3%) for breast augmentations with implants and 50.9% (95% CI 45.7% −56.0%) for breast augmentations with fat grafting. Figure 2 in Appendix 3 shows a linear regression of the relationship between the measured increase in breast volume and the volume of the breast augmentation (implant or injected fat graft volume). The measurement error in milliliters increased with the volume of the breast augmentation volume, but the percentage measurement error remained relatively unchanged from the smallest augmentation to the largest augmentations. The measurement error did not depend on the type of breast augmentation (i.e., whether the augmentation was performed with implants or with fat grafting).
The mean inter-observer variation was 55.25 mL (95% CI 47.13–63.37 mL, range 0.28–268.23 mL) (n = 168). The mean intra-observer variation was 19.77 mL (95% CI 15.87–23.67 mL, range 0.05–75.80 mL) (n = 72).
Measurement of the implant volumes by direct delineation had an average measurement error of 15.5 mL and a systematic error of -1.8%, which was not statistically significant.

Discussion of the results and their relation to previous research

MRI is arguably the most accurate non-invasive technique for measuring tissue volume. This is exemplified by the high accuracy and precision of the measurements of implant volume when directly delineating the implants in this study and many previous studies.[63,66,67,69,70] However, our study showed that the way MRI scans are used to measure a change in breast volume between scans may be subject to a large systematic error. The current approach of measuring changes in breast volume is to first to measure the total breast volume before and after a change in breast volume. The difference between the two measurements is then considered to be equal to the change in breast volume. This may seem logical in theory but may introduce systematic error if the

measured change in breast volume is interpreted as the residual volume of a fat graft for the following reasons: when the breast is augmented with fat, the surgeon will distribute the graft in the breast, but the augmentation will also expand the breast borders laterally, inferiorly, medially and superiorly.[48] Therefore, subcutaneous tissue that was outside the breast before the surgery may be included as part of the augmented breast after the surgery. In theory, this will be counted as part of the increase in breast volume, which will overestimate the volume of the fat graft. If this error is not accounted for by the measurement technique, then the fat graft volume retention might be systematically overestimated.

Perspectives for additional research

The results of this study prompted us to design a new technique developed specifically to measure changes in breast volume. The technique was designed to account for the theoretical overestimation described above. This work is described in study 4.

Study 4 - *Lipovol: Validated Software for Measuring Fat Graft Volume Retention in the Breast with MRI*

Study objectives

To design an MRI-based method to measure fat graft volume retention in the breast and to validate the ability of the method to measure changes in breast volume.

Study objectives in relation to the PhD thesis

To validate a new method designed to measure fat graft volume retention in the breast that can be used to measure fat graft volume retention in the RCT, which is intended to be the primary outcome.

Project summary

Background

We have previously shown that one of the most widely used MRI-based methods for measuring fat graft volume retention had a large systematic error of approximately 50% when used to measure changes in breast volume.[3] We hypothesized that one of the potential reasons for this overestimation is that the approach of measuring total breast volume before and after a change in breast volume to deduct the difference is incorrect if the objective is to measure fat graft volume retention. Although the difference in total breast volume must indeed be calculated as described above, we theorized that the change in total breast volume is the sum of two components. The first component is the added volume of the fat graft, but there may be another component of the change in total breast volume that stems from expansion of the breast borders. The breast is a structure most often defined by its contours. Breast augmentation entails expansion of the breast projection but also lateral, superior and inferior expansion. The expansion of the breast borders will make tissue that was previously outside of the breast, by definition, now a part of the breast. If a method does not account for this phenomenon, there is a risk that this added volume will be interpreted as part of the residual fat graft.

To account for this risk of overestimation, we designed a new method based on MRI scans that can be used to measure changes in breast volume but only changes that arise from the added volume and not from tissue that may be included in the augmented breast due to an expansion of breast borders. An algorithm based on this method was constructed and translated into software that would make most steps automatic. The software was validated by measuring changes in breast volume in 28 women who underwent breast augmentation with a known volume.

Methods

The new software method is called “Lipovol” and will be made freely available for use on the MeVisLab platform.[71] None of the authors have any financial disclosures regarding the Lipovol software or MeVisLab. The validation study was approved by the Regional Ethics Committee of Copenhagen (nr. H-1501379) and the Regional Data Protection Agency. All participants provided written informed consent before inclusion in the study.

Two MRI scans are needed to use the Lipovol software: one recorded before the volume change of interest and another recorded after the volume change has taken place. We used a Dixon sequence for our validation study, but all scans that can be used for volumetric measurements should be useable, including CT scans. The two scans are first aligned based on osseous markers in the thorax in the registration step. Then, a rectangular region-of-interest that encompasses the highest expected volume is drawn around the breast, thus, expanding the breast borders. This rectangular region-of-interest is then mirrored by the software on the other scan which is possible because of the registration. Then, the skin of the patient is delineated automatically with a region-growing algorithm, and the tissue volume inside of the region-of-interest is calculated by the software. The volume within the region-of-interest is then shown in a 3D reconstruction, and the change in breast volume that comes from the augmentation is calculated. See Figure 1, Appendix 4 for an illustration of the 3D reconstruction in a patient before and after breast augmentation.

The validation study was performed with 28 women who had undergone breast augmentation with either breast implants (14 women) or fat grafting (14 women). The women who underwent breast augmentation with implants were scanned before the surgery and 4-7 months after surgery at the same time in their menstrual cycle. The women who underwent breast augmentation with fat grafting were scanned the day before surgery and within 3 hours after the surgery. The MRI scans were performed on a 3-Tesla MRI unit (Siemens Magnetom Verio, Germany) using a 4-channel breast coil. The women were examined head-first and in a prone position.

The ability of the method to accurately measure changes in breast volume was assessed by measurement error against the true volume of the augmentation (i.e., the volume of the breast implant or the volume of the injected fat graft). The precision of the method was assessed by assessing the inter-observer and intra-observer variation.

Measurements were performed by four observers (MH, MØ, JH and MNH) who were all blind to the true volume of the augmentation and to the measurements of the other observers.

Comparisons of continuous outcomes were performed with t-tests and evaluated at a 5% significance level. Measurement errors were calculated with the breast as the independent sampling unit, and an average of the four observers was used to assess the measurement. Inter- and intra-observer variation were calculated with individual measurement as the independent sampling unit. The analyses were performed in R, version 3.6.1 (www.r-project.org, R Foundation for Statistical Computing, Vienna, Austria).

Results

Twenty-eight women who underwent bilateral breast augmentation with either implants (14 women) or fat grafting (14 women) between 2014-2018 were included in the validation study. The four observers made a total of 280 individual measurements of changes in breast volume in the 28 women (56 breasts), with 96 repeated measurements. The women who underwent breast augmentation with fat grafting received a median fat graft volume of 300 mL per breast (range 250-350). The women undergoing breast augmentation with implants chose implants with a mean volume of 348 mL. The average measurement error for the women who received fat grafting was 29.2 mL, with a systematic average measurement error of 6.3% overestimation. The average measurement error for the women who underwent breast augmentation with implants was 92.2 mL, with a systematic measurement error of 23.9% overestimation. The measurement error was significantly higher when measuring the women who received implants than when measuring the women who received fat grafting. The measurement errors in both groups were significantly lower than that achieved with a similar validation of the technique by Herold et al.[43] (study 3 of this thesis)[3]. The median inter-observer variation was 4.8% (IQR 2.7%-9.8%) for the women undergoing fat grafting and 4.9% (IQR 2.4%-8.2%) for the women who underwent breast augmentation with implants. The median intra-observer variation was 4.2% (IQR 2.0%-8.7%) for the women who received fat grafting and 6.2% (IQR 2.4%-10.1%) for the women who received implants. The results of the validation study are summarized in Table 1, Appendix 4. Linear regression showed a high correlation between the measured changes in breast volume and the true volume of the augmentation (i.e., injected fat graft volume or implant volume), with $r^2 = 0.97$ for the women who received fat grafting, and $r^2 = 0.94$ for the women who received implants. Figure 2, Appendix 4 shows linear regression with a 95% prediction interval. A Bland-Altman plot is shown in Figure 3, Appendix 4, indicating that the total measurement error increased with the volume of the augmentation.

The study was presented in part at Plastic Surgery "The Meeting" in San Diego 2019.[72] On the 22nd of June, 2020, the first half of the study was accepted by PRS Global Open after peer-review. Based on the review process, we were encouraged to divide the study into two parts where the first would present the new method and a validation based on the patients undergoing fat grafting. The second study will include the patients undergoing breast augmentation with implants along with supplementary investigations that will validate our new method for wider use in clinical research. The supplementary investigations have yet to be conducted and therefore, I have maintained the original structure of the study in the PhD thesis.

Discussion of the results and their relation to previous research

To the best of our knowledge, this study is the first to validate a technique for measuring fat graft volume retention in the breast by validating the ability of a technique to measure changes in breast volume after breast augmentations. Herold et al.[43] validated their technique with measurements of the volume of breast implants in women who had undergone breast

augmentation. However, only post-operative scans were available for this validation, and the validation was performed through simple delineation of the implant and not by measuring the change in breast volume. With excellent accuracy, other studies have validated the accuracy of breast volume measurement techniques based on 3D photography by comparing the measurement of total breast volume before radical mastectomy with the volume of the mastectomy specimen.[47,63,66,67] However, these assessments compared total breast volume measurements with mastectomy specimen measurements rather than comparing the *change* in total breast volume and the original mastectomy specimen. In our view, the latter is closer to how fat graft volume retention is measured in the breast using current techniques.

Perspectives for additional research

The validation of the new software "Lipovol" in this study has some limitations that should be explored further. First, the technique should be tested on more patients and by other researchers to ensure its reproducibility between research groups. We also plan additional studies to investigate the effect of weight changes on total breast volume which could be incorporated into the software for longitudinal studies where the patients' weight cannot be assumed to remain constant. This would enable us to isolate the effect of weight changes on the fat graft itself. Furthermore, the Lipovol should be compared head-to-head to the more commonly used 3D imaging techniques[68], to explore the strengths and weaknesses of the two imaging modalities. Finally, we hope that the technique will be used for future studies of fat graft volume retention in the breast by other researchers. The research field would benefit if the research community could agree on a few standardized and validated measuring techniques, which would allow for a more direct comparison between studies across research groups.

Study 5 - ***PROJECT PROTOCOL: Fat Grafting with Ex-Vivo Expanded Adipose-Derived Stem Cells for Breast Reconstruction Following Mastectomy, EudraCT: 2016-005186-31***

Study objectives

To investigate if the addition of adipose-derived stromal cells (ASCs) to fat grafting for breast reconstruction can increase the volume retention of the graft and secondly, decrease the number of procedures needed to complete a breast reconstruction with fat grafting as the only treatment modality.

Study objectives in relation to the PhD thesis

This randomised controlled trial is the centre of the work that I have presented in this PhD thesis. The previous four studies were designed to investigate aspects necessary for designing the randomised controlled trial.

Summary of the study protocol

Background

Breast cancer is estimated to be the most frequent cancer, and the most frequent cause- of cancer-related death amongst women world-wide[73] Breast cancer incidence in Denmark is approximately 4700 per year and it constitutes the largest group of patients who needs reconstruction as a part of their cancer treatment. The main surgical treatments of breast cancer patients are mastectomy or breast-conserving surgery (BCS). Especially the mastectomy is a highly invasive procedure and it has a very negative influence on the quality of life for the patient.
The most common negative psychological effects after breast cancer surgery are on body-image and the social and sexual function of the patient. Breast reconstruction can improve the quality of life and, to a large degree, restore a positive body image for the patient.[74]

Reconstructing the breast after mastectomy

For most women who undergo a mastectomy due to breast cancer or a genetic predisposition to breast cancer, the reconstructive procedure is based on tissue flaps and/or the use of prosthetic implants. Flap procedures are often effective in restoring breast contour, but they have the disadvantages of donor site morbidity, and a long recovery period, including several days as an inpatient. Many women find the use of breast implants unacceptable. In a study of women reconstructed with breast implants after prophylactic mastectomy, 51% had a feeling of the reconstructed breast not being their own.[75] Also, when implants are used, they are prone to complications such as implant rupture, displacement, infection, and capsular contracture at high rates, up to 10-13%.[76–79]

A less invasive procedure for breast reconstruction after a mastectomy is to use autologous fat grafting. Fat is transplanted as micro ribbons between the muscle fibres of the pectoralis major muscle via a syringe.[58]
Transplantation of autologous fat has shown promising results in soft tissue reconstruction, however, conventional fat grafting has an inherent disadvantage of an unpredictable fat resorption rate of 25-80%[16,80,81], which makes it difficult to achieve the desired volume and aesthetic result. Reconstructing the breast after mastectomy is possible with fat grafting, however, the patient must endure multiple procedures over a long time span to build up volume. Previous studies report that an estimated 3 treatment sessions are necessary for completing a breast reconstruction after a skin-sparring mastectomy.[2] The reason multiple sessions are necessary is due to resorption of the fat graft between sessions and because only a limited amount of fat can be transplanted in one session as to avoid fat necrosis.

Fat transplantation with adipose-derived-stem/stromal cells

Our research group is working on improving the field of breast reconstruction with a minimally invasive technique based on fat transplantation enriched with adipose-derived stromal cells. In a previous randomised clinical trial, the trial group showed that enrichment of fat grafts with ex-vivo expanded adipose-derived stem cells (also called adipose-derived stromal cells) significantly enhanced the fat graft retention rate, compared to non-enriched grafts.[8] The study was performed with a small quantity of fat (30 mL) injected as a bolus into the subcutaneous tissue of the upper arms of healthy volunteer participants. Each patient had one arm randomly allocated to ASC-enriched fat grafting and the contralateral to non-enriched fat grafting. This study is a proof of concept – that ASC may improve fat graft retention in humans.

Adipose-derived stem/stromal cells

ASCs belong to the group of fibroblast-like mesenchymal stem cells (MSCs).[26] They have a high proliferation potential and the ability to differentiate into adipocytes, osteocytes, chondrocytes, and myocytes.[82–84]
There are no specific markers for MSCs but the International Society of Cellular Therapy (ISCT) has recommended a common set of minimal criteria for the definition of MSCs: adherence to a plastic culture surface; expression of the surface markers CD73, CD90, CD105 and absence of the surface markers CD14, CD34, CD45; and the ability to differentiate into adipocytes, chondroblasts and osteoblasts in-vitro, when subjected to the proper inductive factors and culturing media.[85,86]

Trial design

Primary endpoint:

1. Graft retention of the total volume of injected grafts one year after the final reconstructive procedure, determined by MRI of the breast.

Secondary endpoints:

1. The number of treatments needed for complete breast reconstruction.
2. Radiological changes in the breast after conventional and stem cell-enriched fat injection based on MRI one year after ended treatment with fat grafting to the breast.
3. The graft retention rate of the first, second and any subsequent grafting sessions.

The clinical study is designed as a double-blind, randomised, paired, controlled trial of fat grafting with Adipose Derived-Stromal Cells (ASCs) for breast reconstruction following prophylactic bilateral skin/nipple-sparing mastectomy due to a genetical predisposition to breast cancer.
Each included participant will undergo a minor liposuction in local anaesthesia 15-20 days before undergoing bilateral, prophylactic, skin/nipple-sparing mastectomy and immediate breast reconstruction with fat grafting with/without ASC enrichment. The patients will be randomized to fat grafting with Adipose Derived-Stem Cells to either the right breast or the left breast and fat grafting without any supplement to the contralateral breast.

Inclusion criteria

1. Age 25 - 45 years (25 and 45 included)
2. Females who are offered prophylactic, bilateral nipple-sparing mastectomy due to an estimated familial predisposition to develop breast cancer.
3. Patients who are deemed fit for breast reconstruction with fat grafting by a plastic surgeon in terms of available fat supply, breast symmetry, and who have a small, non-ptotic breast mound.
4. Signed informed consent by the patient
5. Mutual agreement on acceptable breast volume for point of complete reconstruction between patient and surgeon. (defined in METHODS)

Exclusion criteria

1. Smoking.
2. Diabetes or other comorbidities that may increase the risk of surgical complications assessed by the surgeon.
3. Pregnancy, planned pregnancy or breastfeeding during the study period
4. Genetic syndromes associated with a high risk of cancers other than breast- and ovarian cancer.
5. Previous- or planned radiotherapy.
6. Asymmetry of the breasts or thorax.
7. Contraindications against MRI examination (i.e. implanted objects containing metal or claustrophobia)

8. Allergy towards necessary anaesthesia.
9. Allergy towards penicillin or streptomycin
10. Current or previous participation in other clinical trials of medicines.

Criteria for excluding subjects during the study:

- Pregnancy.
- Withdrawal of consent by subjects.
- Microbiological contamination of the cell culture or in the transplanted tissue.

The participants must agree to use contraceptives to pregnancy during the trial period and until one year after the breast reconstructive procedure to be included in the trial. Contraceptives will be defined as hormonal oral pills or hormonal intrauterine devices. Contraceptives are not required if the participant is not fertile. All included patients will be tested for pregnancy by a HCG blood sample.
For this trial, a woman is considered of childbearing potential, i.e. fertile, following menarche and until becoming post-menopausal unless permanently sterile.

Power calculation

Based on previous studies, we expect an absolute difference in volume retention of the ASC enriched fat graft compared to the volume retention of a conventional fat graft of at least 20 percentage points within women with a standard deviation of 15 percent. The sample size was estimated with $\alpha = 0.05$ and power $(1-\beta) = 0.95$.
This gave an estimated sample size of 7 participants for a paired t-test.
Three additional participants will be included to compensate for drop-out during the study period. Drop out can occur by the withdrawal of consent, by the initiative of the primary investigator due to serious side effects (described later in the protocol), or by death.

Choice of design

The variation in fat graft retention is high between individuals but low within the individual.[48] Therefore, we will conduct this study with a paired design where each patient will receive fat grafting with ASC to one side and fat grafting without ASC to the contralateral side. This design will decrease the risk of making a Type I error (the study shows a positive effect even though this effect is random and not caused by the intervention) or Type II error - (the study shows no difference, though there is a positive effect associated with the intervention) and reduce the number of participants needed to investigate the efficacy of the intervention. The control side will receive fat grafting without ASC with structural fat grafting technique[13] which is considered the gold standard for fat grafting.

Randomisation/Unblinding of the study

The allocation sequence is generated using www.randomization.com and kept by a designated person who will not be involved with the data analysis or treatment of the patients. The

sequence will be printed and kept on a data storage device which will be locked away during the study period.
Participants, study personnel and outcome data assessors will be blind to treatment allocation, and the blinding is kept until all the data is analysed. Unblinding of the study can be performed in the case of an emergency at any time if it is considered necessary by the investigator or the sponsor.
If no adverse events or unexpected reactions occur during the study period, unblinding will be performed after the data analysis of all participants after the last visit of the last patient.

Extracting SVF and expanding ASCs

The patients will undergo a minor liposuction under local anesthesia as an outpatient.
Through small incisions, a wetting solution is infiltrated into the subcutaneous fat. The lipoaspirate is procured with a standard liposuction device and sealed in a sterile container/canister and brought to the Cell Therapy facility at the Department of Clinical Immunology.

Isolation of ASCs
ASC isolation and ex vivo expansion will be performed in a laboratory approved by the Danish Patient Safety Authority and the Danish Medicines Agency for Good Manufacturing Practice (GMP) and clinical stem cell expansion at the Cell Therapy Facility, the Department of Clinical Immunology, Rigshospitalet.
The lipoaspirate from the first liposuction will be washed with Hanks Buffered Saline Solution (HBSS) (Gibco, Life Technologies, USA) and centrifuged for five minutes at 100 g. The washed fat will be digested with collagenase NB 6 GMP Grade (SERVA electrophoresis GmbH, Heidelberg, Germany) for 60 min at 37°C to extract the Stromal Vascular Fraction (SVF). The collagenase will be neutralised by adding cell culture medium with 10% pooled Human Platelet Lysate (pHPL) (culturing media). Then, the suspension will be filtered using 100-µm Steriflip filters (Millipore) and centrifuged for 10 minutes at 1200 g. The cell pellet will be resuspended in culture medium, and then the SVF cells will be counted with a NC3000 Nucleocounter) (Chemometec). We expect to isolate approximately 1 x 10^6 SVF cells/ml lipoaspirate.

P0 Culture-expansion of ASCs
The isolated SVF cells will be seeded in cellstack culture flasks, with a seeding density of 10,000-40,000 SVF cells/cm^2.
The culture medium will consist of Dulbecco's modified Eagle's medium (DMEM) (PAA Laboratories, Pasching, Austria), 1% penicillin-streptomycin (GIBCO-Invitrogen, Taastrup, Denmark), 2 IU/mL preservative-free heparin (LEO Pharma, Ballerup, Denmark) and 10% pHPL (produced by the Blood Bank, Rigshospitalet). The primary cultures (P0) will be incubated in a humidified atmosphere containing 95% air and 5% CO^2 at 37°C as described previously.[87] The P0 ASCs will be harvested with trypsin (e.g. TrypLE Select (Gibco, Life Technologies) to detach the cells

from the plastic surface. The harvested cells will be divided into three equal parts. One of these parts will be used immediately for further expansion in P1 in sterile cell culturing flasks. These cells will be harvested at confluency and used for the first fat grafting session. The two remaining parts of the P0 ASCs will be cryopreserved with standard technique of the Cell Therapy Facility. Then, at the time of the second, and possibly third, fat grafting sessions the frozen P0 ASCs will be thawed, seeded in cell culturing flasks and expanded in P1 as described below. (see figure 1).

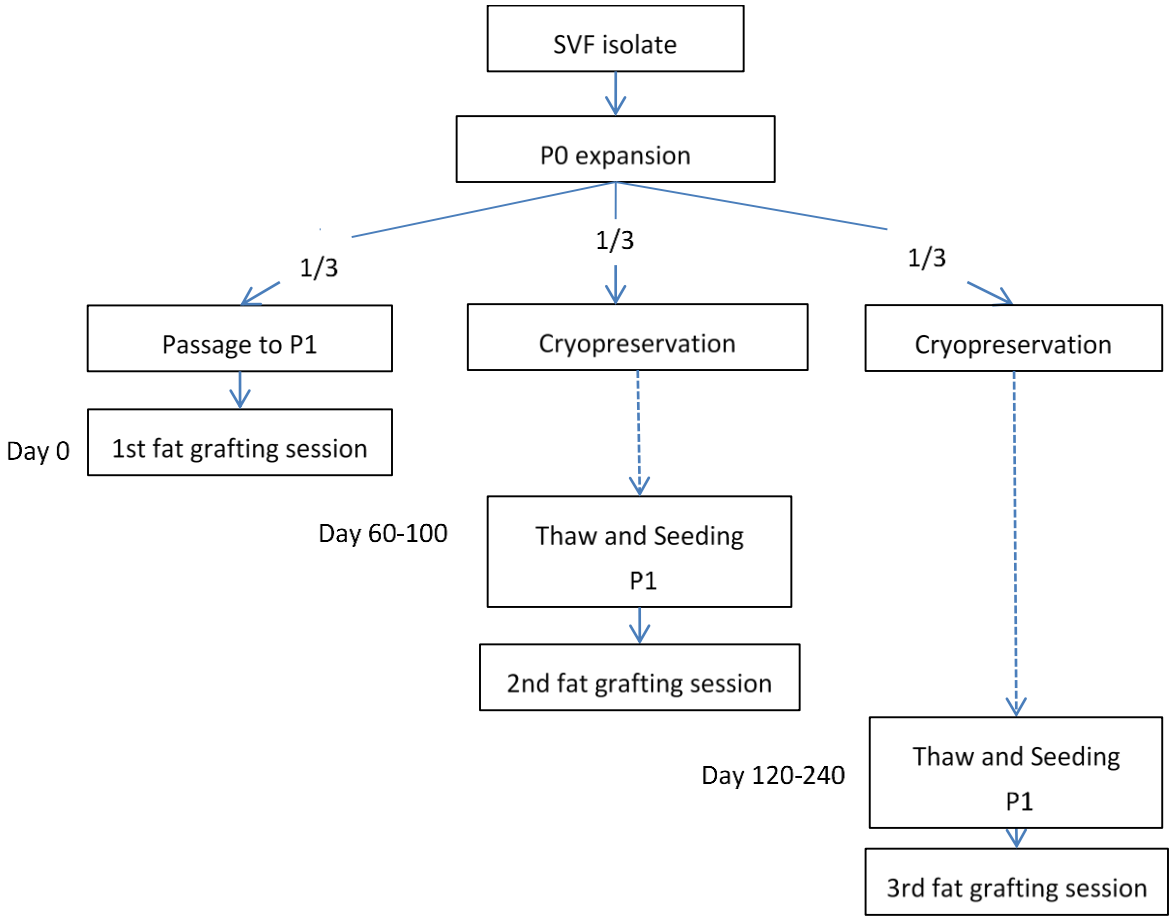


Figure 1: Flowchart illustrating the protocol for ASC expansion

<u>P1 Culture of ASC</u>

P0 ASCs will be seeded in sterile culture flasks to obtain enough P1 ASC. In each ASC enriched fat grafting session, a dosage of 10-20 x 10^6 P1 ASC will be used per mL fat transplant. Based on results from previous studies we expect to harvest approximately 8 x 10^4 ASC/cm^2. Combined, this gives a minimum need of 125 cm^2 culturing surface area / mL of estimated fat graft. The culturing surface area is depending on the estimated fat graft volume for each fat grafting session. E.g. if the surgeon estimates that 150 mL fat graft is needed for each breast, we would need a surface area for cell expansion of:

150 mL fat graft x 125 cm^2 culturing surface area / mL fat graft = 18.750 cm^2

To ensure having enough cells we will add a margin of 30% to the total cell culturing surface area: 18.750 cm^2 x 1.3 = 24.375 cm^2
When the cells reach confluency (expected 5-12 days after passage depending on seeding density and required cell amount) the ASCs will be harvested for transplantation.

pHPL production

Platelet concentrates (PCs) will be produced by a standard buffy coat method and stored in the platelet additive solution InterSol (Fenwal, Inc.). After 7 days of storage, the PCs will be centrifuged at 1677g for 15 min and the platelet pellet will be resuspended in AB plasma.
The pHPL are subsequently manufactured by the freeze-thaw method as described by Schallmoser et al.[88], with minor modifications[89]: after the Intersol is replaced with plasma, the units of platelet rich plasma (PRP) will be frozen at -40°C. Ten units of the PRP will be thawed in a 37°C water bath (Plasma Thawing System, Helmer, Noblesville, Indiana, USA), pooled into a single PRP batch (3-4 L), aliquoted into 300 mL portions and frozen at -40°C. Then, each of the 300 mL bags will be thawed in a 37°C water bath, centrifuged at 4000 g for 15 min to sediment the platelet fragments and transferred into new 300mL bags and stored at -40°C for later use in the preparation of the cell culture medium.
The blood donors will be traceable to the recipients by the electronic Blood Bank IT system, Blodflödet, used at the Blood Bank, Rigshospitalet.

ASC harvesting procedure

The ASCs will be harvested on the day of surgery. The cell culture flasks will be incubated with an enzymatic agent (i.e.TrypLE Select (Invitrogen, Taastrup, Denmark) or Accutase) that will detach the cells from the plastic surface. Then, the enzyme will be neutralised by adding culture medium in a 1:1 ratio. The enzyme will be removed by centrifugation of the cell pellet and discard of the supernatant. The cell pellet will be resuspended in saline with 4 % albumin (CSL Behring ref. 110124 50 g/L, Clinical grade). And brought to the operating theatre for enrichment of the fat transplant.
The ASCs will be controlled for the absence of pathogen contamination in a sample taken 7 days before the surgery. The viability of the ASCs must be greater than 85% and the morphology assessed by microscope must show cells with characteristics of ASCs before the cells are released for the transplantation.
The ASC suspension will be mixed with the fat graft in 100 cc syringes and used for the breast allocated to ASC enriched treatment by the randomization sequence (see description of the surgery on the next page). Documentation of randomization is kept in each patient case report file (CRF) by signature to comply with GCP standards.
Reference samples, from the ASC cell suspension harvested for each treatment, will be collected, and kept frozen at – 150 ˚C in a suspension of 90% culture medium and 10% dimethyl sulfoxid (DMSO). These reference samples will be kept for storage up to 10 years after the follow-up of the

study has ended. The samples are stored in the Cell Therapy Facility, the Blood Bank, Department of Clinical Immunology.

Surgery and follow-up with MRI

MRI to measure the primary endpoint

Magnetic Resonance Imaging (MRI) of the fat grafted breasts will be performed with the use of standard technique at the Department of Radiology. The Lipovol software will be used to measure the fat graft volume retention over time in the reconstructed breasts. The scanning will include the anterior chest wall to ensure the inclusion of the entire breast in the image. MRI will be used for volume assessment of the fat transplants and the assessment of the fat graft volume will be based on the change in the total volume of the breast over time.

Step-by-step description of the study

- Minor liposuction under local anesthesia as an outpatient (15-22 days before the 1st fat grafting procedure) up to 200 CC of centrifuged fat. All obtained fat will be used for isolating SVF.

- Preoperative MRI of the breasts (0-14 days before the 1st fat grafting procedure) and weighing of the patient.

- Mastectomy and 1st fat grafting procedure in one session (day 0)

Liposuction, graft preparation, ASC enrichment and fat grafting procedure

The areas to undergo liposuction on the abdomen/thighs/hips will be marked while the participant is in an upright position. Markings on the breasts for grafting will be done at the same time. Breast surgeons will perform bilateral skin- and nipple-sparing mastectomy by standard technique. The excised breast tissue will be volume measured via water displacement technique for each breast.

Alongside the mastectomy procedure, a wetting Klein's solution will be infiltrated at the donor site. The liposuction will be performed to obtain lipoaspirate for the transplantation and will also contour the body shape of the donor sites. Liposuction will be performed with the use of standard equipment for fat transplantation. The lipoaspirate will be harvested by standard technique and standard equipment for liposuction and fat grafting.

The fat tissue will be mixed with the harvested ASCs corresponding to 10-20 x 10^6 ASC/mL of fat transplant, this step will be simulated to the control side by adding additional lipoaspirate from a mock ASC syringe corresponding to the volume of ASCs.

The ASC-enriched fat tissue will be transferred from the Signet syringes to 10 cc luer-lock syringes for transplantation. The fat enriched with ASC and the fat that is mock-enriched will be kept on two separate tables in the operating room. After the grafts are prepared, a surgical drape will be put up by two nurses to block the view for the surgeons and research personal. Thereafter, a sealed envelope will be opened by the appointed nurses, which will contain the allocation of the ASC side to either the "right" or the "left" breast. The nurses will write "right" and "left" on two separate tables on surgical drape and allocate the ASC enriched syringes and the mock-enriched syringes to the two sides according to the instructions provided by the envelope from the randomized allocation sequence.
Blunt 14-G cannulas will be used for injection, one for each side to prevent residue transplant being mixed from one breast to the other. The fat grafts will be distributed in the subcutaneous-, intra-muscular- and submuscular parts of the breasts in as many channels and planes as possible.

- MRI and clinical evaluation (0-14 days before 2nd procedure)

- 2nd fat grafting procedure (Minimum 8 weeks after the 1st fat grafting session and mastectomy)

After a minimum of 8 weeks after the 1st fat grafting session and mastectomy, the patients will be offered a second fat grafting session. The fat grafting procedure will be conducted via the same technique as described in session 1. Another envelope containing the same allocation as in the first procedure will be used in the same way as described for fat graft session no. 1. ASC enriched fat grafting will be allocated to the same breast in each session, while keeping the surgeons and researchers blinded, by using the same allocation for each patient in all fat grafting sessions.

- MRI and clinical evaluation (0-14 days before 3rd procedure)

- 3rd fat grafting procedure

After a minimum of 8 weeks after the 2nd fat grafting session, the patients will be offered a final fat grafting session if deemed necessary for one or both breasts. A copy of the allocation will be kept in a sealed envelope as used in session 1&2 and it will be opened as described in session 1. Hereby, it is ensured that the breast that received ASC enrichment in session 1&2 will be the one receiving a third ASC enriched fat graft while keeping the surgeons and researchers blinded.

- Subsequent fat grafting procedures will be performed with 8-16 weeks intervals as described above. This will continue per protocol until the point of complete reconstruction (POCR) is achieved. POCR is defined below.

- <u>Follow-up</u> from point of complete reconstruction
    - MRI 6 months from POCR

- MRI 1 year from POCR
- MRI 2 year from POCR

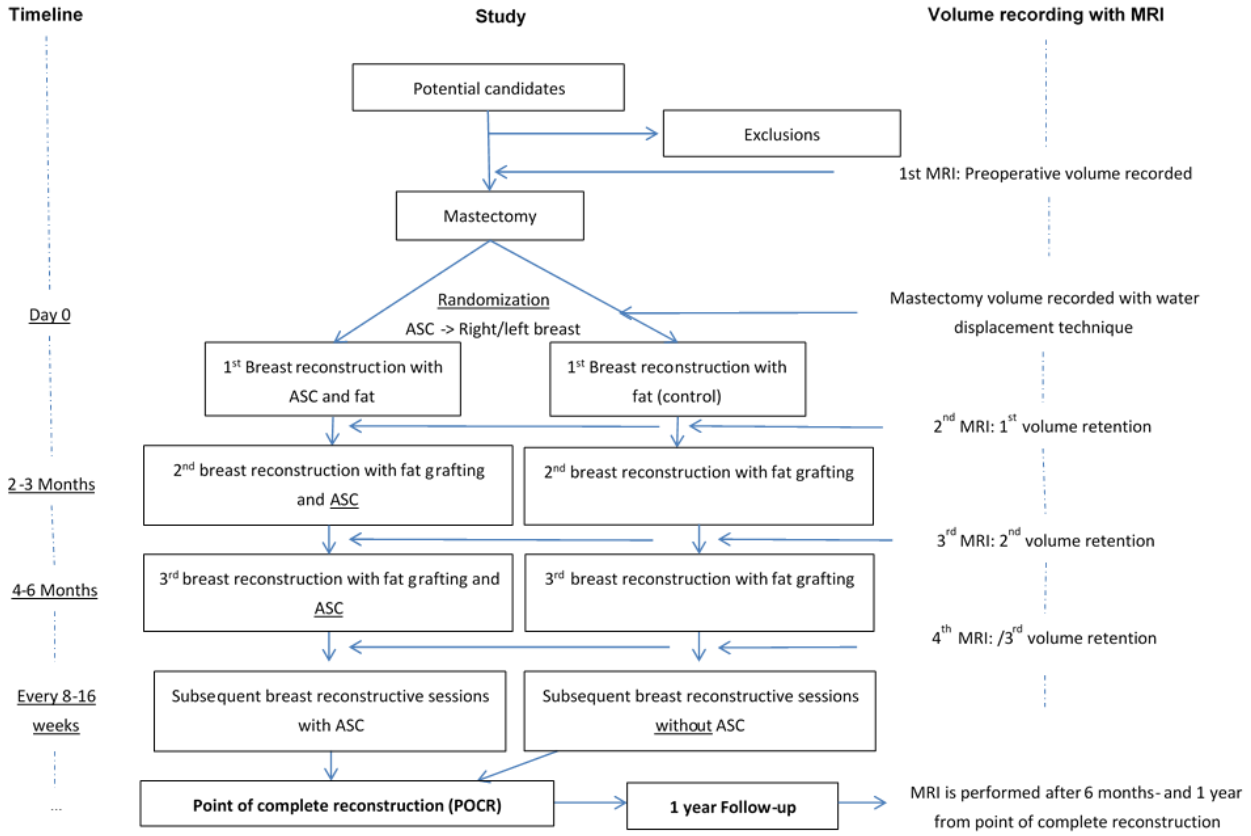


Figure 1: flow-chart illustrating the study timeline and MRI.

Point of Complete Reconstruction (POCR)

At the first visit between the patient and the reconstructive plastic surgeon, the POCR will be agreed upon. This will be done by the plastic surgeon explaining what can be achieved by normal fat grafting with the aid of cup size estimation with transparent plastic cups used in the clinic.[90] POCR will be defined as the achievement of the estimated volume (+/- 15%) and symmetry (+/- 10%).

If more than three treatment sessions are needed to achieve POCR

The treatment sessions will be with stem cell enrichment to one side. If only one breast needs additional treatment sessions after a number of treatment sessions, it will not be known whether the breast needing additional volume is the one allocated to stem cell enriched fat grafting or not. If the breast needing additional treatment is the breast allocated to fat grafting alone, there will be no need for stem cell enrichment and therefore no need for stem cell expansion. The blinding of the treatment at this procedure is maintained by having the keeper of the randomization sequence look at the allocation for the patient in question. Here it will be determined whether

stem cell expansion is needed. If the breast needing additional surgery is the one allocated to fat grafting without ASC enrichment, there will not be a cell expansion procedure.
At the point of surgery, the responsible person for the stem cell expansion will deliver an opaque syringe containing either ASCs or another liquid, which will not be used for transplantation (pHPL will be used, as it has similar appearance to ASC when contained in an opaque syringe). The mixing of fat and stem cells will be performed by trained nurses, and the surgeons and trial group members will be blinded by a surgical drape between them and the mixing table. The nurses will receive an envelope with the allocation sequence as described previously. If the allocation dictates ASCs for the breast that is about to receive fat grafting the nurses will use the ASC contained in the syringe for enrichment. If the allocation dictates ASCs for the breast that is not about to undergo surgery, the syringe will be discarded and a different syringe containing regular fat graft will be used for "mock" mixing.

Revision surgery

Other (surgical) procedures than volume replacement performed on reconstructed breasts such as nipple reconstruction, breast lift or use of implant will not be performed before the end of the primary study period.

Statistical analysis

Differences in the primary endpoint will be assessed by a paired t-test.
The number of treatments needed for complete reconstruction and graft retention rate within sessions will be analysed simultaneously by a joint longitudinal model. Specifically graft retention rates will be modelled by a mixed linear model stratifying on session number and "breast" and "person" as random effects. Moreover, the number of sessions needed to complete the reconstruction will be modelled using a shared frailty grouped-data event time models including "person" as a shared frailty. Changes in radiological markers within the patient (i.e. between breasts) will be assessed by paired t-tests.
The impact of potential dropout will be assessed by sensitivity analyses based on various inverse probability weighting schemes.
All statistical analyses will be made in R (www.r-project.org) using the add on packages survival and multcomp. Adjustment for multiple testing will be done by means of the single step procedure available in the R-package multcomp. P-values will be evaluated at a 5% significance level.

Ethical considerations

The protocol is approved by the Danish National Committee on Health Research Ethics, the Danish Data Protection Agency and the Danish Medicines Agency.
Each test subject must provide written informed consent before they can be included in the study. Information obtained on the subject's health, other purely private matters and confidential information is covered by professional secrecy. Project data will be kept securely locked during the study period.

Risk assessment

Fat transplantation is an established treatment in the field of plastic surgery and is increasingly used for reconstruction after breast cancer surgery[15,16,58]. Fat transplantation to the breast, which includes liposuction of donor sites and injection of the fat to a recipient site, has a low risk of complications (less than 4%) such as infection, bleeding, minor pain, irregularities and loose skin.[15,34]

Fat transplantation can cause non-malignant radiological changes in the breast, which have been reported in 17 studies with an overall occurrence of 13%.[91] The radiological changes are mainly small cysts or calcifications that occur as a result of fat necrosis after conventional fat transplantation.[34] These changes can be differentiated from malignant changes by experienced radiologists and previous studies of fat grafting for breast reconstruction has not shown any increase in the risk of cancer[50,92–97].

Ex vivo expanded ASCs have not been shown to undergo malignant transformation when they are utilized at early passages. The tumorigenesis of ASCs and MSCs has been investigated in several studies, which have not shown any increased tumor formation.[98–100] However one in vitro study has shown that certain breast cancer cell lines increase their migratory properties in coculture with ASCs although the ASCs had no effect on tumour cell proliferation or invasive properties.[101] Another study has shown that ASCs in co-culture with squamous cell carcinoma cell lines increased the proliferation rate of the cancer cells.[102]

Several experimental studies with immunodeficient mice and in vitro studies have been conducted to investigate a wide range of cancer cells that were co-transplanted or stimulated with human MSCs. One study showed that MSCs inhibit the growth of some breast cancer cell lines (MCF-7).[103] Another study showed that MSCs are stimulatory to the HMT-3522 T4-2 breast cancer cell line.[104] Human trials of ex vivo expanded MSCs/ASCs have been performed with a combined population of more than 1000 patients, and none of these trials have reported serious adverse events in response to the cell treatment.[33,105–108] Studies on the effect of ASCs on locoregional recurrence or distant metastasis of breast cancer after breast reconstruction have yet to be conducted. However, two large cohort studies with matched controls did not show an increase in recurrence or a decrease in survival in patients treated with fat grafting.[109,110]

The patients in this trial will be monitored by the screening program at the University Hospital of Copenhagen for patients who are genetically predisposed to breast cancer and who have received bilateral prophylactic nipple-sparing mastectomy.

The patients will receive ASC-enriched fat grafting in only one breast and conventional fat grafting in the contralateral breast. Therefore, the potential beneficial effect of ASCs in volume retention will lead to an asymmetric breast volume in the patients.

Any participant who shows breast asymmetry after follow-up will be offered additional conventional fat transplantation procedures to the breast with a lower volume to achieve symmetry. Patients with an asymmetry of less than 10% between the breasts may not be offered

correctional surgery unless the surgeon assess that an attempt to correct the asymmetry has a good chance of benefitting the aesthetic outcome.

We have no reason to believe that the patients treated in this study will have to endure more procedures or more side effects than patients who are treated with standard fat grafting for breast reconstruction. The participants will benefit from any positive effect in the fat resorption rate in one breast (either the right or the left) caused by the addition of ASCs, resulting in a decreased need for fat harvesting from donor sites, which are often in limited supply in these types of reconstructions. It is expected that both breasts (the breast treated with conventional fat grafting and the breast treated with fat and ASCs) will require two or more treatments before the reconstruction is completed.[58]

Discussion of the protocol in the context of the current evidence

To the best of my knowledge, this study will be the first to evaluate the efficacy of ASC-enriched fat grafting for breast reconstruction after mastectomy. The main strength of the study is the paired study design in which the included patients who undergo a bilateral prophylactic nipple-sparing mastectomy are reconstructed with ASC-enriched fat grafting on one side and placebo-enriched fat grafting on the contralateral side. The paired design balances most factors that influence fat graft retention to isolate the effect of ASC-enrichment. Both breasts are affected equally by physiological factors within the patient (i.e., weight change, smoking or comorbidities), and therefore, the differences in fat graft resorption between the individual patient's breasts can most likely be attributed to ASC-enrichment. Furthermore, the study provides the highest quality evidence because it is randomized, blinded and uses a validated and objective measurement technique.

The effect of ASC/SVF-enrichment on fat graft volume retention has been investigated in several reviews of both preclinical and clinical evidence, and generally they conclude that while cell-enrichment has a great potential, evidence is still lacking.[111–113] Most previous studies of cell enrichment have focused on patients undergoing breast augmentation[32] or for facial contouring[111]. The first study to investigate the retention rate of ASC-enriched fat grafts in a mouse model was Matsumoto et al.[114] Next, Yoshimura et al. were the first to investigate "cell-assisted lipotransfer (CAL)" in a clinical cohort study where they included patients to undergo SVF-enriched fat grafting to the breast.[115] However, this study did not included a control group. Controlled clinical studies of CAL were later conducted by Peltoniemi[32], Gentile[29], Tissiani[30] and Chiu[33]. Of these four studies, two[29,30] found a significant effect of the SVF enrichment but the other two studies[32,33] did not. However, none of the four studies were randomized or blinded, making them susceptible to bias. Furthermore, none of the four studies used a validated measurement technique to measure the fat graft volume retention. The problem with using a method that is not validated and standardized is especially important in studies with no control group.[2] The studies without control groups are very difficult to compare with other studies if they do not use identical methods for measuring the fat graft volume retention and even if they use the same method, there is a risk of interobserver variation that may bias the comparison. These limitations in the study designs of the previous studies could explain the heterogeneity of some previous results.

Few studies have investigated the effect of ex-vivo expanded ASCs for enrichment of fat grafts which can be used to achieve higher concentrations of ASCs per mL fat. The first published study of ex-vivo expanded ASCs for fat grafting was conducted by Koh for Parry-Romberg disease in which ASC-enriched fat grafting was used to correct contour deformities in the face. The second study to investigate ASC-enriched fat grafting was conducted by Kølle et al., which is the first trial investigating ASC-enriched fat grafting by our group. Both aforementioned studies[8] showed a significant higher retention rate in the ASCs-enriched fat grafts than in the control fat grafts.

The main difference between expanded ASC-enrichment and SVF enrichment is that the SVF is a heterogenous cell population that contain ASCs, whereas expanded ASCs comprise a homogenous cell population that has the potential to provide substantially higher ASC dosages. In a study of bolus fat grafts in a porcine model led by Bo Sonnich Rasmussen from our research group, we investigated the dose-response of ASC-enriched fat grafting. The study indicated that the optimal concentration of ASCs was 10 x 10^6 ASCs per mL of fat[116], which is lower than the concentration used in the first RCT conducted by Kølle el al., in which a dose of 20 x 10^6 ASCs per mL fat graft was used. The study found a significantly higher retention rate in the cell-enriched groups compared with the control group (13.0% vs. 9.2%), but the magnitude of the difference was substantially lower than what was found in the study by Kølle et al.[8] (80.9% vs. 16.3%). Interestingly, the study by Rasmussen et al.[116] did not show a significantly difference between SVF-enriched fat grafts and ASC-enriched fat grafts in terms of fat graft retention rate. In the study by Glowinski et al.[9] (EudraCT 2014-000510-59) we used the 10 x 10^6 ASCs per mL of fat concentration for the breast augmentations of healthy female patients with breast hypotrophy. The latter trial was to be concluded before we initiated the third RCT (EudraCT 2016-005186-31) of ASC-enriched fat grafting for breast reconstruction following skin-sparing mastectomy, which is described in study 5.

<u>Preliminary results from a randomized controlled trial of breast augmentation with ASC-enriched fat grafting</u>

The randomized controlled trial (RCT) of ASC-enriched fat grafting for breast augmentation in women with breast hypotrophy (EudraCT 2014-000510-59) which was conducted by our group and led by Peter Vester-Glowinski as the Principal Investigator was recently finalized. The results were presented at the American society of plastic surgery (ASPS) conference Plastic Surgery "The Meeting"[9] and the main paper is in the final stages before submission to a peer-reviewed journal. In this section, I will briefly summarize the trial and its results because it is important in the context of this PhD thesis and in the discussion that follows in the next section.

Ten healthy women with breast hypotrophy were included in the trial. First, the women underwent a small liposuction of approximately 200 cc of fat after local analgesia. This fat was used to isolate the stromal vascular fraction (SVF), which was used to culture-expand ASCs. The ASCs were harvested after 17 days of culture expansion with a minimum yield of 3 x 10^9 ASCs. These cells were used to perform breast augmentation with ex vivo expanded, autologous ASCs to one of the breasts of each patient; the contralateral breast was augmented with normal fat grafting. The ASC-enrichment dose was 10 x 10^6 ASCs per mL of fat graft. The placebo consisted of a non-enriched fat graft that was mixed with the main graft using a mixing procedure identical to that used for the ASC-enriched graft. All the syringes with fat w/o ASCs were prepared prior to draping to cover the view of the operating table with the graft syringes. Then, the envelope containing the allocation was opened behind a drape, and two nurses handed the

syringes to the surgeon according to the allocation. The surgeons were blinded to which breast would be treated with ASC-enriched fat grafts.
The women were examined via MRI performed the day before surgery and 4 months and 1 year after the surgery. These scans were used to measure fat graft volume retention over time using the Lipovol software[72] (described in study 4). Three data assessors blinded to the treatment allocation performed the fat graft volume retention measurements in parallel.
The 10 women underwent breast augmentation with an average fat graft volume of 310 mL per breast (range 300-350 mL). All women received the same stem cell or placebo volume in both breasts. After 4 months, the average fat graft volume retention rate was 54.3% in the breasts treated with ASC-enriched fat and 56.2% in the breasts treated with normal fat grafting. After 1 year, the average fat graft volume retention was 54.0% in the ASC-enriched breasts compared with 55.9% in the breasts treated with normal fat grafting. The difference in fat graft volume retention at 1 year was 1.9% lower in breasts treated with ASC-enriched fat grafting compared with those treated with non-enriched fat grafts, and this difference was not statistically significant (95% CI, -9.11% to 5.31%).[9]

<u>Differences between the first two RCTs and implications for the protocol described in study 5</u>
The preliminary results from the RCT of ASC-enriched fat grafting for breast augmentation (EudraCT 2014-000510-59) led us to postpone initiating the RCT that would investigate ASC-enriched fat grafting for breast reconstruction (EudraCT 2016-005186-31). These two RCTs use the same protocol for isolation and expansion of autologous ASCs and a very similar method of delivering ASCs to the patient. The most important difference between the two trials is the recipient site; the former trial used ASC-enriched fat grafting for breast augmentation, and the next trial will use ASC-enriched fat grafting for breast reconstruction following a prophylactic mastectomy.
Before initiating a trial of ASC-enriched fat grafting for breast reconstruction (EudraCT 2016-005186-31), we need to explore the differences between the RCT of bolus-injected fat grafts to the upper arm (EudraCT 2010-023006-12) and the RCT of ASC-enriched fat grafting for breast augmentation (EudraCT 2014-000510-59) (See the illustration of the trials of ASC-enriched fat grafting in our group in the background section). In the first trial (EudraCT 2010-023006-12), which was published in 2013[8], the authors found a significantly higher retention rate in the ASC-enriched fat graft group compared with the non-enriched graft group (80.9% vs 16.3%). In the second trial (EudraCT 2014-000510-59) which was presented in 2019[9], we did not find any effect of ASC-enrichment (54.0 % retention rate in the breasts treated with cell-enriched fat grafting (n = 10) and 55.9 % in the contralateral breasts treated with normal fat grafting and placebo (n = 10)).
The two first trials (EudraCT 2010-023006-12 and EudraCT 2014-000510-59) relied on similar principles for the ASC-enrichment, but there were differences between the two trials that need to be explored. In the trial of ASC-enrichment for breast augmentation, we used 8 pcs. of CellStack-10 (Corning Life Science, New York, USA) with a combined surface area of 50,880 cm^2

per breast, whereas in the first trial of ASC-enriched fat grafting to the upper arm 10 pcs. of T1000 cell flasks (Nunc A/S, Roskilde, Denmark) with a combined surface area of 10,000 cm^2 were used. Second, the dose varied between the two trials, with the first trial[8] using 20 x 10^6 ASCs/mL fat graft for a total dose of 0.6 x 10^9 ASCs per upper arm and the second trial[9] using 10 x 10^6 ASCs/mL fat graft for a total dose of 3.0 X 10^9 ASCs per breast. Third, the first trial used a bolus injection of the fat graft into the upper arm as the recipient site and the second trial used structural fat grafting to the breast as the recipient site.

Research plan for further studies before initiation of the final trial

The methodological differences between the first two trials conducted in our group have guided the research plan for how to explore the discrepancy in results.

We need a thorough analysis of the cellular characteristics of the ASCs expanded with the protocol that was used in the first trial (EudraCT 2010-023006-12) compared with an analysis of the cell characteristics of the ASCs from the second trial (EudraCT 2014-000510-59). The cells in the two first trials fulfilled the criteria for mesenchymal stem cells according to Bourin et al.[86], confirmed by surface marker analysis. In addition, the protocols for ASC-expansion used in the two trials have both been shown to produce cells that can differentiate into adipocytes, chondrocytes and osteocytes.[8,9,87,117] However, further analyses may show differences in the cell product that might explain the difference in results between the two first trials. One could hypothesize that ASCs, although viable, can be inactivated so that they do not support fat graft retention. In the second trial of ASC-enriched fat grafting for breast augmentation (EudraCT 2014-000510-59), we obtained reference samples from all included patients, which are stored in a biobank. These samples can be compared with cells expanded according to the protocol used in the first trial (that unfortunately does not have reference samples available) to investigate differences in cell characteristics (e.g., transcriptome analysis, Cell differentiation assays and in vitro proliferation potential). Second, further animal studies could be used to investigate the effect of ASCs in bolus grafts versus structural fat grafts. We have developed a porcine model in our group that could be used for this purpose.[118]

Conclusion

This thesis consists of four studies that share a common foundation in that they are all concerned with the evaluation of fat grafts in humans. The first two studies investigated the resorption dynamics of fat grafts; and study 3 and 4 evaluate methods to measure fat graft volume retention in the breast. Furthermore, the four studies are all necessary supporting studies in the design of a protocol for a randomized controlled trial of ASC-enriched fat grafting for breast reconstruction, which is described in study 5.

In study 1 of this thesis[1], the co-authors and I demonstrated that the time to steady state-graft retention of excised fat grafts in the mastoid cavity is 2.2 years on average according to our model. This highlights the need for a long follow-up period when studying fat graft volume retention. In

study 2, we modelled the number of fat grafting sessions needed to complete a breast reconstruction in five groups of patients that have undergone surgery for breast cancer. Our model estimated that approximately three sessions are needed to complete a breast reconstruction following a non-irradiated skin-sparring mastectomy. We also found that significantly more fat grafting sessions are needed to reconstruct an irradiated breast than a non-irradiated breast. In study 3, we evaluated the most commonly used MRI-based method for measuring fat graft volume retention in the breast and found it to overestimate changes in breast volume by approximately 50%. This has important implications for the ability of the method to measure fat graft volume retention and for previous studies that have used this method. In study 4, the co-authors and I presented, and validated, an automated software tool for measuring fat graft volume retention in the breast with high accuracy and precision. The new method can be used in future studies of fat grafting to the breast. The results and the scientific experience gained from the first four studies of this thesis were used to design a randomized controlled trial of ASC-enriched fat grafting to the breast which has been approved by the Danish Medicines Agency and the National Committee on Health Research Ethics.

Recently, our group published the preliminary results of another randomized controlled trial of ASC-enriched fat grafting for breast augmentation (EudraCT 2014-000510-59) which did not find a significantly higher fat graft volume retention in breasts treated with ASC-enriched fat grafting than in breasts treated with normal fat grafting. Based on these results, the study group behind the next RCT of ASC-enriched fat grafting for breast reconstruction following a skin-sparing mastectomy (EudraCT 2016-005186-31) has postponed the trial initiation. Further studies are needed to investigate the optimal procedure for isolation of SVF, expansion of ASCs, ensuring that the ASCs have the appropriate characteristics to support fat graft volume retention before delivery to patients. The research plan for these studies are outlined in this thesis although the plan is not yet final. The heterogeneous results from studies of ASC-enriched fat grafting highlights the need for more exploration into the exact mechanism by which ASCs may support fat graft volume retention in vivo. Nevertheless, methods to increase the volume retention of fat grafts are still warranted. The studies presented in this thesis has contributed to our understanding of the resorption dynamics of fat grafts. Furthermore, the studies of methods to measure fat graft volume retention in the breast, including the one that I presented in study 4, can be used to ensure reproducible results, that can be compared between research groups in future studies. We expect that the studies described in the research plan will provide us with the necessary foundation to initiate the third RCT of ASC-enriched fat grafting for breast reconstruction. We hope that this final study will lead us to our goal, which is the ability to offer our patients in need of breast reconstruction a better treatment in the future.

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Appendix 1

Quantifying Long-Term Retention of Excised Fat Grafts: A Longitudinal, Retrospective Cohort Study of 108 Patients Followed for up to 8.4 Years

Quantifying Long-Term Retention of Excised Fat Grafts: A Longitudinal, Retrospective Cohort Study of 108 Patients Followed for Up to 8.4 Years

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Background: Predicting the degree of fat graft retention is essential when planning reconstruction or augmentation with free fat grafting. Most surgeons observe volume loss over time after fat grafting; however, the portion lost to resorption after surgery is still poorly defined, and the time to reach steady state is unknown.
Methods: The authors compiled a retrospective, longitudinal cohort of patients with vestibular schwannoma who had undergone ablative surgery and reconstruction with excised fat between the years 2006 and 2015. Fat volume retention was quantified by computed tomography and magnetic resonance imaging and used to model a graft retention trajectory and determine the volumetric steady state. In addition, the authors evaluated the association between graft retention and secondary characteristics, such as sex and transplant volume.
Results: A total of 108 patients were included. The average baseline graft volume was 18.1 ± 4.8 ml. The average time to reach steady state was 806 days after transplantation. By this time, the average fat graft retention was 50.6 percent (95 percent CI, 46.4 to 54.7 percent). No statistically significant association was found between baseline graft volume and retention. Fat graft retention over time was significantly higher in men than in women (57.7 percent versus 44.5 percent; $p < 0.001$).
Conclusions: The authors' data provide evidence that the time to reach fat graft volumetric steady state is considerably longer than previously expected. Fat grafts continue to shrink long after the initial hypoxia-induced tissue necrosis has been cleared, thus indicating that factors other than blood supply may be more influential for fat graft retention. (*Plast. Reconstr. Surg.* 139: 1223, 2017.)
CLINICAL QUESTION/LEVEL OF EVIDENCE: Therapeutic, IV.

Fat grafting is a very commonly used tool for correcting soft-tissue defects in many surgical specialties.[1] Few studies have quantified long-term fat graft retention, and the results of those studies are inconsistent.[2] This inconsistency may be because of limited access to high-quality imaging, such as computed tomography or magnetic resonance imaging, and the logistic challenges associated with long-term follow-up. The majority of studies quantifying fat graft retention have been conducted in animals with a short follow-up of 3 to 12 months and a limited population size.[3–10] Few human studies have used objective measures to quantify the volume retention of fat grafts, and

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to the best of our knowledge, none of these have had a follow-up period longer than 1 year.[11–14] Published studies with a follow-up period longer than 1 year have relied on a surgeon's assessment/experience and did not quantify graft volume.[15–17]

The ability to predict the long-term outcome of reconstructive procedures, such as fat grafting, is important to ensure the desired aesthetic outcome. To improve knowledge regarding the development of free fat grafts over time, we quantified long-term graft volume retention in a retrospective longitudinal cohort study of patients treated with excised fat to fill dead space in the mastoid cavity after ablative surgery for vestibular schwannoma.

Approximately 50 percent of vestibular schwannomas are removed by means of the translabyrinthine approach[18] at our institution. This approach starts with a retroauricular incision followed by a mastoidectomy. Thereafter, the dura is exposed and can be opened. The tumor is found on the inner side of the dura and is removed by dissection. After removal of the tumor, an excised fat graft from the lower abdominal area is used to fill the dead space left by the mastoidectomy (Fig. 1).[19] The role of the fat graft is to prevent cerebrospinal fluid leakage and to avert a large hollowing behind the patient's ear.[20]

After surgery, the patients are offered postoperative computed tomography and two follow-up magnetic resonance imaging scans during the first year, followed by one yearly magnetic resonance imaging scan to detect recurrence. This large collection of imaging material had captured the destiny of fat grafts over time, and we saw this as a unique opportunity to document long-term outcomes of excised fat grafts.

PATIENTS AND METHODS

Study Design

In a retrospective longitudinal cohort study, fat graft retention was quantified over time by analysis of computed tomographic and magnetic resonance imaging scans from 108 patients. The data were used to model long-term fat graft retention trajectory and to estimate the time to steady state in graft volume.

The data were obtained from patients who had undergone reconstruction with excised fat to fill dead space in the mastoid cavity after ablative surgery for vestibular schwannoma. The collection of data without consent was approved by the Danish Health and Medicines Authority. The protocol for handling patient data was approved by the Regional Danish Data Protection Agency of Copenhagen.

Study Population

The cohort consisted of patients treated surgically for vestibular schwannoma with a translabyrinthine approach and who underwent reconstruction

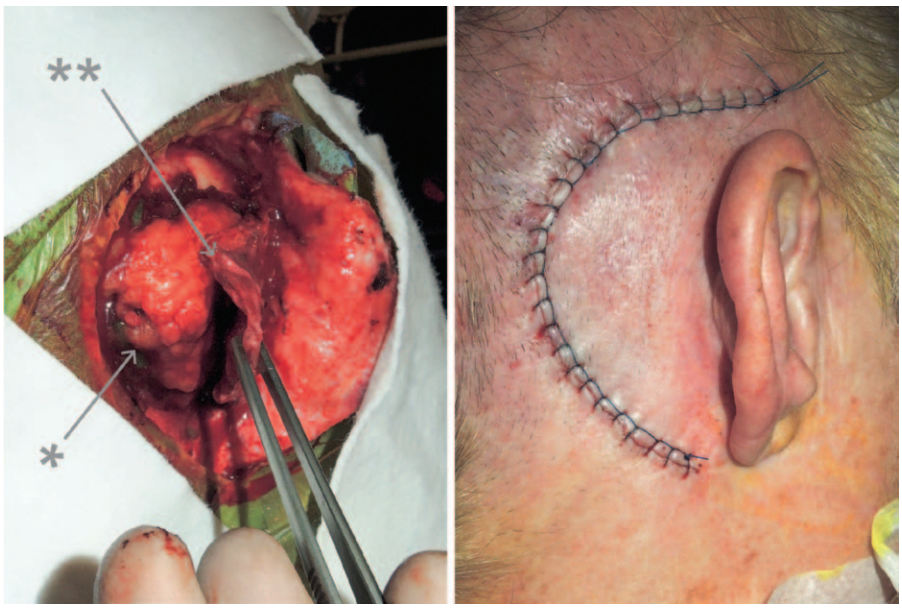

Fig. 1. A 74-year-old woman. (*Left*) After removal of vestibular schwannoma and placement of fat graft. The fat graft is marked with an *asterisk*; the pericranial flap used to cover the fat graft is marked with *double asterisks*. (*Right*) Same angle as seen on the left after closure. The location of the incision behind the ear is shown without the draping.

with excised fat grafting between October of 2006 and September of 2015 at the Copenhagen University Hospital. Patients were excluded if they had undergone a secondary operation or radiotherapy in the area of fat grafting or if the first follow-up scan was obtained more than 14 days after surgery. Furthermore, patients were excluded if visualization of the entire fat graft was prevented by technical issues, such as scans that showed only part of the transplant. The observation started with the first scan and ended with the last follow-up scan.

Neurosurgical treatment of vestibular schwannoma in Denmark is performed solely at Copenhagen University Hospital, Rigshospitalet. Thus, the included patient population originates from the entire Danish population (5.6 million inhabitants).[21] Vestibular schwannoma is a benign, slow-growing, and generally nonhereditary tumor originating from the Schwann cells of the vestibular nerve or, more rarely, the acoustic nerve.[22–24] Previous reports on Western patients with vestibular schwannoma state that the demographics of these patients are similar to those of the background population in age and distribution of the sexes.[18,22] Vestibular schwannoma is not related to any significant risk factors or comorbidities, and microscopic free-margin surgery (single treatment) is typically curative, with a 0.05 percent recurrence rate.[25,26]

Primary Outcome

The primary variables of interest were volume retention of fat grafts over time and the time to reach volumetric steady state. Volume retention was measured by analysis of computed tomographic and magnetic resonance imaging scans obtained after transplantation. The baseline fat graft volume was measured from the postoperative computed tomographic scan obtained within the first few days after ablative surgery to exclude postoperative bleeding. The baseline volume was set to 100 percent and served as the comparison for all subsequent examinations. The volumes recorded from subsequent follow-up magnetic resonance imaging scans were calculated as a percentage of the baseline volume and plotted against the number of days after surgery. These data were used to model a fat graft retention trajectory. Steady state was defined as a graft resorption rate of less than 5 percent of the original volume per year.

Covariates and Secondary Outcomes

To investigate the association between covariates and fat graft retention, the patients were divided into high- and low-baseline-volume groups, with a cutoff volume of 16 ml. This value was chosen by the authors before reviewing the follow-up data, to avoid post hoc analysis bias. The patients were also stratified by sex.

To give an impression of the available blood supply to the investigated grafts, we measured the distance from the nearest edge of the recipient bed to the graft center in all patients at time 0. Also, the recipient bed was evaluated by calculation of the distribution of tissues comprising the graft boundary in 10 randomly selected scans at time 0. Finally, the histologic composition of the investigated fat grafts was assessed by collecting biopsy specimens from patients undergoing revision surgery because of bleeding (early cases) or recurrence (late cases) from July of 2015 to May of 2016 and by radiologic analysis of magnetic resonance imaging sequences that enabled characterization of the tissue composition.[27,28]

Measuring Fat Graft Volume by Computed Tomography and Magnetic Resonance Imaging

The fat transplant volumes were calculated by plotting a region of interest around the fat graft on all slices containing the fat graft as shown in Figure 2. The area was multiplied by slice thickness, and all slices containing the fat transplant were summed to determine the graft volume. The described technique has been demonstrated to be highly accurate and precise.[29–32]

Pathologic Analysis of Graft Composition

Biopsy specimens were collected from patients undergoing revision surgery between October of 2015 and October of 2016 to evaluate the histologic composition of fat grafts after transplantation. The biopsy specimens were formalin-fixed and paraffin-embedded. They were then cut into 4-μm-thick sections and stained with hematoxylin and eosin. To validate the conclusions of the pathologic analysis, a subset of slices were stained immunohistochemically for CD68 PGM1 (macrophages), with van Gieson for connective tissue and S-100 for fat tissue.

Statistical Analysis

Fat graft retention over time was modeled with a four-parameter log logistic dose-response curve.[33] Evidence of significant resorption, total resorption over time, and the time to steady state (defined as a yearly resorption less than 5 percent of original volume) were evaluated with maximum-likelihood methods. Confidence intervals were obtained with a parametric bootstrap

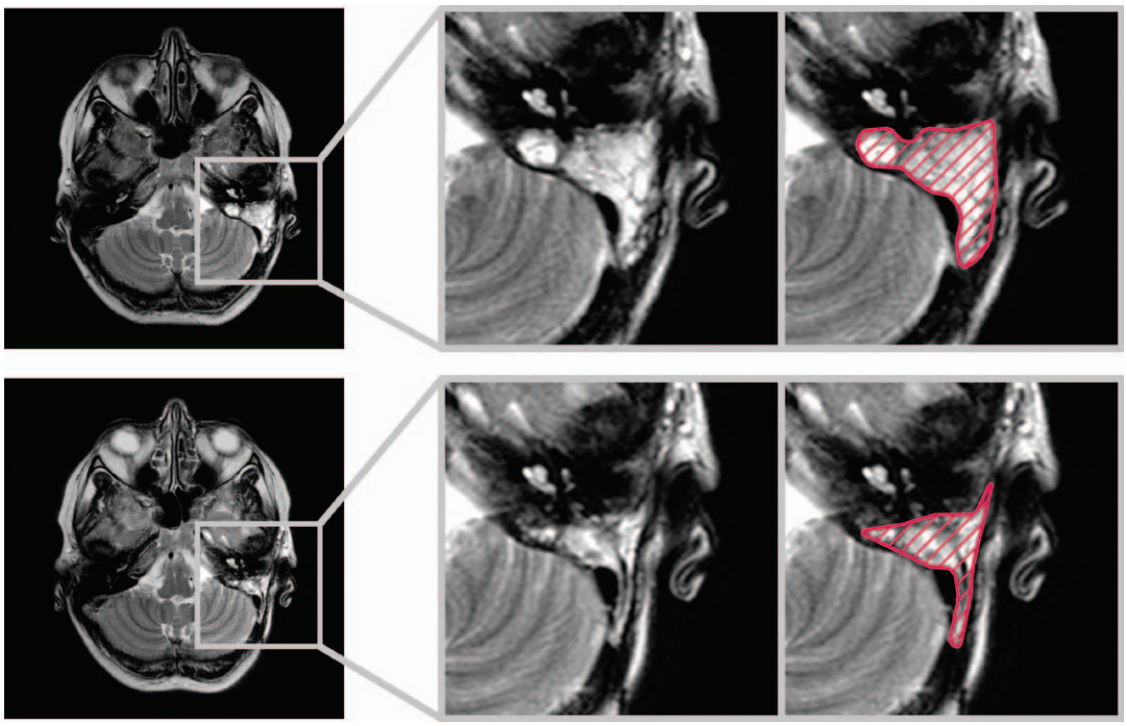

Fig. 2. A 48-year-old woman. (*Above*) Second scan, magnetic resonance imaging, 128 days after surgery. A region of interest is drawn around the fat transplant, and the volume is calculated to be 85.9 percent of the original volume. (*Below*) Third scan; a region of interest is drawn around the same fat transplant at day 863, at which point graft retention was 44.8 percent of the original volume.

analysis based on 5000 bootstrap samples; p values were evaluated at the 5 percent significance level.

The fit of the model was assessed by an accumulated residual-type goodness-of-fit test. The normality of the residuals was assessed graphically with a quantile-quantile plot and numerically with the Kolmogorov-Smirnov test for normality.

All analyses were performed in R (www.r-project.org) using the add-on package drc. The original technical biostatistics report can be found in the Supplemental Digital Content. (**See Document, Supplemental Digital Content 1**, which shows the technical biostatistics report describing the statistical analysis and the results, ***http://links.lww.com/PRS/C143***.)

RESULTS

Study Cohort

A total of 208 patients had undergone fat graft implantation after vestibular schwannoma ablative surgery between October of 2006 and September of 2015 at our hospital. After exclusion, 108 patients (51.9 percent) were included in the longitudinal cohort as shown in the flow diagram in Figure 3.

Overall, 324 imaging sequences distributed across 108 patients were examined (average number of imaging sequences per patient, 3.0; 95 percent CI, 2.9 to 3.1 scans; range, two to six scans). The average age on the day of surgery was 47 years (95 percent CI, 44.7 to 49.7 years; range, 26 to 85 years). The average observational period was 2.7 years (95 percent CI, 2.3 to 3.1 years; range, 17 days to 8.4 years). The graft volume did not reach total resorption during follow-up for any patients. Patient characteristics and study variables are listed in Table 1.

Primary Outcome

The average original fat graft volume was 18.1 ml (95 percent CI, 17.2 to 19 ml). Evidence of resorption over time was significant ($p < 0.0001$). The fat graft retention trajectory was modeled using 324 examinations (Fig. 4). Steady state in volume was estimated to be after 806 days (95 percent CI, 645 to 956 days). At that point in time, fat graft retention was estimated to be 50.6 percent (95 percent CI, 46.4 to 54.7 percent).

Association between Baseline Graft Volume and Graft Retention Rate

The patients were stratified according to baseline graft volume. The mean volume in the low-baseline-volume group ($n = 40$) was 13.6 ml (95 percent CI, 13 to 14.3 ml), and the mean volume

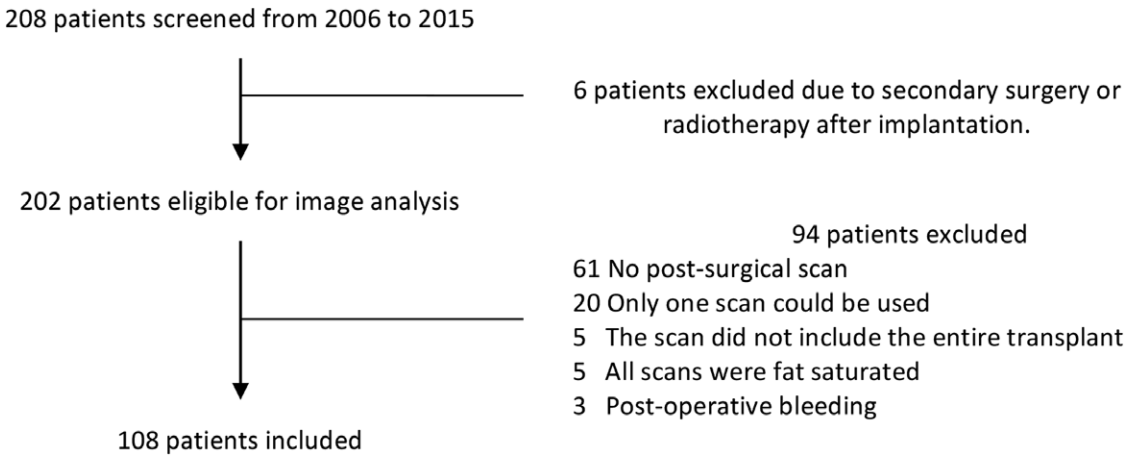

Fig. 3. Flow diagram of patient inclusion.

Table 1. Patient Characteristics and Study Variables (108 Patients)

Characteristic	Value (%)
Patients	108 (100)
Male	49 (45.4)
Female	59 (54.6)
Age at surgery, yr	
Mean	47
95% CI	44.7–49.7
Follow-up, yr	
Mean	2.7
Range	17 days–8.4 yr
95% CI	2.2–3.1
Image series, total	324
Image series per patient	
Mean	3.0
Range	2–6
95% CI	2.9–3.1
Patients with 2- to 3-image series	74 (68.5)
Patients with 4- to 5-image series	33 (30.5)
Patients with 6-image series	1 (1)
Baseline graft volume, ml	
Mean	18.1
95% CI	17.2–19.0
Low-volume group	
No.	40 (37)
Mean volume, ml	20.1
95% CI	19.7–21.7
High-volume group	
No.	68 (63)
Mean volume, ml	13.6
95% CI	13.0–14.3
Recipient-site composition, %	
Bone	
Mean	39
95% CI	34.8–43.2
Skin	
Mean	36
95% CI	30.9–41.8
Dura	
Mean	25
95% CI	19.2–30.1

in the high-baseline-volume group ($n = 68$) was 20.1 ml (95 percent CI, 19.7 to 21.7 ml). The fat graft retention trajectory over time was not significantly different between the two groups ($p = 0.18$).

Association between Sex and Graft Retention Rate

Fat graft retention over time was significantly higher in male patients than in female patients ($p < 0.001$). For men, steady state was estimated to be 714 days after surgery (95 percent CI, 475 to 963 days), and at that point in time, fat graft retention was estimated to be 57.7 percent (95 percent CI, 51.4 to 63.2 percent). For women, steady state was 874 days after surgery (95 percent CI, 684 to 1038 days), and at that point in time, retention was estimated to be 44.5 percent (95 percent CI, 39.5 to 50.1 percent) (Fig. 5).

Histologic Analysis

To evaluate graft composition, five specimens obtained 22 days, 1.3 years, 3.3 years, 4.4 years, and 4.6 years after the primary operation were analyzed histologically. In the four late grafts, mature fat tissue with limited amounts of fibrosis was found. Macrophages were present with fine vacuolated cytoplasm indicative of lipid digestion. Some samples had hemosiderin content as a result of prior bleeding. In the newly transplanted "young" biopsy specimen (22 days postoperatively), focal necrosis with surrounding macrophages was found. Figure 6 shows a specimen collected 22 days after transplantation versus a specimen collected 4.4 years after transplantation.

Recipient-Site Analysis

The recipient sites were evaluated by calculating the distribution of tissues comprising the boundaries by means of imaging analysis. The mean recipient site was found to consist of bone (39 percent), skin (36 percent), and dura mater (25 percent).

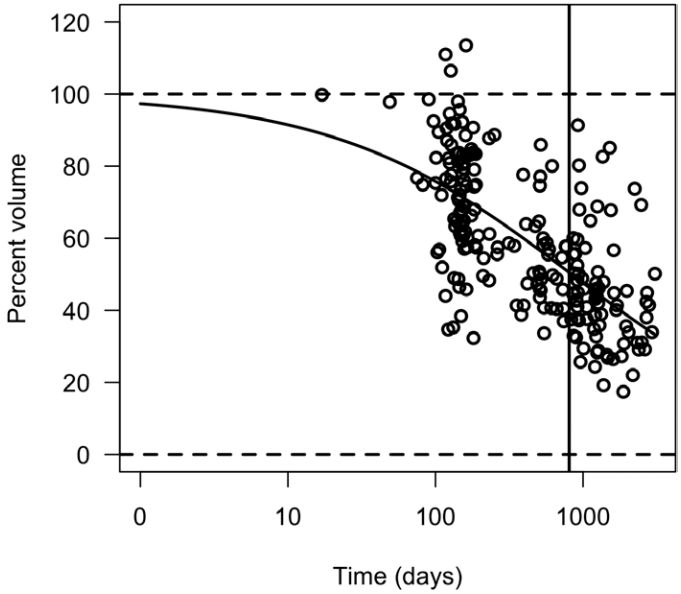

Fig. 4. Estimated and observed percentage volume as a function of time on a logarithmic scale. *Dashed lines* indicate the upper and lower plateaus of the model; *solid vertical line* shows the point of steady state (<5 percent resorption of the original volume per year).

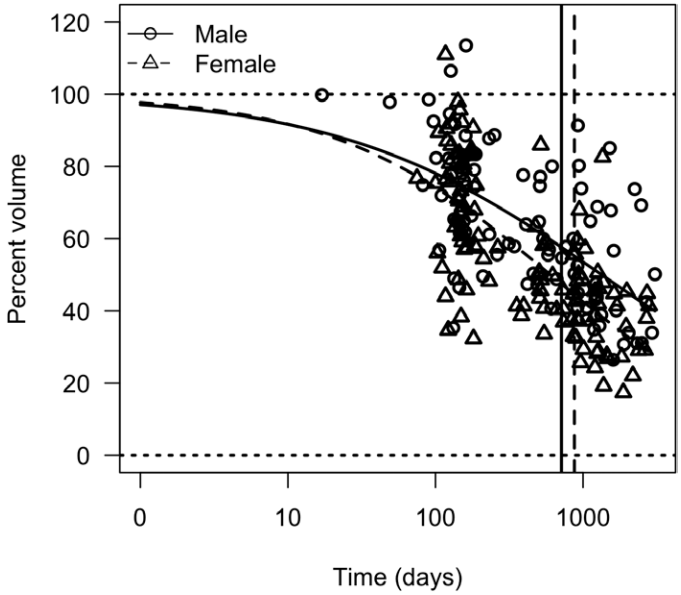

Fig. 5. Estimated and observed percentage volume retention as a function of time on a logarithmic scale stratified for sex. *Dashed lines* represent the upper and lower plateaus of the models; *vertical lines* show the point of steady state for both sexes.

Graft Center–to–Nearest Edge of Recipient Bed Distance

To give an estimate of the theoretical distance from the central adipocytes to the nearest blood supply, we measured the distance from the graft center to the nearest boundary of the recipient bed. The mean distance from recipient tissue to the graft center was 9.59 mm (95 percent CI, 9.31 to 9.87 mm) ($n = 108$).

DISCUSSION

In this longitudinal retrospective cohort study of 108 patients who were representative of the general Danish population, we determined the

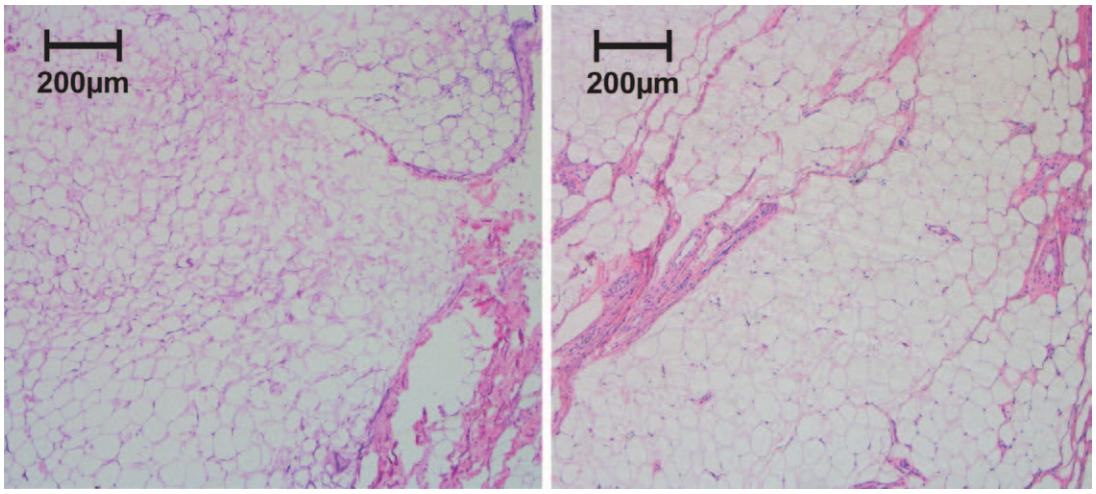

Fig. 6. Hematoxylin and eosin staining of biopsy specimens from the middle portion of the fat grafts (4-μm sections). (*Left*) Biopsy specimen of fat graft collected 22 days after transplantation. The biopsy specimen shows a focal necrosis and macrophages with vacuoles containing lipid as a sign of lipid digestion. (*Right*) Biopsy specimen of fat graft collected 4.4 years after transplantation. The biopsy specimen shows mature fat tissue with streaks of fibrosis and few mature macrophages.

first reported long-term fat-graft retention trajectory of excised fat grafts. We found that graft resorption continued 2.2 years before steady state was reached. When steady state was achieved, the fat graft volume was reduced to approximately 50 percent of the original volume. The time to steady state was much longer than expected. Most previous studies have concluded that resorption is complete within 6 months after surgery, by which time the body is thought to have removed all the dead cells, leaving a stable cell population.[34,35] It is crucial for surgeons to know the amount of time required to achieve steady state in fat graft resorption and to have a qualified prediction of total graft retention to plan appropriate overcorrection or revision.

This study features important differences in study design and analyses compared with prior studies. Fat graft resorption is a continuous process; therefore, it should be evaluated through continuous outcomes.[36] Other studies have dichotomized the resorption process by assessing graft retention at only a single point in time after transplantation. With the single-point approach, it cannot be known whether graft retention has reached the steady state. This study provides a baseline for the fat graft resorption process and may be used as a guideline for future studies on fat grafting.

Despite the high statistical power of the present study, there are potential limitations concerning the conclusions that can be drawn from the results, because of the study design. We present these possible limitations in three categories discussed below: (1) study type, (2) recipient site, and (3) fat graft type.

Limitations in Study Design

Retrospective cohort studies have an inherent risk of post hoc analysis bias, such as selection bias, whereby follow-up or inclusion/exclusion criteria are influenced by the variable of interest (e.g., fat graft retention).[37] As the present study was retrospective, we were also prevented from gathering useful information on secondary characteristics such as smoking status and body mass index, which could have had an influence on graft retention.[38]

To avoid bias in the present study, the inclusion and exclusion criteria were planned before the data were analyzed. The reasons for exclusion were related primarily to technical problems associated with evaluating fat graft volume from the follow-up imaging (n = 91). Technical issues were expected because the primary reason for follow-up was to screen for recurrence of vestibular schwannoma or postoperative bleeding and not to evaluate the fat graft retention. The technical problems associated with evaluating graft volume were random and not related to the resorption rate of the fat graft. Therefore, we expect that the study design was not a source of bias.

Influence of the Recipient Site

The graft retention rate is presumably influenced by the characteristics of the recipient area.

To assess the recipient site in the present study and how it might relate to other known recipient sites for fat grafting, we evaluated 10 random image sequences. We found that the investigated recipient site consisted of bone (39 percent), skin (36 percent), and dura (25 percent). The dura is known to be highly vascularized.[39,40] We suggest that the well-vascularized dura combined with bone and skin represents the vascularization and tissue composition of the craniofacial region in general. Fat tissue grafted to other regions, such as the breast, would have different vascularization and therefore would possibly have different graft retention.

Limitations in Fat Graft Type

Fat can be grafted in solid form (i.e., excised fat) or as lipoaspirate (lipofilling); the latter is gaining popularity because of easier extraction and transplantation by means of syringe. The present study investigated excised fat grafts; thus, whether our results apply to lipofilling demands further evaluation. Only three studies, which were conducted in animals, have quantified and compared fat graft retention in excised fat and lipofilling. Two of these studies have reported significantly higher retention rates when using excised fat compared with lipofilling,[3,5] and the third study has found no significant differences in retention rate when comparing excised fat with lipoaspirate.[4] The studies used graft volumes of 1 to 2 ml and had 6 to 12 months of follow-up.[3–5] The higher retention rate achieved when using excised fat appears to be attributable to a higher cellular viability in newly excised fat compared with lipoaspirate.[41] Recently, a study by Bourne et al.[42] has shown that fat grafting with the Coleman technique[43] leads to pooling of fat deposits along native tissue planes, as evaluated histologically in an excised pannus. Therefore, the theoretical distance to the nearest blood supply may be longer than anticipated.[42] The studies investigating difference in graft retention between the two techniques indicate that excised fat is similar or superior to lipofilling; however, there is a need for cohort studies of lipofilling with sufficient follow-up to show that steady state has occurred.

Previously, it has been proposed that excised fat tissue gradually necrotizes and is replaced by fibrosis over a 9-month period.[44] In theory, this outcome could explain the relatively high graft retention and the very lengthy resorption process observed in the present study, but it was not supported by our analyses. The histologic analysis revealed that normal mature fat tissue was the predominant component of the grafts, with only sporadically located fibrosis, many years after transplantation. Also, we were able to evaluate the graft tissue composition by means of T1-weighted and fat-saturated magnetic resonance imaging sequences.[27,28] This analysis revealed that the grafts consisted of viable fat tissue (distinguishable from oil cysts) many years after transplantation (Fig. 7). No oil cysts were found in the images.

Scientific Implications

We have studied fat graft retention of relatively low-volume grafts (mean, 18.1 ml) in humans. However, because the investigated grafts were excised, solid fat grafts, grafted into a dead space in the skull base, there was a long distance to the nearest blood supply for a large part of a given graft. The average distance from the recipient tissue to the graft center was almost 1 cm at the time of transplantation. This distance would theoretically cause a very large part of the graft to necrotize and subsequently be replaced by scar tissue according to the hypothesis of Carpaneda and Ribeiro, which suggests that fat tissue located more than 0.15 cm from a blood supply is bound to necrotize.[45] This hypothesis further suggests that an increase in baseline fat volume would markedly increase the amount of fat outside the survival zone and thereby decrease fat retention. Despite high statistical power, the present study did not support this hypothesis. We were unable to find a significant difference in fat graft retention between the low-baseline-volume group (average, 13.6 ml) and the high-baseline-volume group (average, 20.1 ml) (67.7 percent higher baseline volume). This lack of an association indicates that factors besides blood supply may play a more significant role in determining the retention rate of a fat graft.

An additional finding of this study was the significant difference in fat graft retention between men and women ($p < 0.0005$). Men had significantly higher fat graft retention (57.7 percent) than women (44.5 percent). To the best of our knowledge, this study is the first demonstration of a statistically significant association between sex and fat graft retention. We found no plausible confounder explaining this difference; rather, we found that male patients receive slightly larger transplants, which we would expect to work in favor of higher retention rates

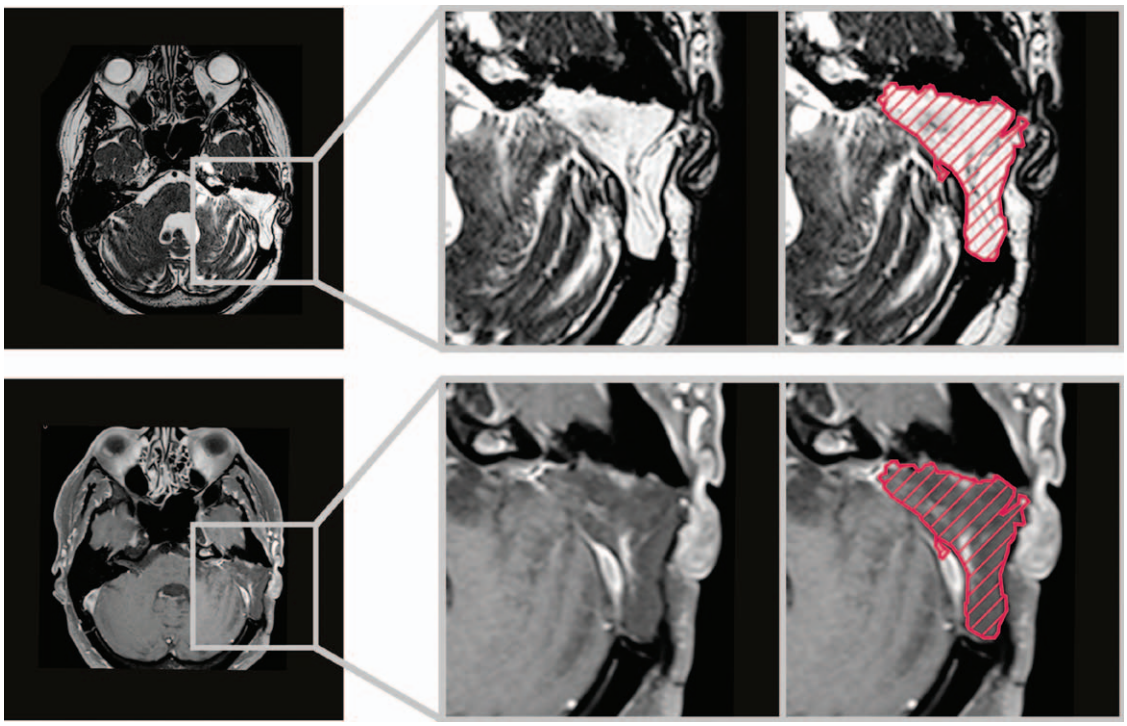

Fig. 7. T1-weighted magnetic resonance imaging scan of a 53-year-old man on the day of surgery. (*Above*) A region of interest is drawn around a fat graft at 2579 days after surgery (6.8 years) on a T1-weighted sequence. (*Below*) The same region of interest is shown on a fat-saturated sequence to verify that the original region of interest contained mostly fat tissue with a few bright streaks of scar tissue.

in the female patients. The current study is not designed to uncover the cause behind this difference in retention rate and therefore we refrain from speculations regarding this matter. Future studies may shed light on the mechanisms underlying this difference in graft retention between the sexes.

CONCLUSIONS

Our data provide evidence that the time to reach steady state for fat graft volume is much longer than previously anticipated. The fat graft retention trajectory showed that steady state was reached 2.2 years after transplantation. The absolute fat graft retention at that time was 50.6 percent. Male patients had a significantly higher fat graft retention rate at steady state (57.7 percent) compared with female patients (44.5 percent). No statistically significant association was found between transplant volume and graft retention rate. The lack of a correlation between transplant size and retention rate and the long time required to reach steady state suggest that factors other than blood supply may play a more influential role in fat graft retention than previously thought.

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Appendix 2

Efficacy of breast reconstruction with fat grafting: A systematic review and meta-analysis

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Review

Efficacy of breast reconstruction with fat grafting: A systematic review and meta-analysis

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KEYWORDS
Fat grafting;
Breast reconstruction;
Breast cancer;
Meta-analysis;
Mastectomy;
Breast-conserving surgery

Summary *Background:* Breast reconstruction with fat grafting is a new alternative to prosthetic implants and flaps for women with breast cancer. In this study, we investigate the efficacy of fat grafting for breast reconstruction in a meta-analysis.
Methods: The study followed the PRISMA and MOOSE guidelines for systematic reviews and meta-analyses. Studies were included if the patients underwent complete breast reconstruction with fat grafting as the only treatment modality. The number of fat grafting treatments needed to complete a breast reconstruction was modeled in a meta-analysis for five treatment categories: modified radical mastectomy, skin-sparing mastectomy, and breast-conserving surgery; the two mastectomy groups were subdivided into nonirradiated and irradiated.
Results: Twenty-one studies were included in the meta-analysis. The studies comprised 1011 breast reconstructions in 834 patients. The estimated numbers of treatments to complete a reconstruction were 2.84–4.66 in the mastectomy groups and 1.72 in the breast-conserving surgery group. The number of fat grafting sessions needed to complete a breast reconstruction was significantly higher for the irradiated patients than for the nonirradiated patients ($p < 0.05$). There was no significant difference in the number of fat grafting sessions needed

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to complete a breast reconstruction after a modified radical mastectomy versus a skin-sparing mastectomy.
Conclusions: This study provides an evidence-based foundation for several practical issues related to breast reconstruction with fat grafting. The analysis showed that radiotherapy is the most important factor associated with the number of treatment sessions needed to complete a breast reconstruction and with the rate of complications.

Contents

Breast cancer is the most common malignancy in women worldwide, with approximately 1671,149 new cases of breast cancer and 521,907 breast cancer deaths predicted in 2012.[1] The percentage of US women who opted to undergo breast reconstruction after breast cancer was estimated at 43.3% based on data from NSQIP 2014.[2] Fat grafting in its current form has been used for more than 30 years,[3] but it remains a relatively new procedure for a complete breast reconstruction.[4] Breast reconstruction with fat grafting provides a new surgical alternative to implants or flap-based breast reconstructive procedures, though most often, fat grafting is used in combination with the two.[5] The oncological safety of fat grafting for breast reconstruction has been discussed and reviewed extensively, and no study has shown evidence for an increased incidence of recurrence.[4,6,7]

When fat grafting is performed, one can expect a portion of the injected fat to be lost to resorption after surgery. Although many studies suggested that the time to steady-state volume maintenance is 2–4 months after grafting,[8–13] one study indicated that the time to steady state in fat graft retention can be as long as 2.2 years.[14] The amount of resorption, combined with the fact that the amount of fat that can be injected in a given session is limited by the volume of the recipient site, makes multiple fat grafting procedures necessary before one breast reconstruction can be completed with fat grafting alone.[15,16] Multiple studies investigated fat grafting for breast reconstruction, but the reported outcomes are quite heterogeneous, and the efficacy of the procedure has not been systematically reviewed or subjected to meta-analysis. A structured and evidence-based overview of the key practical issues with using fat grafting for breast reconstruction would help plastic surgeons and their patients decide whether to proceed with this approach in a given situation.

We conducted a meta-analysis of observational studies that focused on the efficacy of using only fat grafting for breast reconstruction. We compared the number of treatment sessions and the total amount of fat needed to complete a breast reconstruction after a specific type of resection (i.e. radical mastectomy vs. skin-sparing mastectomy) and stratified the data by the use (or nonuse) of radiotherapy.

Methods

This meta-analysis was conducted in accordance with the MOOSE guidelines[17] and the Cochrane Handbook for Systematic Reviews of Interventions[18] when applicable. The selection process, data extraction, and analysis of data were performed by three trained investigators in parallel (MH, MØ, and AL). All discrepancies were discussed until consensus. The protocol for study selection, data extraction, and analysis was designed before initiating the search.

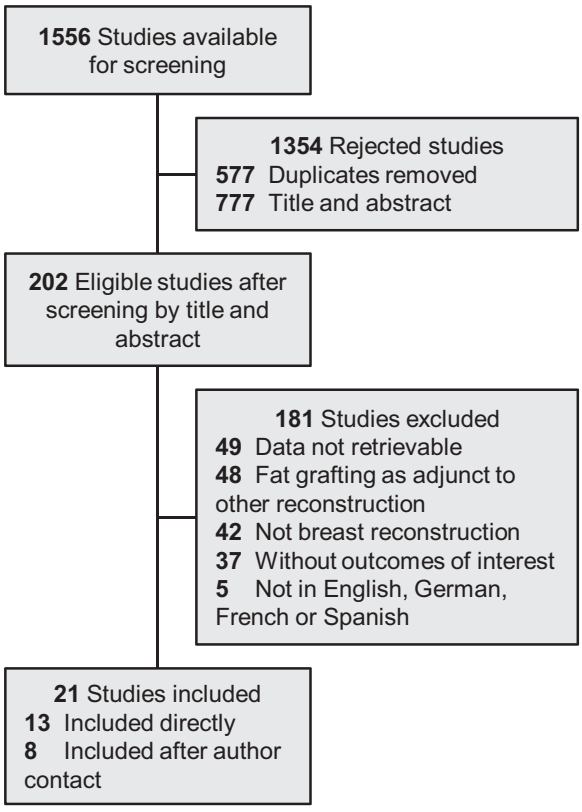

Figure 1 Flowchart of the screening process.

Search strategy and inclusion criteria

A literature search was performed in July 2017 using the PubMed, EBSCO, EMBASE, and Cochrane databases with EndNote X8.

The following search terms were used: (breast reconstruction OR mastectomy OR lumpectomy OR quadrantectomy OR BCS) AND (fat graft* OR lipofilling OR fat transfer OR lipotransfer OR lipografts OR fat transplantation OR lipomodelling). Studies found manually or through the reference lists of included studies were also eligible for inclusion.

Studies were included if the patients underwent breast reconstruction with fat grafting as the only treatment modality after the surgical resection of the breast due to cancer or a genetic predisposition to breast cancer (e.g., BRCA mutation).

The studies were required to specify that the patients were treated until a "complete reconstruction" was achieved with a minimum of 6 months of follow-up. Studies were excluded if the number of treatments was predetermined "per protocol." Patients were excluded if the reconstruction with fat grafting was abandoned for other treatment modalities (e.g., implant or flap).

Authors were contacted for further information if there was doubt regarding the criteria for inclusion or if the data reported were inadequate. In studies with overlapping patient populations from the same group, the article containing the largest population was included. Articles written in English, German, French, Spanish, Swedish, or Danish were eligible for inclusion.

Treatment categories

The patients were divided into the following five treatment categories: modified radical mastectomy, skin-sparing mastectomy, and breast-conserving surgery; the two mastectomy groups were subdivided into nonirradiated and irradiated. All patients in the breast-conserving surgery group were irradiated.

Data extraction and quality assessment

The following baseline characteristics were collected from each study: author, publication year, country of origin, number of patients, number of breasts, age, follow-up, and study design. Data specific to the fat grafting procedure were also collected and included the following: the number of fat grafting treatments needed to complete a reconstruction, accumulated fat graft volume, fat graft volume per treatment, complications, fat graft processing technique, and expansion method of the recipient site. To assess the risk of bias, the Methodological Index for Non-Randomized Studies (MINORS) was used.[19] Aesthetic outcome and patient satisfaction were no assessed in the analysis.

Data synthesis and statistical analysis

A statistical analysis was performed to estimate the mean number of fat grafting treatments needed to complete a reconstruction in each of the five treatment categories. All other endpoints were summarized using basic descriptive statistics. The MINORS score was reported with the mean and confidence intervals. The mean number of fat grafting treatments to complete a reconstruction was modeled by a shifted hypergeometric distribution with a study-specific probability of successful reconstruction in the next treatment. This probability was assumed to follow a beta distribution with parameters depending on the study group.[20] From this model, the expected number of treatments was estimated, along with their standard errors, using the maximum likelihood estimation. Furthermore, the probability of exceeding a certain number of treatments was estimated using the same approach. The thresholds were determined a priori: three treatments for the mastectomy groups and one treatment for the breast-conserving surgery group.

Pairwise comparisons of the expected number of treatments between the mastectomy groups were based on Z-tests. The group comparisons were chosen a priori to maintain a high sensitivity in the analysis. The standard error is usually calculated assuming study independence between treatment categories. However, several studies included patients from multiple treatment categories; therefore, a sensitivity analysis was performed. A Bonferroni correction was applied to adjust for multiple testing. The complication rates were compared with a chi-squared test.

Table 1 Characteristics of the included studies.

Author	Year	Country	Design	Size (No.)	Age (yr)	Follow-up (mo)	Expansion	Fat processing	MINORS
Babin[24]	2017	France	Case report	1	74	48	None	Centrifugation	11
Babovic[25]	2010	USA	Case report	1	54	56	None	Centrifugation	9
Brown[26]	2017	Australia	Retrospective	19	53	-	None	Centrifugation	10
Cheng[27]	2013	Taiwan	Case report	1	37	12	BRAVA	Centrifugation	9
Constantini[28]	2013	Italy	Prospective	9	52.2	12	None	Centrifugation	12
Fabiocchi[29]	2017	Italy	Retrospective	57	49.1	32.5	Expander	Centrifugation	11
Fitoussi[30]	2009	France	Case report	1	47	6	None	Centrifugation	9
Hansen[31]	2015	Denmark	Case report	1	57	19	BRAVA	Centrifugation	8
Ho Quoc[32]	2016	France	Retrospective	17	45	12	BRAVA	Centrifugation	9
Hoppe[33]	2013	Germany	Retrospective	9	52	14.5	None	Sedimentation	11
Hoy[34]	2016	USA	Case report	1	50	8	None	-	6
Jarrah[35]	2013	Spain	Case report	1	41	24	None	Centrifugation	9
Khouri[21]	2015	USA	Retrospective	427	45	30	BRAVA	Centrifugation	9
Longo[36]	2014	Italy	Prospective	21	37.5	46.7	None	Centrifugation	14
Manconi[37]	2016	Italy	Retrospective	11	48.4	27.9	Expander	Both	11
Mestak[38]	2013	Czech Rep.	Case report	1	38	10	BRAVA	Sedimentation	9
Noor[39]	2016	UK	Retrospective	32	50.8	49.1	None	-	10
Semprini[40]	2014	Italy	Retrospective	151	64	69	None	Centrifugation	10
Serra-Renom[41]	2011	Spain	Case report	2	56.5	12	None	Centrifugation	9
Silva-Vergara[42]	2016	Spain	Retrospective	65	52	107	None	Centrifugation	10
Stillaert[43]	2016	Belgium	Retrospective	6	44.2	11.3	Expander	Centrifugation	11

Publication bias was assessed with a funnel plot showing the relationship between study size and deviation from the group mean. The studies were stratified by study group.

All analyses were performed using the statistical software R version 3.3.2 (www.r-project.org) and the add-on package stats4. All p-values were evaluated at a 5% significance level. All figures were constructed with GraphPad Prism version 7.04, GraphPad Software, California, USA, www.graphpad.com.

Results

Search results

The initial search identified 1556 studies. After screening based on title and abstract, 202 studies were eligible for full-text screening. Data were readily extractable from 13 studies. Author contact provided eight additional studies out of 57 author contacts, resulting in a total of 21 studies in the meta-analysis. The reasons for exclusions in the full-text screening were primarily a lack of stratification into the surgical resection categories used in our analysis or no reporting of radiotherapy treatment. The screening process is shown in Figure 1.

Characteristics and quality of included studies

The 21 studies from 11 countries provided data from 1011 breast reconstructions in 834 patients (78.8% unilateral and 21.2% bilateral reconstructions). The mean study size was 39.7 patients per study (range 1–427), and the mean follow-up was 43.8 months (range 6–107 months). The patients had a mean age of 49.8 years (range 37–74). The numbers of studies contributing to each of the five groups were 11 for the nonirradiated modified radical mastectomy group (282 breasts), seven for the irradiated modified radical mastectomy group (168 breasts), seven for the nonirradiated skin-sparing mastectomy group (156 breasts), five for the irradiated skin-sparing mastectomy group (49 breasts), and eight for the breast-conserving surgery group (356 breasts). All included studies were observational: two were prospective, ten were retrospective, and nine were case reports.

The mean MINORS score of the included studies was 9.85 (95% CI 9.18–10.53) out of a maximum score of 16. The study characteristics and individual MINORS scores are shown in Table 1.

Injected volumes and complications

The mean accumulated injected volumes of fat graft per breast were 744.8 mL in the nonirradiated modified radical mastectomy group, 740.8 mL in the irradiated modified radical mastectomy group, 657.7 mL in the nonirradiated skin-sparing mastectomy group, 626.2 mL in the irradiated skin-sparing mastectomy group, and 230.5 mL in the breast-conserving surgery group. The mean injected volumes reported in the included studies are shown in Table 2. The total complication rates in the five groups were as follows: 14.7% in the nonirradiated modified radical mastectomy group, 44.1% in the irradiated modified radical mastectomy group, 10.7% in the nonirradiated skin-sparing mastectomy group, 26.1% in the irradiated skin-sparing mastectomy group, and 21.1% in the breast-conserving surgery group. There was a significantly higher complication rate in the irradiated modified radical mastectomy group than in the nonirradiated modified radical mastectomy group ($X^2 = 47.28$, $p < 0.001$) and a significantly higher

Table 2 Mean injected volumes of fat grafting for breast reconstruction.

Modified radical mastectomy nonirradiated ($n=9$)	No. of breasts	Accumulated	Mean volume per treatment (mL)				
			1st	2nd	3rd	Subsequent	Average
Babovic - 2010[25]	1	1615.8	84.2	78.9	84.2	456.1	403.9
Cheng - 2013[27]	2	430	230	200	-	-	215
Hansen - 2015[31]	1	957	187	150	30	147.5	136.71
Hoppe - 2013[33]	5	984.4	259	298	261.4	205	259.1
Jarrah - 2013[35]	1	720	360	240	120	-	240
Manconi - 2016[37]	4	738.5	288	295.5	310	-	305.3
Mestak - 2013[38]	2	815	235.0	280	300	-	271.7
Silva-Vergara - 2016[42]	9	586.2	218.9	229.3	164.4	210	211
Stillaert - 2016[43]	1	510	-	-	-	-	176
Number of breasts	26	26	25	25	23	16	26
Mean	-	744.8	239.0	246.4	211.2	218.5	243.3
Range	-	430-1615.8	84.2-360	78.9-298	30-310	147.5-456.1	136.71-403.9
Modified radical mastectomy irradiated (n = 6)	No. of breasts	Accumulated	Mean volume per treatment (mL)				
			1st	2nd	3rd	Subsequent	Average
Babin - 2017[24]	1	500	200	150	150	-	166
Fitoussi - 2009[30]	1	380	150	150	-	-	80
Hoppe - 2013[33]	4	888.8	236.3	255	240	205	237
Manconi - 2016[37]	1	1158	96	167	170	362.5	231.6
Silva-Vergara - 2016[42]	18	724.9	196.9	234.6	223.6	195.6	213.9
Stillaert - 2016[43]	1	620	-	-	-	-	155
Number of breasts	26	26	25	25	24	23	26
Mean	-	740.8	197.4	228.4	221.0	204.5	208.9
Range	-	380-1158	96-236.25	150-255	150-240	195.6-362.5	80-237
Skin-sparing mastectomy nonirradiated ($n=6$)	No. of Breasts	Accumulated	Mean volume per treatment (mL)				
			1st	2nd	3rd	Subsequent	Average
Constantini - 2013[28]	1	272	80	81	111	-	90.66
Fabiocchi - 2017[29]	56	706.1	310.4	328.1	280.7	154.3	329.0
Ho Quoc - 2016[32]	6	790	-	-	-	-	-
Longo - 2014[36]	17	410.3	114.4	147.9	147.9	-	136.8
Manconi - 2016[37]	6	792.3	291	375.3	252	-	324.9
Stillaert - 2016[43]	4	728.8	-	-	-	-	168.3
Number of breasts	90	90	80	80	80	56	84
Mean	-	657.7	264.4	290.3	248.2	154.3	279.3
Range	-	272-792.3	80-310.4	81-375.3	111-280.7	-	90.7-329
Skin-sparing mastectomy irradiated ($n=3$)	No. of breasts	Accumulated	Mean volume per treatment (mL)				
			1st	2nd	3rd	Subsequent	Average
Constantini - 2013[28]	1	331	150	116	65	-	110.3
Fabiocchi - 2017[29]	20	658.2	286.2	299.8	316.6	177	335.8
Longo - 2014[36]	12	597.5	67.5	70	139.2	134.2	110.6

(*continued on next page*)

Table 2 (*continued*)

Modified radical mastectomy nonirradiated ($n=9$)	No. of breasts	Accumulated	Mean volume per treatment (mL)				
			1st	2nd	3rd	Subsequent	Average
Number of breasts	33	33	33	33	33	32	33
Mean	-	626.2	202.5	210.7	244.5	160.9	247.1
Range	-	331-658.2	67.5-286.2	70-299.8	65-316.6	134.2-177	110.3-335.8
Breast-conserving surgery irradiated ($n=5$)	No. of Breasts	Accumulated	Mean volume per treatment (mL)				
			1st	2nd	3rd	Subsequent	Average
Brown - 2017[26]	19	88	-	-	-	-	40
Constantini - 2013[28]	7	285.1	149.4	141	104	-	148.1
Ho Quoc - 2016[32]	11	531	-	-	-	-	-
Noor - 2016[39]	32	219.8	210	78	-	-	201.8
Silva-Vergara - 2016[42]	44	215.9	143.6	157.5	141.7	125	146.2
Number of breasts	113	113	83	83	51	44	102
Mean	-	230.5	169.7	125.5	136.5	125	144.0
Range	-	88-531	143.6-210	78-157.5	104-141.7	-	40-201.8

Note: Only studies reporting fat grafting volumes are shown. Hyphens indicate data not available.

complication rate in the irradiated skin-sparing mastectomy group than in the nonirradiated skin-sparing mastectomy group ($X^2=6.73$, $p<0.001$). No significant difference in the complication rate was evident between the modified radical mastectomy groups and the skin-sparing mastectomy groups. The reported complications are presented in Table 3.

Number of treatments needed for complete reconstruction

The mean number of treatments needed to complete a reconstruction was modeled for the five treatment categories using the data from the 1011 included breast reconstructions. The estimated numbers of treatments needed for complete reconstruction were 2.84 (95% CI 2.69-2.98) for the nonirradiated modified radical mastectomy group, 4.27 (95% CI 3.27-5.28) for the irradiated modified radical mastectomy group, 2.93 (95% CI 2.25-3.61) for the nonirradiated skin-sparing mastectomy group, 4.66 (95% CI 3.12-6.19) for the irradiated skin-sparing mastectomy group, and 1.72 (95% CI 1.32-2.12) for the breast-conserving surgery group. The forest plot in Figure 2 shows the reported number of treatments needed to complete a reconstruction for all included studies compared with the pooled estimate of the meta-analysis.

The number of treatments needed to complete a reconstruction was compared by surgical type and radiotherapy status with *Z*-tests. The results are presented as a bar chart in Figure 3. The numbers of treatments needed to complete a reconstruction were not significantly different between the skin-sparing mastectomy group and the Modified radical mastectomy group when the patients were stratified by whether they received radiotherapy or not ($p>0.05$). Conversely, the numbers of treatments needed to complete a reconstruction were significantly higher in the irradiated mastectomy groups than in their nonirradiated counterparts: 4.27 (95% CI 3.27-5.28) vs. 2.84 (95% CI 2.69-2.98) ($p=0.01$) for modified radical mastectomy and 4.66 (95% CI 3.12-6.19) vs. 2.93 (95% CI 2.25-3.61) ($p=0.04$) for skin-sparing mastectomy.

The estimated probabilities of exceeding three treatments for complete reconstruction were 21% (95% CI 17−25%) in the nonirradiated modified radical mastectomy group, 47% (95% CI 33-62%) in the irradiated modified radical mastectomy group, 21% (95% CI 8−35%) in the nonirradiated skin-sparing mastectomy group, and 51% (95% CI 36−65%) in the irradiated skin-sparing mastectomy group. The probability of exceeding one treatment in the breast-conserving surgery group was 39% (95% CI 28−50%).

Fat grafting technique and expansion

The surgical technique varied among the included studies. For the preparation of fat grafts, 16 studies used centrifugation (76.2%), two studies used sedimentation (9.5%), one study used both (4.8%), and two studies did not report their processing methods (9.5%). Two types of recipient site expansion were used in the included studies: internal expansion (i.e., an inflated expander, which could be sequentially deflated with each fat grafting session and eventually removed) and external expansion (e.g., a BRAVA device[21]). In the mastectomy groups, five studies used external expansion (29.4%), three studies used internal expansion (17.6%), and nine studies did not use any expansion (52.9%). No expansion was reported in the breast-conserving surgery group.

Table 3 Complications after fat grafting for breast reconstruction.

Modified radical mastectomy nonirradiated ($n = 8$)[21,27,33,35,37,38,42,43]	No. (%)
Palpable lumps	33 (11.8%)
Ulceration necrosis	3 (1.1%)
Infection	1 (0.4%)
Fat necrosis	1 (0.4%)
Oily cysts	2 (0.7%)
Hematoma	1 (0.4%)
Total	**41/279 (14.7%)**
Modified radical mastectomy irradiated (n = 7)[21,24,30,33,37,42,43]	
Palpable lumps	57 (33.9%)
Ulceration necrosis	9 (5.4%)
Infection	1 (0.6%)
Fat necrosis	4 (2.4%)
Pneumothorax	3 (1.8%)
Total	**74/168 (44.1%)**
Skin-sparing mastectomy nonirradiated (n = 5)[21,29,36,37,43]	
Palpable lumps	9 (6.0%)
Ulceration necrosis	2 (1.3%)
Infection	1 (0.7%)
Oily cysts	1 (0.7%)
Pneumothorax	1 (0.7%)
Aspirated cyst	2 (1.3%)
Total	**16/149 (10.7%)**
Skin-sparing mastectomy irradiated (n = 3)[21,29,36]	
Palpable lumps	6 (13.0%)
Ulceration necrosis	2 (4.3%)
Infection	3 (6.5%)
Hematoma	1 (2.2%)
Total	**12/46 (26.1%)**
Breast-conserving surgery irradiated (n = 4)[21,39,40,42]	
Palpable lumps	50 (15.7%)
Ulceration necrosis	3 (0.9%)
Infection	3 (0.9%)
Fat necrosis	3 (0.9%)
Oily cysts	3 (0.9%)
Pneumothorax	1 (0.3%)
Ecchymosis	3 (0.9%)
Hematoma	1 (0.3%)
Total	**67/318 (21.1%)**

Publication bias

Publication bias among the included studies was assessed with a funnel plot, and the plot shows the percentage deviation from the group mean as a function of study size (Figure 4). This deviation showed an equal distribution around the group mean, except for two case reports that reported a very high number of treatments needed to complete a reconstruction. The analysis did not indicate a significant publication bias.

Discussion

In this meta-analysis involving 1011 breast reconstructions from 21 studies, we present estimates of the number of fat grafting treatments needed to complete a breast reconstruction in five different treatment categories. These estimates may help the patient and the reconstructive surgeon to make an informed decision regarding whether to use fat grafting for breast reconstruction after surgical resection of the breast.

Our analysis found that the number of treatments needed to complete a reconstruction was significantly higher for an irradiated breast than for a nonirradiated breast. Contrary to our expectations, there was no significant difference in the number of treatments needed to complete a reconstruction after a modified radical mastectomy compared with that after a skin-sparing mastectomy. However, this analysis does not account for the difference in the aesthetic outcome, which would likely be superior after a skin-sparing mastectomy.

The complication rate was significantly higher in the irradiated mastectomy groups than in their nonirradiated counterparts. These results are consistent with those of previous reports[5] and seem reasonable from a clinical perspective (Table 3).

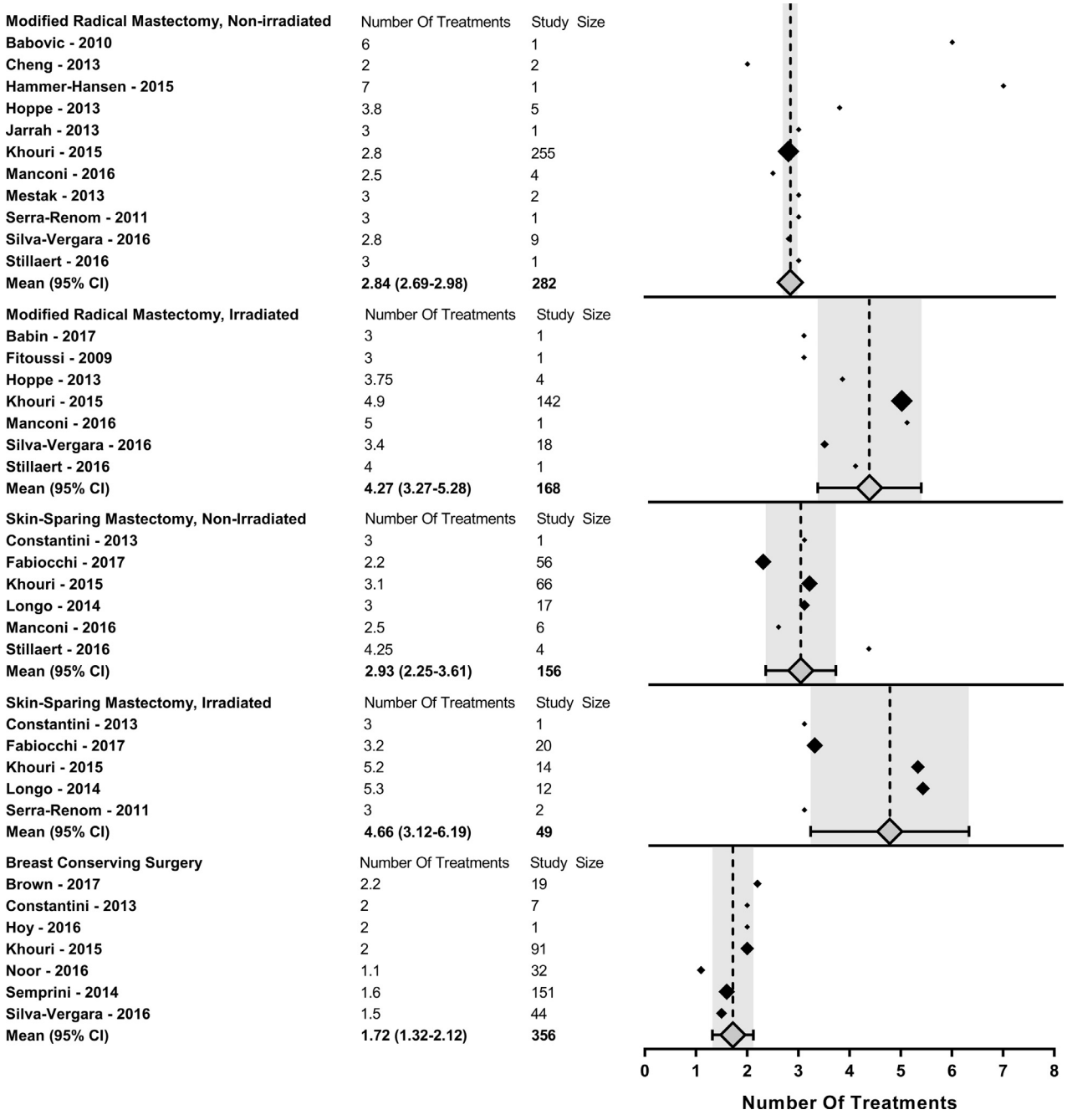

Figure 2 Forest plot showing the mean number of fat grafting treatments needed to complete a reconstruction. The data markers indicate the individual study mean. The size of the mark is proportional to the number of cases. The vertical dashed lines indicate the group means with 95% CIs (shaded interval). Only the studies reporting the average number of fat grafting treatments needed to complete a reconstruction are shown. *CI*, Confidence Interval.

Limitations

The applicability of our analysis is limited by how well the patients included in our analysis represented the background population of women treated with fat grafting for breast reconstruction. We evaluated different sources of bias in our meta-analysis to estimate differences between our sample and the background population and to estimate how this affected the results of our analysis.

The studies with many patients contributed the most to the results of this meta-analysis, representing a risk of bias. In the studies with many patients, one can assume that the involved surgeons were more experienced than the average plastic surgeon in using fat grafting for breast reconstructions. Thus, the results of this review may represent the outcomes achieved by the best surgeons in the field of fat grafting and may therefore be more optimistic than the true average. However, by analyzing the funnel plot, we found no tendency of the larger studies to present better results than average. Rather, the funnel plot was symmetrical, except for two case studies that deviated considerably from the mean, with a high number of treatments needed to com-

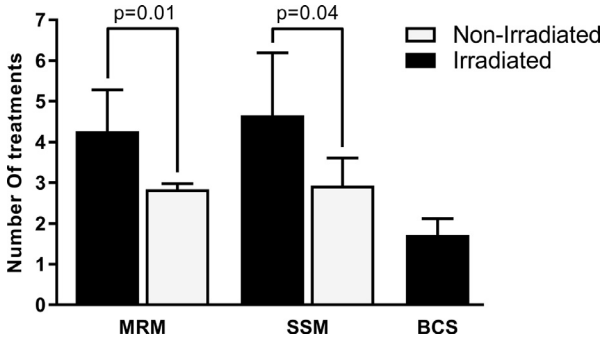

Figure 3 Mean number of fat grafting treatments needed to complete a breast reconstruction. Vertical error bars indicate 95% confidence intervals, and brackets represent *Z*-test comparisons between treatment categories. *MRM*, Modified Radical Mastectomy; *SSM*, Skin-Sparing Mastectomy; *BCS*, Breast-Conserving Surgery.

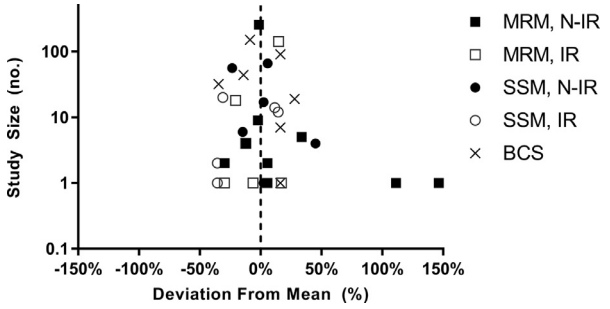

Figure 4 Funnel plot showing the study size and percent deviation from the group mean number of fat grafting treatment sessions needed to complete a breast reconstruction. *Y*-axis is transformed to a logarithmic scale. Each treatment category is represented by its own symbol. *No.*, Number of Breasts; *MRM*, Modified Radical Mastectomy; *SSM*, Skin-Sparing Mastectomy; *BCS*, Breast-Conserving Surgery; *N-IR*, Non-Irradiated; *IR*, Irradiated.

plete a reconstruction. These two case studies were particularly difficult cases, and the case studies as a group ($n = 9$) may have had a slightly higher average as a result. These two outliers had no significant impact on the results of our meta-analysis because of the large sample size.

The investigated outcomes of this meta-analysis were chosen because of their unambiguous nature and clinical applicability (e.g., the number of treatments needed to complete a reconstruction). More complex outcomes, such as the retention rate of the fat graft or percentage volume augmentation,[22] were not included because volume measurements of the breast were calculated very differently between groups and because a validated method has yet to be presented.[23]

We found no effect on the number of treatments needed to complete a breast reconstruction based on the type of mastectomy resection. It should be stressed however that this lack of effect is not proof that the type of mastectomy resection is unimportant for a breast reconstruction with fat grafting. Several important factors are missing from the background data material, including the volume needed to be reconstructed or possible differences in the aesthetic outcome. Generally, the results presented in this study should be used to provide an estimate of the average numbers of treatments needed to complete a reconstruction based on the cancer treatment preceding the reconstruction and the average volume of fat that should be available for fat grafting. The results can be used to inform both patients and surgeons before choosing fat grafting for a breast reconstruction.

Other stratifications such as age, BMI, fat processing technique, efficacy of expansion, and smoking status were not attempted because of small sample sizes or lack of information.

Conclusion

We are the first to report a meta-analysis of the efficacy of fat grafting for breast reconstruction. The analysis provided estimates of the number of fat grafting treatments needed to complete a breast reconstruction in five different patient categories: 2.84 after a nonirradiated modified radical mastectomy, 4.27 after an irradiated modified radical mastectomy, 2.93 after a nonirradiated skin-sparing mastectomy, 4.66 after an irradiated skin-sparing mastectomy, and 1.72 after breast-conserving surgery. The analysis shows that irradiated breasts need significantly more treatments for complete reconstruction than nonirradiated breasts after either modified radical mastectomy or skin-sparing mastectomy. Furthermore, the risk of having to undergo more than three treatments is only 21% in the two nonirradiated mastectomy groups, in contrast to 47–51% in the irradiated mastectomy groups. The total complication rate was also significantly higher in the irradiated groups than in the nonirradiated groups. Surprisingly, the analysis showed no significant difference in the number of treatments needed to complete a reconstruction after a modified radical mastectomy compared with that after a skin-sparing mastectomy when the radiotherapy status was kept constant. Although this review provides an evidence-based foundation for several practical issues related to breast reconstruction using fat grafting alone, the review provides no insights into whether this approach is attractive for the patient. In other words, we are unable to derive any meaningful conclusions about the aesthetic outcome of using fat grafting for total breast reconstruction or the resulting patient satisfaction. Future studies are needed to compare the results of this reconstructive approach to more common techniques.

Conflict of interest

None of the authors have a financial interest in any of the products, devices, or drugs mentioned in this manuscript.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.bjps.2018.08.024.

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Appendix 3

The current gold standard breast volumetry technique seems to overestimate fat graft volume retention in the breast: A validation study

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The current gold standard breast volumetry technique seems to overestimate fat graft volume retention in the breast: A validation study☆

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KEYWORDS
Fat graft retention;
Fat grafting;
MRI;
Breast volume;
Breast volumetry;
Volume retention

Summary *Background:* MRI is generally considered as the gold standard for measuring breast volume because of its high accuracy of the modality. Many techniques used to measure total breast volume have been validated, but none of these techniques have been validated for their ability to measure the volume retention of fat grafts in the breast. In this study, the authors investigated the accuracy of the most common MRI technique used to measure fat graft retention in the breast by measuring the volume changes after breast augmentation.
Methods: Patients undergoing breast augmentation with either breast implants or fat grafting underwent MRI scans before and after surgery. Blinded observers measured the change in breast volume from the MRI scans. The difference between the measured change in breast volume and the volume of the breast augmentation was used to determine the accuracy of the MRI technique.
Results: Twenty-eight patients with a total of 56 breasts were included. In total, 168 measurements of change in breast volume were performed by the observers. The MRI measurements of change in breast volume overestimated the true volumes of the breast augmentations by

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an average of 50.8%, and only 8 of the 168 individual measurements had measurement errors below 50 mL.
Conclusion: The MRI technique, which is considered as the gold standard for the quantification of fat graft volume retention, was associated with a significant measurement error. These findings have potential implications for the interpretation of previously published results of studies based on this technique.

Background

When fat grafting is performed, it is expected that a portion of the fat graft undergoes resorption after surgery.[1-3] Therefore, studies investigating fat grafting often involve measurement of the fat graft volume retention (i.e., what percentage of the graft volume will remain after the resorption process has ended). We hypothesize that the current method used to measure fat graft volume retention in the breast with MRI may be associated with a much larger error than that previously estimated.[4]

The typical procedure used to assess fat graft volume retention in the breast is as follows[5-10]: (1) the total breast volume is measured by MRI before surgery and again after surgery when no further resorption is expected; (2) the preoperative breast volume is subtracted from the postoperative breast volume to calculate the *change* in the breast volume; and (3) the change in the breast volume is divided by the injected fat volume to calculate the volume retention of the fat graft.

For example, if a breast is augmented with a 200 mL fat graft and we measure the preoperative breast volume as 300 mL, then the breast volume one year after fat grafting is 400 mL. The difference between the two measurements is a 100 mL increase in breast volume. The change in volume is then compared to the injected volume of 200 mL, which is interpreted as a fat graft volume retention rate of 50%.

The validity of the approach described above hinges on the presumption that the measured difference in breast volume is identical to the residual volume of the fat graft (i.e., a 300 mL breast plus a 100 mL residual fat graft volume will result in a 400 mL breast). The accuracy of simply subtracting the measurements of the total breast volume to calculate the change in breast volume (e.g., after breast augmentation) has never been convincingly validated.

In this study, we investigated the accuracy of the most commonly used technique to measure fat graft volume retention with MRI scans, which has been described by Herold et al.[5-8] We investigated the efficiency of the MRI technique to measure the change in breast volume after breast augmentation with either breast implants or fat grafts. The agreement between the measured change in breast volume and the true volume of the breast augmentation (i.e., the volume of the breast implant or fat graft) is used to determine the accuracy of the technique.

Patients and methods

The collection of data was approved by the regional Danish Data Protection Agency, and the need for study approval was waived by the Regional Committee on Health Research Ethics. All patients provided written informed consent before participating in the study.

Patients

Patients who were scheduled for bilateral cosmetic breast augmentation with either breast implants or fat grafting were eligible for inclusion in the study. The exclusion criteria were any known breast disease, pregnancy, or contraindications for MRI. Patients were recruited from June 2014 to April 2017 at the Copenhagen University Hospital, Rigshospitalet and Amalieklinikken, Copenhagen. Patients who received breast implants underwent their first MRI examination within 10 days before surgery and the second MRI examination after a minimum of four months, at which time the postoperative edema was expected to have subsided. Patients were examined at the same time during their menstrual cycle to prevent cyclical breast volume changes from influencing the measurements. The patients who underwent fat grafting were examined on the day before surgery and again within three hours after surgery, at which time no fat graft resorption was expected, and minimal postoperative edema would have formed.

MRI examinations

Patients were examined with a 3-Tesla MRI unit (Siemens Magnetom Verio; Erlangen, Germany), except for two patients who were examined with a 1.5-Tesla unit (Siemens Magnetom Avanto; Erlangen, Germany) because of the maintenance of the 3 Tesla unit. A 4-channel breast coil was used in all examinations. Volume assessments were performed on an axial breathhold DIXON sequence, with 72 slices and a 3-mm slice thickness. Patients were examined head-first, with their arms raised and in the prone position, and with the breasts freely suspended in the breast coil.

MRI volume assessment

All volume assessments were performed by three independent observers (JH, MNH, and SS) who were blinded to the true volume of the breast augmentation (i.e., implant size and fat grafting volume).

The total breast volume was measured by delineating the breasts on the axial slices, as described by Herold et al.[5-8] The borders are shown in Figure 1. The skin was used as the superficial border, and the internal costal surface was used

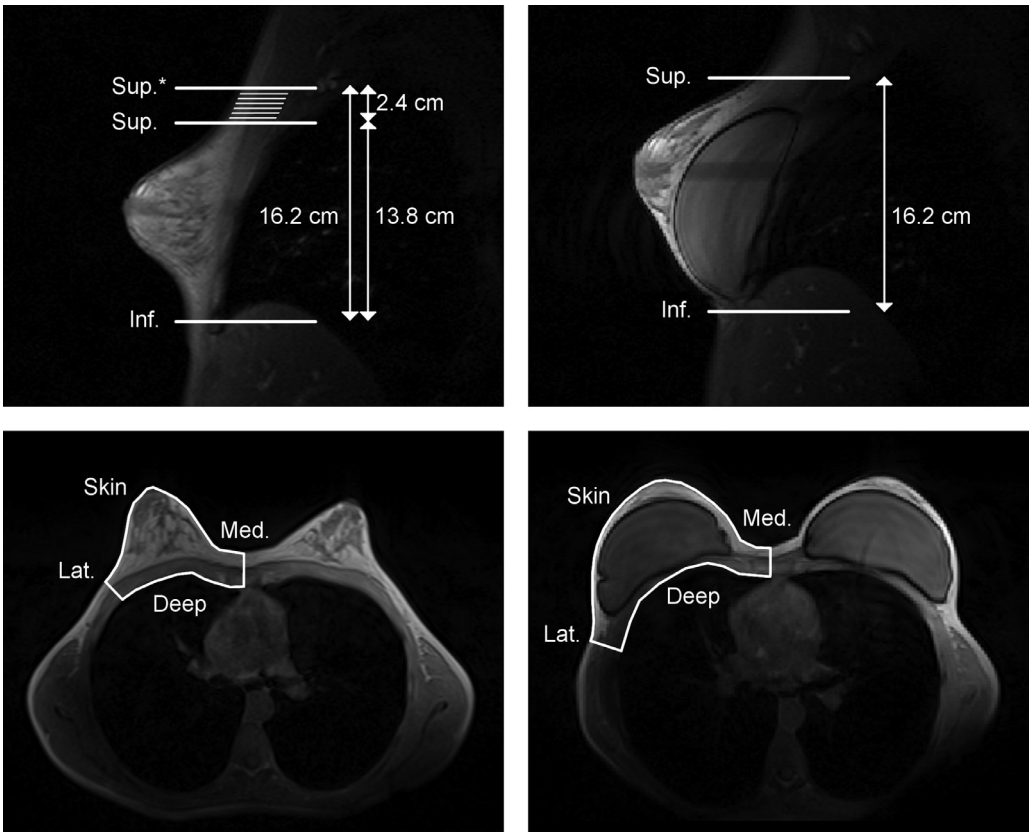

Figure 1 Sagittal and transverse views of the preoperative (left) and postoperative MRI examinations (right) of a patient with breast implants. Sup.* indicates the adjusted cranial border of the preoperative breast, and the hatched area represents the extra tissue added to obtain an equal number of slices on the two examinations. *Sup.*: superior; *Inf.*: inferior; *Lat.*: lateral; Med.: medial.

as the deep border. The center of the sternum was used as the medial border, and the lateral border was placed at the point where the thickness of the breast was equal to the thickness of the surrounding skin. Similarly, the cranial and caudal borders of the breast were placed, where the subcutaneous thickness of the breast was equal to the thickness of the skin identified on sagittal view. The change in breast volume after breast augmentation was calculated by subtracting the preoperative breast volume from the postoperative breast volume. All of the above-mentioned steps were verified by Dr. Herold, who was the corresponding author of the most commonly used MRI technique.[7] Dr. Herold added an additional step to the procedure, which was to include an equal number of slices in the preoperative examination and the postoperative examination. This procedure was performed by adding extra slices to the preoperative scan (because the breast was smaller before the augmentation). The delineation along with the procedure for adding extra slices to the preoperative scan is shown in Figure 1.

To determine the intraobserver variation, the volume measurement was repeated by each observer for 12 patients (six patients with implants and six patients with fat grafting).

The volumes of the breast implants were also measured by delineating the implants directly on the postoperative MRI examination. The measured volume of the implant was compared to the size of the implant stated by the manufacturer to provide a control for the MRI delineation accuracy.

Data analysis

The measurement error was calculated by subtracting the true volume of the breast augmentation (i.e., the implant size or fat graft volume) from the measured change in breast volume between the preoperative and postoperative MRI examinations:

$$\begin{aligned} \textit{Measurement error} = (&\textit{postoperative MRI measurement} \\ &-\textit{preoperative MRI measurement}) \\ &-\textit{true volume} \end{aligned}$$

The percentage of measurement error was calculated by dividing the volume of the measurement error by the true volume of the breast augmentation. The measurement errors were then categorized into three sets of clinically relevant margins of error chosen a priori: 1) less than 50 mL, 2) between 50 and 100 mL, and 3) more than 100 mL.

Statistical analyses

The sample size was calculated using the Clopper-Pearson exact method, which resulted in at least 53 breast augmentations. We targeted a 1:1 ratio between breast augmentations with implants and those with fat grafting. A scatterplot was used to visualize the distribution of measurement errors against the true volume of the breast augmentations. A

simple linear regression fitted through zero and a paired *t*-test were used to assess the accuracy of the measured change in the breast volume compared to the true volume of the breast augmentation. All analyses were performed using R statistical software, version 3.3.3 (www.r-project.org), and the p-values were evaluated at a 5% significance level. All plots were constructed using GraphPad Prism version 7.02 (GraphPad Software, California, USA, www.graphpad.com).

Results

Patients

Twenty-eight patients who underwent bilateral breast augmentation (56 breasts) were included in the study. Fourteen patients underwent breast augmentation with implants, and 14 patients underwent breast augmentation with fat grafting. Patient characteristics and measurement errors are summarized in Table 1. The patients who received implants underwent the second MRI examination after 4.5 months (range: 4–7 months), and all patients who received fat grafting underwent the second MRI examination within three hours after surgery. Patients who received implants had a mean weight gain of 0.54 kg (95% CI −0.3–1.4 kg) at the time of follow-up. The change in weight was not considered for the patients who received fat grafting because the interval between the MRI examinations was less than 24 h.

MRI measurements

Three observers calculated the changes in the breast volume in all 56 breasts from 28 patients and repeated the measurements in 24 breasts. This approach provided a total of 168 individual measurements and 72 repeated measurements of the change in breast volume after breast augmentation. The average time required to measure the change in breast volume for one patient with two breasts was 183 min (95% CI 170–196 min).

Measurement error

The mean measurement error, which was defined as the difference between the measured change in the breast volume and the true volume of the breast augmentation, was 178.4 mL (95% CI 156–201 mL) among the breast implant patients and 153.4 mL (95% CI 138.4–168.5 mL) among the fat grafting patients. These measurement errors correspond to percentage errors of 50.8% (95% CI 45.3%—56.3%) in the implant patients and 50.9% (95% CI 45.7%—56.0%) in the fat grafting patients. When the measurement errors were categorized into clinically relevant margins of error, only 8 of the 168 individual measurement errors (4.8%) were less than 50 mL. Thirty of the 168 measurement errors (17.9%) were between 50 and 100 mL, and 130 measurement errors (77.4%) were greater than 100 mL.

A scatterplot of the measurement errors against the true volumes of the breast augmentation is presented in Figure 2, which shows that 166 of the 168 measured changes

Table 1 Patient characteristics and measurement errors.

Breast augmentation with implants	
Age, yr	28.2
Range	21–39
Weight, kg	57.9
Range	47.5–68
BMI	21.1
Range	18.5–26.2
Implant volume, mL	349
Range	225–420
Measurement error, mL	178.4
95% CI	155.8–201.0
Percentage error, %	50.8
95% CI	45.3–56.3
Breast augmentation with fat grafting	
Age, yr	34.9
Range	30–44
Weight, kg	69.5
Range	59–83
BMI	24.2
Range	22.1–26.8
Fat graft volume, mL	304
Range	250–350
Measurement error, mL	153.4
95% CI	138.4–168.5
Percentage error, %	50.9
95% CI	45.7–56.0
Measured change in breast volume – overall	
Individual measurements, No.	168
Repeated measurements, No.	72
Measurement error, mL	165.9
95% CI	152.2–179.6
Percentage error, %	50.8
95% CI	47.0–54.6
Measurement error, No. (%)	
<50 mL	8/168 (4.8)
50–100 mL	30/168 (17.9)
>100 mL	130/168 (77.4)
Interobserver variation, mL	55.25
95% CI	47.1–63.4
Percentage variation, %	16.4
95% CI	14.1–18.6
Intraobserver variation, mL	19.77
95% CI	15.9–23.7
Percentage variation, %	6.0
95% CI	4.8–7.3
Volume measurement of the implant by direct delineation on MRI examination	
Implants, No	28
Measurement error, mL	15.52
95% CI	9.8–21.2
Percentage error, %	4.4
95% CI	2.9–5.9
Systematic measurement error, mL	−6.72
95% CI	−14.3 to 1.0
Systematic measurement error, %	−1.78
95% CI	−3.9 to 0.34

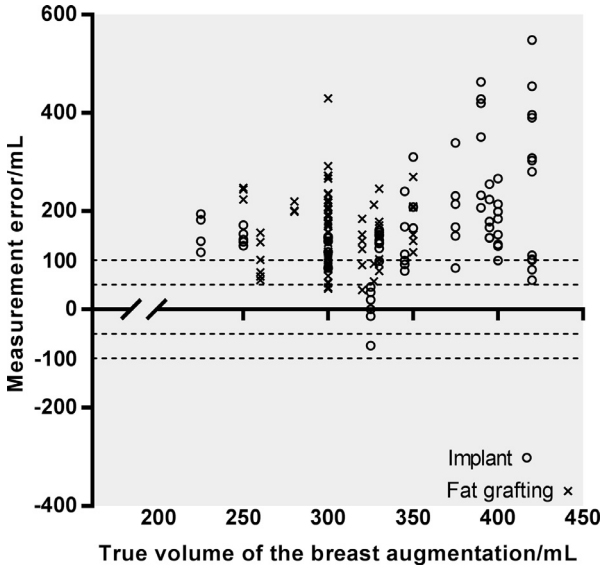

Figure 2 Scatterplot of the measurement error against the true volume of breast augmentation. Measurements close to the true volume are located near the horizontal axis, and the dotted lines show the clinically relevant margins of error for the 50 mL and 100 mL categories. All but two of the 168 measurements overestimated the true volume of the breast augmentation.

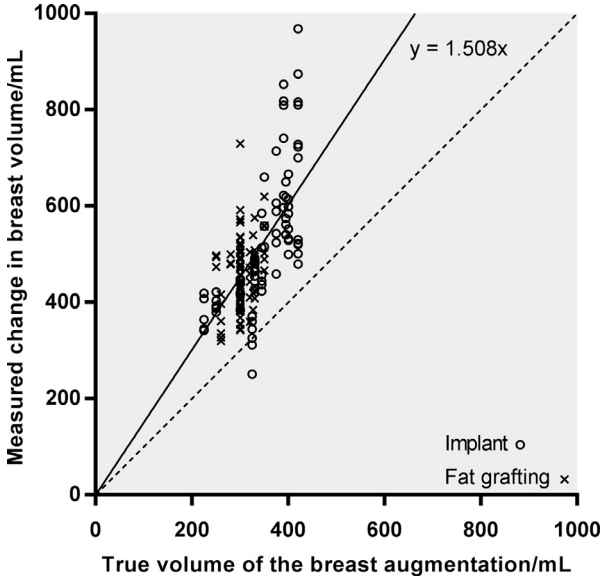

Figure 3 Linear regression showing the relationship between the measured change in breast volume and the true volume of the breast augmentation. The dashed line indicates no measurement error. The regression line has a slope of 1.508.

in breast volume overestimated the true volume of the breast augmentation. The relationship between the measured changes in breast volume and the true volume of the breast augmentation is shown in Figure 3 with a simple linear regression. The slope of the regression line indicates that the measured change in breast volume tended to overestimate the true volume of the breast augmentation by 50.8% ($p < 0.001$). The magnitude of the measurement error increased with larger breast augmentation, but the percentage measurement error (of the true volume of the breast augmentation) did not significantly depend on the true volume of the breast augmentation or on whether the augmentation was performed with implants or fat grafting.

Interobserver and intraobserver variations

The mean difference in the measured change in breast volume among the three observers ($n = 168$) was 55.25 mL (95% CI 47.13–63.37 mL, range 0.28–268.23 mL). The mean difference between repeated measurements of the change in breast volume performed by the same observer ($n = 72$) was 19.77 mL (95% CI 15.87–23.67 mL, range 0.05–75.80 mL).

Volume measurement of the implant by direct delineation on the MRI examination

To test the accuracy of the MRI unit and a straightforward delineation, we measured the volumes of the breast implants directly on the postoperative MRI examinations ($n = 28$). The mean measurement error of implant volume compared to the volume stated by the manufacturer was 15.52 mL (95% CI 9.8–21.2), corresponding to a percentage measurement error of 4.4% (95% CI 2.9–5.9%). The systematic error was negligible at −1.8% of the implant volume (not statistically significant).

Discussion

MRI is probably the most accurate technique used for the noninvasive measurement of tissue volume, and therefore, MRI is considered the gold standard in breast volumetry.[6] The use of MRI as an accurate imaging modality is supported in our study by the low measurement errors, and the absence of a systematic error, when using MRI to measure the total implant volume by direct delineation on the MRI scan. Nonetheless, our findings show that the current technique used to analyze MRI scans to measure the *change* in the breast volume grossly overestimated the expected volume increase after breast augmentations. In our view, the problem is not due to a lack of accuracy in the MRI scans or with measuring the total breast volume, which has been demonstrated to be fairly accurate using MRI, 3D imaging, or CT scans.[11–15] However, it seems that the current technique used to measure the *change* in the breast volume with multiple MRI scans does not measure what is intended to be measured: The current technique of using MRI scans to calculate change in breast volume relies on a presumption that a 300 mL total breast volume plus a 100 mL breast augmentation will result in a 400 mL total breast volume. Our results suggest that the use of the current MRI technique to measure change in breast volume after transferring volume will significantly overestimate the actual transferred volume (e.g., the volume of a residual fat graft).

We hypothesize that the systematic overestimation found in our study is due to physical changes in the

dimensions of the breast that occur when the volume changes as follows: A breast undergoing a volume increase will do so by adding projection anteriorly and also by expansion of the breast borders. The inferior border will be moved downward, the lateral border will be moved more lateral, and the superior border will be moved more upward. Therefore, the measured change in breast volume is the sum of both the transferred tissue (e.g., the breast implant or the fat graft) *and* the tissue surrounding the pre-enlarged breast that will become a part of the breast when the borders expand. We have proposed this principle previously,[4] and it is supported by the results of this study. The current MRI technique, pioneered by Dr. Herold et al. [5] is very accurate in determining total breast volume, but the technique is not appropriate for tracking the volume retention of fat grafts in the breast. A fat graft in the breast is usually dispersed as much as possible during surgery, and therefore, it cannot be directly delineated, instead it has to be determined by measuring the change in breast volume.[16,17] The solution for measuring fat graft volume retention (i.e., the percentage volume of a fat graft that remains after the resorption period) will be to further develop the MRI technique to measure changes in the breast volume that correspond to the change in the fat graft volume.

Limitations

The potential limitations of this study should be addressed. First, there is a possibility that the technique was used incorrectly in this study. To ensure the correct use of the technique, all three observers were individually trained in following all steps of the procedure, as described in the Methods section. Many steps require subjective decision making by the observer, which is a likely explanation for the relatively high interobserver variation (55.25 mL, 95% CI 47.13–61.98 mL), but it does not account for the much higher systematic error of 165.9 mL average overestimation found in our study.

Second, the physical factors of the patients, such as weight changes, postoperative edema, and scar tissue formation, are expected to influence the actual change in breast volume. Weight changes and scar tissue formation are not expected in patients undergoing breast augmentation with fat grafting due to the short follow-up period (less than 3 h). However, some postoperative edema is expected at the time of examination, which may account for some of the overestimation, but it is not reasonable to attribute the entire overestimation to edema.

The patients who received implants were examined 4–5 months after surgery, at which time no edema of significance is expected. Therefore, the measurement overestimation cannot be explained by edema in these patients. The patients who had breast implants experienced a mean weight gain of only 536 g, which is comparable to the weight of two breast implants; hence, we do not expect the changes in bodyweight to systematically influence the breast volume in this study. Overall, the limitations of this study cannot account for the large overestimations of changes in breast volume after breast augmentations found in this study.

Implications

Our findings raise concerns regarding the accuracy of using the most commonly used MRI breast volumetry technique for measuring fat graft volume retention in the breast.[7] Our results should be further investigated by other researchers to either confirm or refute the results. If confirmed, our findings could have potential implications for the interpretation of the results reported in many previous studies using the MRI technique to calculate fat graft volume retention.

Conclusion

This study represents the first attempt to validate the ability of the current MRI-based breast volumetry technique, described by Herold et al.[7] to measure changes in breast volume. The results of this validation study indicate that the current MRI technique is not suited for measuring changes in the breast volume and therefore should not be used to measure the volume retention of fat grafts in the breast. The average measured change in breast volume overestimated the true volume of breast augmentations by 50.8% in patients who had breast augmentation with implants and 50.9% in patients who had breast augmentation with fat grafting. Only 8 of the 168 individual measurements had measurement errors below 50 mL of the true change in breast volume. It is important to note that our study does not question the accuracy of the efficiency of the breast volumetry technique[5] to measure total breast volume, which was the original purpose of the technique.

We propose that a different approach for analyzing MRI scans to measure changes in the breast volume is needed with a higher accuracy to determine fat graft volume retention in breasts.

Conflicts of interest

None of the authors have financial interests related to the content of this manuscript. Activities not related to this article: Felix C. Müller is employed as an industrial Ph.D. student at Siemens Healthineers Danmark, cofounded by the Danish government fund “Innovationsfonden.”

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.bjps.2019.03.029.

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Appendix 4

Lipovol: Validated Software for Measuring Fat Graft Volume Retention in the Breast with MRI

Manuscript

Title:

Lipovol: A new method for measuring fat graft volume retention in the breast with MRI

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Summary

Background: We have previously shown that the current gold standard breast volumetry technique fails to accurately measure changes in fat graft volume retention in the breast and systematically overestimates volume retention by approximately 50%. In this study, we present Lipovol: a new automated software method dedicated to measuring fat graft volume retention in the breast based on magnetic resonance imaging (MRI) scans.

Methods: The software method was validated by four observers using the MRI scans of 28 patients undergoing breast augmentation with fat grafting or breast implants. The changes in breast volume were measured with the software and compared with the injected fat graft volumes/implant volumes.

Results: The software method measured the injected fat graft volumes with an average systematic overestimation of 6.3% (SD = 10.5). Significantly higher measurement errors were recorded for the implant patients, with a systematic overestimation of 23.9% (SD = 18.1), than for the fat grafting patients. The median interobserver variation was less than 7% for both groups.

Conclusion: This new software method is significantly more accurate and precise than the previous gold standard technique. We propose our new method for measuring fat graft volume retention in the breast for clinical research. The software will be available free of charge and it can be used on the MeVisLab platform.

Keywords

Fat grafting; breast augmentation; breast; MRI; volume retention

Introduction

One of the greatest challenges in using fat grafting in the breast is that a variable part of the graft volume is resorbed in the time after surgery.[1] An accurate measurement technique to assess the fat graft volume retention in the breast after breast augmentation[2,3] and breast reconstruction[4,5] is needed to investigate the resorption dynamics of fat grafts in the breast, which can be used to develop new strategies for improving the procedure.[6–8] We have previously shown that the measurement of total breast volume[9] is a very different task than measuring the volume retention of fat grafts that are dispersed in the breast and that the current gold standard technique for measuring fat graft volume retention[10,11] was subject to a systematic error of more than 50%.[12,13] The problem with fat grafts in the breast is that they cannot be delineated on the MRI scan and therefore, we must rely on the measurement of a change in breast volume between two MRI scans of the breasts recorded before the surgery and after the volume resorption has taken place. This change in breast volume can then be compared with the injected volume to calculate the fat graft volume retention rate. E.g. a fat graft of 300 mL is injected and after 1 year the retention rate is 60% corresponding to a 180 mL augmentation. To measure the retention rate, we would have a pre-operative scan and a 1-year-post-operative scan where the expected increase in breast volume would be 180 mL. The accuracy of the measurement technique should be judged by the technique's ability to estimate the exact change in breast volume from one scan to another. However, to our knowledge, previous techniques have not been validated for their ability to measure a change in breast volume. In this study, we present a new software method (Lipovol) that is dedicated to the measurement of changes in breast volume and specifically the volume retention of fat grafts in the breast using magnetic resonance imaging (MRI) scans. Additionally, we present a validation study of the methods's ability to measure changes in breast volume on 28 women who have undergone a breast augmentation with a known volumes. Lipovol will be freely available and can be used on the MeVisLab[14] platform which is an open source software platform for radiological analysis that is also free to use for noncommercial purposes.

Patients and Methods

Lipovol software

Two MRI scans are required to measure fat graft volume retention with the Lipovol software method: one acquired before surgery and one acquired after surgery when the fat graft has reached volumetric steady state. First, the software is used to align the two scans on top of each other using osseous pointers in the thorax. This is the key step that sets Lipovol apart from previous techniques: the alignment allows the user to outline a region-of-interest on the second scan (containing the residual fat graft) which is automatically mirrored on the first scan which ensures that the two regions-of-interest will delineate the same block of tissue on the two scans. Lipovol will then automatically delineate the skin in the two regions-of-interest and calculate the volumes. The difference in volume between the two regions-of-interest will be equal to the residual fat graft volume because the regions-of-interest contain the same tissue block at the two time points (with and without the residual fat graft). The retention rate is calculated by dividing the residual fat graft volume with the initially injected fat graft volume. A demonstration of the software method's workflow is shown in figure 1 and as a video in the supplementary material.

Validation

The study was approved by the regional ethics committee of Copenhagen (nr. H-1501379) and the Regional Data Protection Agency. All participants provided written informed consent before inclusion in the study.

The accuracy of the software was validated using three types of patients with known changes in breast volume: 1) Patients undergoing breast augmentation with fat grafting who were scanned before surgery and within 3 h after surgery, at which time minimal edema formation is present, and the resorption of the fat graft has yet to begin. 2) Patients undergoing breast augmentation with breast implants scanned before surgery and 4-5 months after surgery (i.e., after edema formation). 3) Patients scanned twice on the same day whose second scan was performed after repositioning (no expected change in breast volume).

All measurements of the changes in breast volume were performed by four observers (MH, MØ, JH, and MNH) who were blinded to the transplanted volume.

Patients

The patients were included from the University Hospital Copenhagen and Amalieklinikken, Copenhagen. The MRI scans were acquired on a 3 Tesla MRI unit (Siemens Magnetom Verio, Germany) with a 4-channel breast coil. The patients were examined in a head-first prone position. Parts of the MRI scans have been used in a previous study.[9] Lipovol works on the inphase and outphase images of a 2-point Dixon sequence. The patients were scanned at the same point in their menstrual cycle to prevent cyclical breast volume changes from influencing the measurements.

Statistical analysis

Continuous variables were presented as the mean (SD) when normally distributed and as the median (IQR) when non-normally distributed. The distributions were visualized with histograms and QQ-plots and tested for normality with the Shapiro-Wilk test.[15] Comparisons between normally distributed data were performed with t-tests and evaluated at a 5% significance level. Analyses of measurement errors were calculated with breast as the independent sampling unit and an average of the four reads were used as the measurement. Inter- and intra-observer variation were calculated with the individual measurement as the independent sampling unit. All analyses were performed in R, version 3.6.1 (www.r-project.org, R Foundation for Statistical Computing, Vienna, Austria).

Results

Between 2014-2018, we included and performed MRI on a total of 56 breasts in 28 patients. The patients underwent bilateral breast augmentation with either fat grafting (n = 14) or implants (n = 14), and all patients were scanned twice on the first day to calculate the measurement error at baseline. Four

independent observers provided a total of 280 individual measurements of the changes in breast volume and 96 repeated measurements.

Patients undergoing fat grafting received a median fat graft volume of 300 mL per breast (range 250-350, n = 28), whereas the mean measured change in breast volume was 322 mL (range 242-403, n = 112). The systematic measurement error in the fat grafting patients was 6.28% (SD = 10.5), which is significantly more accurate than the previous gold standard technique[10] that had a systematic error of 50.9% (SD = 24.3)[9] ($p < 0.001$). The median interobserver variation was 4.8% (IQR, 2.7-9.8), and the median intraobserver variation was 4.2% (IQR, 2.0-8.7). Patients undergoing breast augmentation with implants received a median volume of 348 mL per breast (range 225-420, n = 28), whereas the measured change in breast volume was 432 mL (range 268-638 mL, n = 28). The systematic measurement error in the implant patients was 23.9% (SD = 18.1). The median interobserver variation was 4.9% (IQR, 2.4-8.2), and the median intraobserver variation was 6.2 (IQR, 2.4-10.1). The systematic measurement error was significantly higher for the implant patients than for the fat grafting patients when using Lipovol ($p < 0.001$), but Lipovol was still significantly more accurate than the current gold standard technique[10], which had a systematic error of 50.8% (SD = 27.3) in the implant patients[13] ($p < 0.001$). A summary of the validation results is provided in table 1. When measuring the volume difference between repeated measurements recorded on the same day (expected volume change = 0 mL) for the 28 patients (56 breasts), we found a systematic measurement error of only -0.07 mL (SD = 6.2). The linear regression with a 95% prediction interval is shown in figure 2 and indicates a high correlation between the measured volumes and the true volumes with $r^2 = 0.97$ for the fat grafting patients and $r^2 = 0.94$ for the implant patients. The measurement errors are analyzed in a Bland-Altman plot[13] in figure 3, which shows a trend of higher measurement errors with higher augmentation volumes.

Discussion

The new software method for measuring fat graft volume retention in the breast was significantly more accurate than the current gold standard technique.[7] Lipovol was particularly accurate and precise for the analysis of fat graft volume changes (6.28% systematic error, SD = 10.5).

Limitations

The software was validated in patients with known changes in breast volume. Half of the patients had undergone fat grafting and were examined before and directly after surgery at which time no fat graft resorption would have occurred (<3 h). The other half had undergone breast augmentation with implants and were examined before and 4-5 months after surgery. None of the patients experienced any considerable weight changes during this period (average weight gain of 536 g). The advantage of using fat grafting patients for validation is that the primary purpose of the software is to measure the residual volumes of fat grafts. The disadvantage is that we cannot wait for the edema to subside before performing the second scan because we do not know the true resorption dynamics of the fat grafts; therefore, we must accept that some edema is present at the time of the second scan (<3 h after surgery). The expected edema may in part account for the systematic overestimation of 6.28% in these patients. Additionally, the range of fat graft volumes was relatively narrow in our patient population (range, 250-350 mL). However, this range is in accordance with the most frequently used fat graft volumes for primary breast augmentation in the literature.[16,17] The implant patients have the advantage of stable implant volumes, which allow us to perform the second scan after the edema was expected to have subsided. The disadvantage is that the measured changes in breast volume after breast augmentations with implants might be different from those of breast augmentations with fat grafts due to the different placement of the implants compared to that of the dispersed fat grafts. Overall, we do not know how much of the overestimation that stems from edema in fat grafting patients and physiological changes in the breast after implants affected the error of the software measurements. Therefore, we do not suggest that the systematic overestimation found in this validation study should be deducted from future measurements using the software method. We cannot

explain why the measurement errors for the implant patients were so much higher than those for the fat grafting patients based on this study. We speculate that the implants work like an expander, by drawing lax tissue into the breast from the surroundings, the degree of which would vary between patients based on the thickness of the subcutaneous tissue and the elasticity of the tissues.

Conclusion

The new software method was able to measure the fat graft volume of newly operated patients with high accuracy across four observers, a systematic error of only 6.28% and an interobserver variation of 4.8%. The fat graft volumes measured with the software method were highly correlated with the true fat graft volumes (r^2 = 0.97), and the variation was low (SD = 10.5). The results of this validation study can be used to perform power calculations for future comparative studies that will use fat graft volume retention measured with the new software method as the primary endpoint. Further studies are needed to investigate the accuracy of the software in measuring smaller or larger fat graft volumes than those used in this validation study. Overall, we propose that the Lipovol software method can be used for unbiased and accurate scientific measurement of fat graft volume retention in the breast.

Conflicts of interest statement

None of the authors have a financial interest in any of the products, devices, or drugs mentioned in this manuscript.

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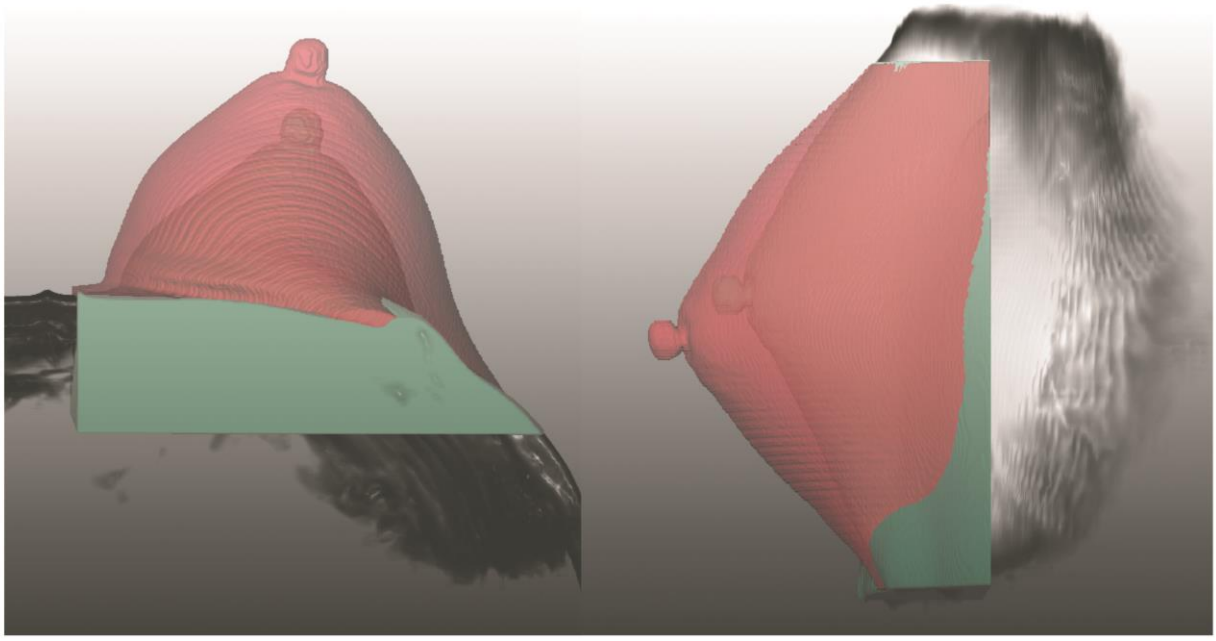

Figure 1: 3D reconstruction from Lipovol. The postoperative scan (red color) is aligned and superimposed on the preoperative scan (green color).

Table 1: Title: Summary of the validation results

Breast augmentation with fat grafting	
Individual breasts, No.	28
Individual measurements, No.	112
Repeated measurements, No.	48
Median fat graft volume, mL	300
Range, mL	250-350
Mean measured fat graft volume, mL	322
Range, mL	242-403

Mean systematic error, pct	6.28
SD, pct	10.5
Median interobserver variation, pct	4.8
IQR, pct	2.7-9.8
Median intraobserver variation, pct	4.2
IQR, pct	2.0-8.7
Breast augmentation with implants	
Individual breasts, No.	28
Individual measurements, No.	112
Repeated measurements, No.	48
Median implant volume, mL	348
Range, mL	225-420
Mean measured implant volume, mL	432
Range, mL	278-624
Mean systematic error, pct	23.9
SD, pct	18.1
Median interobserver variation, pct	4.9
IQR, pct	2.4-8.2
Median intraobserver variation, pct	6.2
IQR, pct	2.4-10.1
Same-day repeated measurements	
Individual breasts, No.	56
Individual measurements, No.	56
Mean measurement error, mL	-0.07
SD, mL	6.2

Footnote: Abbreviations: No. = Number, IQR = Interquartile range, SD = Standard deviation, pct = Percentage

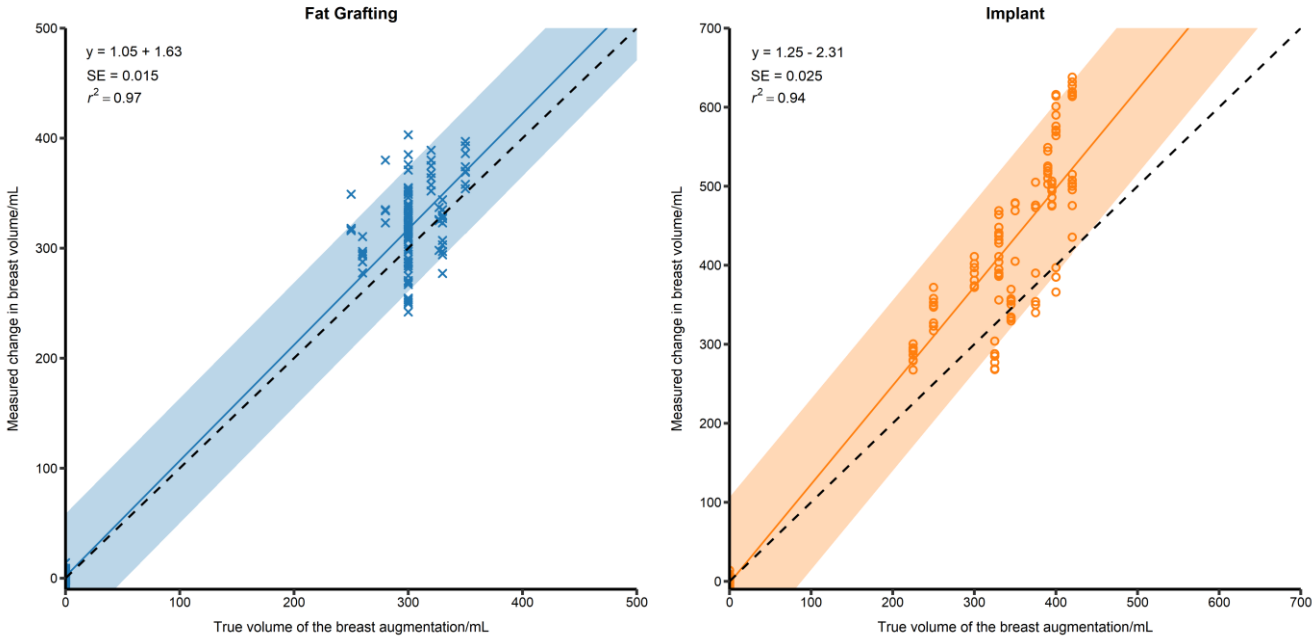

Figure 2: Linear regression analysis with a 95% prediction interval of the measured changes in breast volume compared to the true volumes of the breast augmentations.

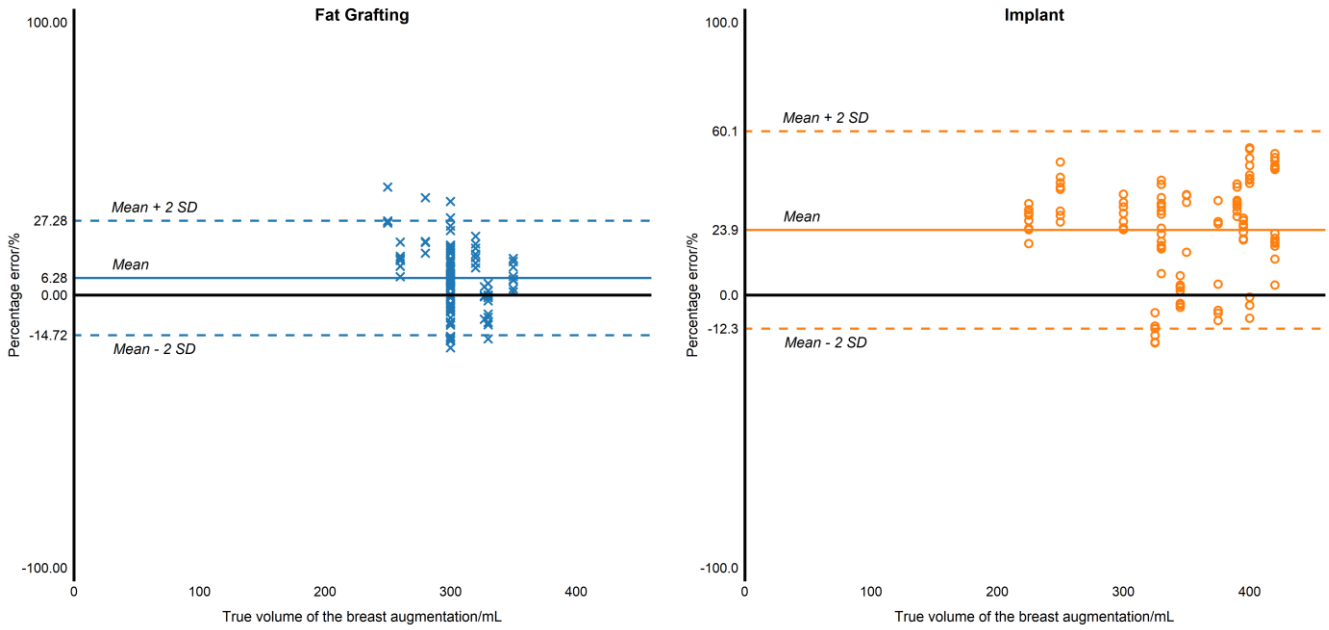

Figure 3: Bland-Altman plot of the percentage measurement errors compared to the true volumes of the breast augmentations.