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FRANCISCO CLARO DE OLIVEIRA JUNIOR

AVALIAÇÃO DA SEGURANÇA ONCOLÓGICA DA LIPOENXERTIA AUTÓLOGA PARA O CÂNCER DE MAMA

ASSESSING THE ONCOLOGICAL SAFETY OF AUTOLOGOUS FAT GRAFTING FOR BREAST CANCER

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Tese apresentada à Faculdade de Ciências Médicas da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Doutor em Ciências da Saúde, na área de Oncologia Ginecológica e Mamária.

Thesis presented at School of Medical Sciences at State University of Campinas as part of demanded requires for the title of Doctor in Health Sciences, in Gynecology and Mammary Oncology Field

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A uma pessoa que tornou esta tese possível.

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Orientador compreensivo e incentivador;
Médico atencioso e humano;
Pesquisador ousado e persistente.

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RESUMO

Introdução: A lipoenxertia para reconstrução da mama é uma técnica efetiva, de baixa complexidade e baixo custo. Entretanto, a associação entre obesidade e câncer de mama, além de evidências laboratoriais de que as adipocinas podem estimular a proliferação celular e a diferenciação de células-tronco mesenquimais (ASC), suscita dúvidas sobre a segurança oncológica do procedimento para a prática clínica. **Objetivo:** Avaliar o risco oncológico da lipoenxertia autóloga para o câncer de mama. **Métodos:** Esta tese trata de quatro estudos interligados. No primeiro, foi desenvolvido um modelo experimental para o estudo da lipoenxertia em ratas da raça Sprague-Dawley avaliando características adaptativas do tecido adiposo de omento pediculado por meio de avaliações macroscópica, histológica e morfologia de adipócitos. No segundo, este modelo foi aplicado para comparar o potencial oncogênico do tecido adiposo exposto à dieta hipercalórica com o procedimento de lipoenxertia, através de marcadores de inflamação crônica (CD68 em macrófagos), proliferação celular (Ki67) e níveis de PAI-1. No terceiro estudo foram comparadas as mamas que receberam lipoenxertia de omento e de subcutâneo, e as que não foram manipuladas, através de avaliações histológica, imunoistoquímica e PCR-RT de CD68, Ki67, PAI-1 e receptores de estrógeno (ER, marcador 1D5). O quarto estudo foi uma revisão sistemática da literatura sobre a utilização de tecido adiposo do omento para o tratamento das afecções mamárias. **Resultados:** No primeiro estudo, o tecido adiposo do omento pediculado sofreu processo adaptativo resultante de modificações no seu interstício, sem alteração morfológica dos adipócitos (p=0,27) e sem atipias celulares, quando estimulado pela translocação. No segundo estudo, o tecido adiposo não manipulado exposto à dieta hipercalórica produziu proliferação celular (representada por elevação do Ki67, p=0,046) e elevação dos níveis de PAI-1 (p<0,001) quando comparado aos controles e à lipoenxertia sem dieta hipercalórica. No terceiro estudo, o número absoluto, a estrutura histológica e a morfologia celular dos ductos terminais lobulares mamários, assim como a expressão e o padrão dos marcadores CD68, PAI-1, Ki67 e RE, não se modificaram nos grupos de enxertia com tecido adiposo do subcutâneo ou do omento, quando comparados ao tecido mamário não manipulado, p>0,05. Na revisão sistemática foram identificados 60 estudos que envolveram 985 mulheres. Oito estudos analisaram o risco oncológico para recidiva de câncer de mama, sendo

que em sete a enxertia foi realizada em estádio avançado e a recorrência local foi de 35,5% (143/403). Um destes estudos avaliou pacientes com câncer em estádio inicial (89 mulheres) e nenhuma recidiva foi identificada em *follow-up* médio superior a cinco anos após a reconstrução. **Conclusão:** Pelos estudos experimentais *in vivo*, foram identificados o potencial oncogênico dos adipócitos nativos sem manipulação quando expostos à dieta hipercalórica. Entretanto, o tecido adiposo enxertado ou as células do sítio receptor (incluindo as células ductais) não mostraram qualquer alteração potencialmente oncogênica, quando analisado o processo de enxertia isoladamente. Os achados experimentais deste estudo estão perfeitamente alinhados com as conclusões da literatura para lipoenxertia e com a revisão sistemática de estudos clínicos em tecido adiposo de omento, os quais sustentam a segurança oncológica da lipoenxertia para reconstrução da mama.

Palavras-Chave: mama; tecido adiposo; omento; câncer de mama; transplante.

ABSTRACT

Introduction: Lipofilling for breast reconstruction is an effective procedure, with low complexity and low cost. However, its oncological safety was questioned when obesity was identified as a risk factor for breast cancer along with some *in vitro* and preclinical studies that identified the oncogenic potential of adipocytes to cellular proliferation and differentiation of adipose stromal cells (ASC). **Objective**: To assess the oncological safety of autologous fat grafting for breast cancer. **Method:** The present research is composed by four studies. In the first one, it was proposed an experimental model for lipofilling using adipose tissue of pedicle omental flap in Sprague-Dawley rats to assess its adaptative properties through histopathological analyses and adipocyte morphology. In the second, this model was used to compare the oncogenic potential of samples of native unmanipulated adipose tissue when exposed to high-energy diet to samples of fat exposed only to the lipofilling procedure through markers of chronic inflammation (CD68 in macrophages), cellular proliferation (Ki67), and levels of PAI-1. In the third, lipofilled breasts (grafted with subcutaneous or omental fat tissue) were compared with unmanipulated breasts (control) through histopathological analyses, immunohistochemical and PCR-RT tests of CD68, Ki67, PAI-1 and 1D5 markers. The forth study was a systematic review of treatment of breast disorders with adipose tissue of pedicle omental flap. **Results:** In the first study, the adipose tissue of transposed pedicle omental flap underwent an adaptative transformation with intertitial changes, without changes of adipocyte morphology (p=0.27), without cellular atypia. In the second, the unmanipulated fat exposed to high-energy diet showed cellular proliferation (overexpression of Ki67, p=0.046) and increased levels of PAI-1 (p<0.001) when compared to controls and samples of grafted fat of tissue not exposed to high-energy diet. In third study, the number and immunohistochemical analyses of mammary terminal lobular ducts, as well as expression and pattern of markers CD68, PAI-1, Ki67 and ER, did not change in the breasts lipofilled with subcutaneous or omental fat if compared to control breasts, p>0.05. The systematic review identified 60 studies with 985 women. Eight studies analyzed the oncologic risk for breast cancer relapse, seven of them described breast lipofilling after treatment of advanced disease and their recurrence rate was 35.5% (143/403). The eighth study in this series, analyzed 89 women treated for non-advanced disease and found no cancer recurrence during

a mean follow-up longer than five years. **Conclusion:** Our experimental studies *in vivo* identified the oncogenic potential of native unmanipulated fat when exposed to a high-energy diet. However, this potential was not oberved in grafted fat or in its host microenvironment (including the ductal lobular cells) when the procedure of lipofilling was analyzed alone. Our experimental results are in accordance to clinical results reported in literature for breast lipofilling and to the clinical data identified in our systematic review for adipose tissue of pedicled omental flap. Thus, they sustain the oncological safety of fat grafting for breast reconstruction.

Key words: breast; adipose tissue; omentum; breast cancer; transplantation.

LISTA DE ABREVIATURAS E SIGLAS

ARRIVE – *Animal Research: Reporting of In Vivo Experiments*

ASC – *Adipocyte Stromal Cell (*Células-tronco Derivadas de Tecido Adiposo)

ASPRS – *American Society of Plastic and Reconstructive Surgeons*

ASPS – *American Society of Plastic Surgeons*

cDNA – *Complementary Deoxyribonucleic Acid* (Ácido Desoxirribonucleico Complemetar)

CAPES – Coordenação de Aperfeiçoamento de Pessoal de Nível Superior

CEUA – Comitê de Ética no Uso de Animais da Universidade Estadual de Campinas

CO^2 – *Carbon Dioxide* (Dióxido de Carbono)

DAB – *3,3'-Diaminobenzidine* (3,3 Diamino-benzidina)

DNA – *Deoxyribonucleic Acid* (Ácido Desoxirribonucleico)

dNTPs – *Deoxyribonucleotide Phosphates* (Desoxirribonucleotídeos Fosfatados)

EDTA – *Ethylenediaminetetraacetic Acid* (Ácido Etilenodiamino Tetra-acético)

ER (RE) – *Estrogen receptor* (Receptor de Estrógeno)

GRADE – *Grading Recommendations Assessment, Development, and Evaluation*

HE – Hematoxilina–eosina

IB-UNICAMP – Instituto de Biologia da Universidade Estadual de Campinas

MEDLINE – *US National Library of Medicine*

OM – *Omentum* (Omento)

PAI-1 – *Plasminogen Activator Inhibitor-1* (Inibidor da Atividade de Plasminogênio-1)

PBS – *Phosphate-buffered Saline* (Solução de Tampão Fosfato)

PCR - RT – *Polymerase Chain Reaction– Real Time*

PRISMA – *Preferred Reporting Items for Systematic Review and Meta-Analyses*

PROSPERO – *International Prospective Register of Systematic Reviews*

RNA – *Ribonucleic Acid* (Ácido Ribonucleico)

RNAm – *Messenger RNA* (Ácido Ribonucleico Mensageiro)

SciELO – *Scientific Electronic Library Online"*

SC – *Subcutaneous* (Subcutâneo)

SD – *Standard Deviation* (Desvio Padrão)

TRAM – *Transverse Rectus Abdominis Musculus* (Retalho Transverso do Músculo Reto-abdominal)

uPA – *Urokinase-type Plasminogen Activator* (Uroquinase)

WAT – *Write Adipose Tissue* (Tecido Adiposo Branco)

SUMÁRIO

1. INTRODUÇÃO

O grande avanço da oncologia mamária, nas últimas décadas, permitiu maior compreensão da fisiopatologia do câncer de mama, que possibilitou a detecção precoce desta doença com consequente aumento no número de casos tratados, assim como cirurgias mais conservadoras que permitem a reconstrução mamária imediata através de diversas técnicas(1-7). Devido a estes fatores, a reconstrução mamária, procedimento que melhora a autoestima e pode influenciar positivamente a qualidade de vida das mulheres mastectomizadas por câncer, vem sendo realizada com mais frequência (8-12).

Muitas técnicas de reconstrução mamária foram desenvolvidas ao longo dos anos e suas indicações, muitas vezes, são baseadas em fatores relacionados à sequela da mastectomia, às características físicas das pacientes, ao prognóstico do câncer de mama, à qualificação da equipe médica e aos recursos institucionais disponíveis. Apesar de indicações específicas dependentes das situações supracitadas, em muitos casos pode-se optar por diversas técnicas para a reconstrução mamária imediata ou tardia. Dentre os procedimentos mais comumente empregados em nosso meio estão os retalhos cutâneos locais (13), os retalhos miocutâneos pediculados - como os do músculo grande dorsal (14-16) e o retalho transverso do músculo retoabdominal (TRAM) (17-19) -, os retalhos miocutâneos a distância (microcirúrgicos)(20, 21) e o uso de materiais aloplásticos, como expansores teciduais temporários ou definitivos e implantes de silicone(22-24).

Cada uma dessas técnicas de reconstrução mamária apresenta suas limitações e, independente de sua complexidade, resultam em complicações específicas com diferentes graus de morbidade(25). Os retalhos cutâneos locais apresentam grandes limitações, pois só podem ser utilizados para reconstrução de pequenas deformidades. Como complicações são observadas cicatrizes inestéticas, correção inadequada de deformidades, deiscências, necrose total ou parcial do retalho, que podem resultar em cistos ou calcificações visíveis em exames de imagem(25-27).

Dentre os retalhos miocutâneos pediculados, os mais utilizados são o transverso do músculo retoabdominal (TRAM) e o do músculo grande dorsal. Este tem como principal limitação a incapacidade de proporcionar grandes volumes, sendo que para a reconstrução de mamas volumosas é necessária a associação de

implantes mamários. O TRAM, por sua vez, por ser uma cirurgia de grande complexidade, não deve ser realizado em pacientes com comorbidades como diabetes, cardiopatias, obesidades e, por apresentar pedículos localizados no abdome superior, deve ser evitado em pacientes com antecedentes de cirurgias abertas em abdome superior.

No que diz respeito às complicações específicas destas técnicas de reconstrução, a exemplo dos retalhos cutâneos locais, também são observadas cicatrizes inestéticas, correção inadequada de deformidades, deiscências e, principalmente no TRAM, necrose total ou parcial do retalho, que podem resultar em cistos ou calcificações visíveis em exames de imagem. Em adição, estes retalhos apresentam complicações adicionais na área doadora, como seroma (mais frequente nos retalhos de músculo grande dorsal) e herniações ou abaulamentos abdominais (específico do TRAM) (18, 19, 28-31) (32-35).

Em casos de grandes deformidades e graves sequelas de radioterapia, o retalho pediculado de omento, descrito inicialmente em 1963 (36), permanece como uma opção disponível. Como limitações, esta técnica não reestabelece isoladamente o volume mamário, necessitando de um implante e, principalmente, não fornece cobertura cutânea, devendo receber uma cobertura de enxerto de pele. Como principais complicações são descritas a necrose parcial e total do retalho, infecção, hérnia abdominal, e alterações do sistema digestivo resultantes do processo de extração do omento da cavidade abdominal(37-42).

Os retalhos microcirúrgicos são hoje uma ótima opção a todas as técnicas de reconstrução mamária, podendo ser utilizados até mesmo nos casos mais complexos. Apresentam como grande limitação a necessidade de uma equipe cirúrgica altamente especializada, estrutura hospitalar complexa e bem equipada, além do alto custo(21, 43-47). Estes retalhos possuem as mesmas complicações discutidas previamente nos retalhos pediculados(43-45, 48-50).

Os materiais aloplásticos, como expansores teciduais temporários ou definitivos e implantes de silicone, apresentam como limitações a necessidade de boa cobertura tecidual no sítio receptor, sendo sua implantação não recomendada em tórax previamente irradiado. Esta técnica apresenta, ainda, um alto índice de complicação em mamas que serão posteriormente submetidas à radioterapia, se comparada às técnicas que utilizam exclusivamente retalhos autólogos para a reconstrução(25). Suas complicações específicas são a extrusão, contratura

capsular, mau posicionamento e movimentação do implante, interações com doenças do tecido conectivo, além da remota possibilidade de desenvolvimento de linfoma anaplásico de grandes células(51-56). Apresentam também complicações comuns a outras técnicas de reconstrução como o seroma, a infecção e a deiscência cutânea(35, 57-59).

Uma alternativa a estas técnicas convencionais de reconstrução mamária é a lipoenxertia mamária autóloga, também denominada *lipofilling*(60-67). Nesta técnica, a gordura autóloga é obtida por meio de lipoaspiração do tecido subcutâneo, principalmente nas regiões abdominal e quadril, e, uma vez preparado, o tecido adiposo é infiltrado na região mamária (60-62, 66). Tem sua principal indicação para correção de pequenas deformidades ou reconstruções parciais, mas pode ser utilizada isoladamente ou associada a outras técnicas convencionais para reconstrução total(61, 68, 69).

Alguns estudos demonstraram que a lipoenxertia na mama foi capaz de tratar a radiodermite, revertendo úlceras e proporcionando elasticidade a áreas fibróticas(61,70-74). Apesar de o tecido adiposo ter sido descrito para a reconstrução mamária há mais de cem anos(61, 66), apenas nos últimos anos ressurge como um procedimento promissor para o tratamento das deformidades mamárias; entretanto, ainda cercado de dúvidas quanto à sua indicação e segurança (61, 67, 75-80). A técnica chegou a ser banida nas décadas de 1980 e 1990(81) por elevada incidência de complicações, risco oncológico duvidoso e possível dificuldade no seguimento pós-operatório, devido à formação de cistos e calcificações resultantes da liponecrose que poderiam interferir na detecção precoce do câncer de mama.

Apesar das complicações clínicas do *lipofilling* de mama atualmente serem menores do que as das técnicas convencionais - pela recente evolução na técnica do procedimento de lipoenxertia- e do seguimento pós-operatório não ser mais afetado pelas alterações radiológicas - também presentes em técnicas de reconstrução com retalhos cutâneos e miocutâneos locais e a distância -, o seu risco oncológico ainda não está claro (61, 67, 69, 76, 80, 82). A lipoenxertia mamária ainda apresenta mínima comorbidade no sítio doador (podendo até trazer melhora), o que não ocorre com os retalhos cutâneos e miocutâneos, e por utilizar o tecido do próprio paciente, não apresenta complicações específicas dos materiais alógenos como rejeição, contratura capsular, exclusão, entre outros(61, 67, 69, 83).

Mesmo consideradas as inúmeras vantagens da lipoenxertia mamária (baixo custo, com poucas complicações, o que favorece uma recuperação pós-operatória mais rápida), a técnica não é recomendada pela *American Society of Plastic Surgeons* (ASPS) (75) devido ao teórico potencial oncogênico. A dúvida em relação à lipoenxertia mamária é baseada em dados relacionados ao conhecido risco da obesidade para o câncer de mama e de evidências laboratoriais de que as adipocinas produzidas pelos adipócitos podem estimular a proliferação celular, com consequente diferenciação das células-tronco mesenquimais do tecido adiposo (*adipocyte stromal cell,* ASC), produzindo resposta inflamatória crônica, consequente recrutamento de macrófagos e aumento dos níveis da proteína Inibidora de Atividade de Plasminogênio-1 (PAI-1) proveniente dos adipócitos (84-94).

Apesar de todo este apelo teórico acerca do risco de câncer de mama decorrente da lipoenxertia, estudos clínicos e de revisões não conseguiram reproduzir, na prática, este resultado(61, 67, 68, 80). Por falta de evidências clínicas do potencial oncogênico da lipoenxertia mamária, o procedimento é um dos mais realizados hoje no mundo para o tratamento estético e reparador das mamas, apesar de não recomendado por algumas associações de especialidades médicas.

Com base nestas informações, foi proposta uma investigação do potencial risco oncológico da lipoenxertia autóloga nas mamas através da busca de mais evidências clínicas e da análise laboratorial dos elementos questionados (fatores relacionados à obesidade, proliferação celular e inflamação crônica).

2. OBJETIVOS

2.1. Objetivo geral

Avaliar a segurança oncológica da lipoenxertia autóloga para o câncer de mama.

2.2. Objetivos específicos:

2.2.1. Analisar os fatores adaptativos histológicos e morfológicos do retalho pediculado de omento translocado.

2.2.2. Comparar o potencial oncológico do tecido adiposo quando exposto exclusivamente a fatores de risco, como a dieta hipercalórica e a translocação (modelo experimental de lipoenxertia).

2.2.3. Analisar *in vivo* o comportamento do tecido mamário em ratas, quando exposto à lipoenxertia.

2.2.4. Avaliar o risco oncológico clínico do tecido adiposo na topografia mamária, por meio de revisão sistemática sobre o uso da gordura intra-abdominal pediculada (comportamento no sítio receptor semelhante à lipoenxertia) para o tratamento das afecções/reconstruções mamárias.

3. METODOLOGIA

Esta tese consiste de quatro estudos interligados. Os três primeiros são avaliações experimentais da associação da lipoenxertia com a formação de neoplasias da mama, enquanto o último estudo é uma revisão sistemática da literatura pertinente à segurança oncológica da transposição de tecido adiposo autólogo na topografia mamária. Uma síntese das características metodológicas de cada estudo é apresentada a seguir, enquanto os detalhes podem ser avaliados nas publicações referentes a cada um.

3.1. Modelo animal e tamanho amostral para o estudo da lipoenxertia e análise do risco oncológico do tecido adiposo

Foram realizados três estudos experimentais em ratas da raça Sprague-Dawley. O cálculo do tamanho amostral e o animal escolhido foram baseados em trabalhos experimentais já realizados para este tipo de estudo, acrescido de 20% de animais, pensando em possíveis perdas durante o experimento(95-98). Estes estudos resultaram do projeto desenvolvido sob as diretrizes propostas pela Animal Research: Reporting of In Vivo Experiments (ARRIVE)(99), e foi aprovado pelo Comitê de Ética no Uso de Animais (CEA) da Universidade Estadual de Campinas (Unicamp), sob o protocolo de número 2210-1 (Anexo 1). Os ratos foram obtidos no Centro de Reprodução Central da Unicamp com 56 dias de vida. Eles permaneceram no biotério do Instituto de Biologia (IB) da Universidade até a coleta do material para análise. As ratas ficaram isoladas em caixas separadas, limpas diariamente sob um ciclo de luz de 12h/dia, em temperatura constante de 22 ± 2°C. Em cada estudo foram utilizadas ratas de uma mesma ninhada que nunca apresentaram prenhez, com o objetivo de se obter o maior controle possível em relação ao material genético, e com a finalidade de se evitar vieses secundários a fatores que não fossem relacionados ao elemento estudado.

3.2. Estudos experimentais desenvolvidos em retalho de omento para o estudo da lipoenxertia

3.2.1. Modelo experimental para o estudo da gordura enxertada

O tecido adiposo do omento pediculado foi utilizado para simular o enxerto livre de gordura do tecido celular subcutâneo, a fim de se evitar vieses relacionados à absorção imprecisa (de 10% a 90%) na amostra colhida do sítio receptor. Este modelo foi definido utilizando-se os seguintes critérios:

1 - Discussão entre as equipes de fisiologia do IB da Unicamp e docentes de alguns Departamentos da Faculdade de Ciências Médicas da Universidade atuantes em laboratórios de pesquisa (laboratório de patologia especializada, laboratório da sinalização celular) referente à adoção do tecido adiposo do omento para simular o tecido adiposo de subcutâneo para os marcadores analisados;

2 - Revisão da literatura referente à similaridade entre estes dois tecidos(100-105);

3 - Controle amostral de similaridade tecidual (amostra do tecido celular subcutâneo para comparar resultados encontrados com o omento de controle) a fim de se evitar vieses não relatados na literatura ou resultante de qualquer alteração fisiológica específica de um dos tecidos adiposos no decorrer do experimento. Este controle tecidual foi adotado no segundo estudo também para comparar os níveis de proliferação e inflamação entre os tecidos adiposos do omento e do subcutâneo, uma vez que é sugerido um maior potencial inflamatório do omento se comparado ao subcutâneo, e assim evitar resultados falsos-positivo na análise deste estudo(100-102, 104, 106);

4 – Revisão da literatura e experiência pessoal quanto à similaridade de resposta adaptativa do tecido adiposo de omento pediculado quando comparado ao enxerto de gordura livre do subcutâneo(70, 86, 107-113);

5 – Desenvolvimento de um estudo experimental (Experimento 1) para observar, e confirmar, na prática, se a translocação é capaz de exercer um estímulo adaptativo como o ocorrido com o enxerto livre de gordura (não pediculado). Neste estudo foi feita uma análise e comparação do comportamento do tecido adiposo enxertado com vascularização (pediculado) com um seguimento pediculado de omento mantido em seu leito habitual (intra-abdominal).

3.2.2. Desenho do primeiro e segundo estudos experimentais

3.2.2.1. Grupos:

A. **Tecido adiposo exposto à dieta hipercalórica:** tecido adiposo de omento de ratos que receberam oito semanas de dieta de cafeteria.
B. **Tecido adiposo exposto à translocação:** tecido adiposo de omento enxertado no meio subcutâneo de ratos em dieta regular para roedores.
C. **Tecido adiposo de controle:** tecido adiposo de omento dos mesmos ratos que forneceram a amostra exposta à dieta hipercalórica e que oito semanas antes tiveram a dieta substituída por ração regular para roedores.
D. **Tecido adiposo de controle tecidual:** tecido adiposo do subcutâneo de ratos com dieta regular para roedores (dos mesmos animais das amostras anteriores e exposto às mesmas condições das amostras B e C) que foi utilizado como controle para identificar similaridade entre a gordura do subcutâneo e do omento para os testes realizados. A enxertia de gordura do subcutâneo não foi utilizada nestes experimentos com a finalidade de se evitar vieses secundários à reabsorção imprecisa deste tecido nas amostras coletadas de seus respectivos sítios receptores.

3.2.2.2. Dieta:

Com 56 dias de vida (oito semanas), os ratos iniciaram a dieta de cafeteria exclusiva. Esta dieta consistiu de substituição de água por refrigerante *ad libitum* (Coca-cola®) e ração regular para roedores por pastilhas feitas de 37,5% de ração regular para roedor, 25% de amendoim, 25% de chocolate e 12,5% de *cookies*, oferecidas juntamente com *wafer, snacks,* bolos industrializados e pão (4,41 kcal/g, sendo 43,1% de carboidratos; 12,1% de proteína e 46,9% de gordura). A ração regular para roedores Nuvilab® CR-1 (Nuvital, Brasil) tem 2,63 kcal/g(114). A dieta de cafeteria foi mantida por oito semanas (mais 56 dias). Neste período, aos 112 dias de vida, uma amostra de tecido adiposo dos ratos expostos à dieta hipercalórica foi extraída para análise e os ratos passaram a receber dieta regular para roedores por mais oito semanas. Com 168 dias de vida, e após oito semanas de dieta regular para roedores, os ratos foram submetidos à eutanásia e foram coletadas outras amostras teciduais.

Com a dieta de cafeteria foi possível utilizar um controle antes-depois de cada animal e verificar que as alterações teciduais, quando houve, foram decorrentes exclusivamente da dieta e não de outros fatores.

3.2.2.3. Procedimento de coleta do tecido exposto à dieta hipercalórica e translocação do omento pediculado

Foi realizado sob anestesia geral, por meio de injeção intraperitoneal de 0,5 mL de Quetamina (Vetaset® Fort Dodge, 1g/10ml) e Xilesin (Rompum® Bayer, 2g/10ml) na proporção de 3:2, conforme protocolo do IB da Unicamp.

Em seguida, o rato foi colocado em uma plataforma de plástico e contido através de fixação de suas patas com fita adesiva. Foi realizada a tricotomia de uma área de 4X7 cm da região abdominal. Antissepsia com Iodopovidona Tópico com 1% de Iodo Ativo (Riodeine®, Rioquímica) e posicionamento de campo estéril fenestrado descartável. O procedimento foi então realizado da seguinte forma:

1. Incisão mediana da pele de 4 cm de extensão com bisturi, lâmina n^{o}. 15;
2. Dissecção de loja subcutânea de todo hemiabdômen direito, com tesoura romba de Metzembaun curva;
3. Laparotomia com incisão da musculatura abdominal com 3 cm de extensão, bisturi lâmina n$^{o.}$ 15 para acesso à cavidade abdominal;
4. Localização e exteriorização do epíplon da cavidade abdominal com duas pinças de Adison-Brown;
5. Divisão do omento em 3 partes, sendo que:
    a. 1/3 esquerdo: foi mantido na cavidade abdominal (controle);
    b. 1/3 médio: foi coletado para análise (tecido adiposo exposto à dieta hipercalórica);
    c. 1/3 direito: foi translocado para o meio subcutâneo do hemiabdômen direito e fixado à parede abdominal em suas extremidades (para futura identificação) com fio de *nylon* 5.0. O epíplon foi mantido pediculado em sua arcada vascular direita (tecido enxertado).
6. A musculatura foi fechada com pontos contínuos de *nylon* 5.0, exceto o 0,3cm superior, que foi deixado aberto para a passagem do pedículo do omento translocado;
7. A pele foi fechada com pontos contínuos invertidos de *nylon* 5.0.

3.2.2.4. Procedimento de coleta das amostras de controle e do tecido translocado:

1. O rato, após oito semanas da translocação do omento, foi submetido à eutanásia por decapitação através de guilhotina específica, após perda de consciência em câmara de CO_2. (7 L/min).
2. Todo o omento translocado foi identificado e retirado para análise (modelo experimental de tecido adiposo enxertado), assim como todo o omento intra-abdominal remanescente (1/3 esquerdo - controle) e uma amostra de tecido subcutâneo dos flancos.

3.3. Estudo experimental da lipoenxertia mamária em ratas

3.3.1. Desenho do terceiro estudo experimental

3.3.1.1. Grupos:

A. Ratas que não receberam lipoenxertia (Sham): ratas da linhagem Sprague-Dawley, com 16 semanas de vida e que nunca tiveram prenhez (totalizando 18 mamas em três animais).

B. Ratas que receberam lipoenxertia de gordura subcutânea nas seis mamas torácicas: ratas com 16 semanas de vida que receberam lipoenxertia mamária de gordura subcutânea com oito semanas de vida (18 mamas em três animais).

C. Ratas que receberam lipoenxertia de gordura de omento nas seis mamas torácicas: ratas com 16 semanas de vida que receberam lipoenxertia mamária de gordura de omento com oito semanas de vida (18 mamas em três animais).

3.3.1.2. Dieta:

Dieta regular para roedores.

3.3.1.3. Procedimento cirúrgico

As ratas com oito semanas de vida foram divididas em três grupos, totalizando 54 mamas torácicas. Trinta e seis mamas torácicas receberam enxerto de gordura autóloga (de omento ou subcutâneo) preparada por centrifugação. O procedimento foi realizado sob anestesia geral, com injeções intraperitoneais de cetamida (Vetaset®, Fort Dodge Laboratories) e xilazina (Rompum®, Bayer), ambas na dose de 50 mg/kg. Após o procedimento, as ratas ficaram em observação por oito semanas, a fim de se analisar a ação da lipoenxertia mamária sobre o epitélio mamário.

A gordura autóloga de cada rata foi extraída em bloco. O tecido adiposo do omento foi extraído através de uma laparotomia mediana de aproximadamente 4 cm. A gordura de subcutâneo foi coletada através de incisões cutâneas de 2 cm sobre as fossas ilíacas direita e esquerda. Para simular o procedimento da gordura lipoaspirada realizada no ser humano, a gordura em bloco do rato foi microfragmentada manualmente com uma lâmina de bisturi, alocada em uma seringa de 10cc e, em seguida, a sua homogeinização foi complementada através de múltiplas passagens por um emulsificador (Tulip Medical Produts, San Diego, CA, EUA), que dividiu os blocos de gordura em fragmentos homogêneos e pequenos o suficiente para serem injetados com uma cânula de até 22G (0,7mm de diâmetro). Na seringa de 10cc, a gordura foi centrifugada por três minutos a 3000rpm (força centrífuga de aproximadamente 1000G). O conteúdo hidrossolúvel (inferior) e a gordura livre (superior) foram desprezados e apenas as células viáveis (nível médio) foram transferidas para uma seringa de 1cc. Finalmente, aproximadamente 0,2ml do tecido adiposo autólogo preparado foi enxertado, com uma cânula de 0,9mm de diâmetro, em cada uma das seis mamas torácicas de cada rata.

As ratas, após oito semanas da translocação do omento, foram submetidas à eutanásia e, em seguida, foram dissecadas e removidas todas as glândulas mamárias, a pele e o tecido celular subcutâneo de uma área de 1cm de diâmetro ao redor dos mamilos torácicos.

3.4. Obtenção dos dados dos estudos experimentais

3.4.1. Estudo Macroscópico (Experimento 1):

Foram avaliadas as características macroscópicas do tecido adiposo de cada uma das amostras coletadas.

3.4.2. Estudo Histológico

O material obtido foi imerso em soluções de formol tamponado a 10% e enviado ao Laboratório Experimental de Anatomia Patológica da Unicamp. Logo após, foi realizado processamento para inclusão em parafina, e o material foi cortado em micrótomo com 4μm de espessura, colocado em lâminas e corado pela técnica de hematoxilina – eosina (HE). A análise histológica incluiu análise e comparação entre as amostras para o aumento de 400x.

3.4.2.1. Experimento 1:

1) A viabilidade do tecido (presença de necrose);
2) Grau de fibrose;
3) Presença de atipias celulares;
4) Estudo morfológico dos adipócitos.

3.4.2.2. Experimento 2:

Avaliação de atipia celular.

3.4.2.3. Experimento 3:

1) Determinação do número de unidades lobulares ductais terminais;
2) Número de camadas de células epiteliais nas unidades ductais lobulares;
3) Morfologia das células ductais;
4) Análise da presença de ectasia ductal, lesões papilares (projeções) e atipias nucleares.

3.4.3. Marcadores utilizados

3.4.3.1. Ki67

O Ki67 é uma proteína celular presente em células em proliferação e ausente em células em repouso(115-118). A sua utilização nos nossos estudos foi adotada para avaliar a taxa proliferativa das células do tecido adiposo enxertado (por meio de análise da expressão gênica por PCR-RT) e do tecido glandular mamário (por meio do estudo imunoistoquímico).

3.4.3.2. CD68

O CD68 é uma glicoproteína que se expressa predominantemente em macrófagos e está presente em processos inflamatórios e no tecido tumoral(119-127). Desta forma, este marcador foi utilizado para a identificação de inflamação crônica. Como este marcador não é específico dos macrófagos, foi realizada, por um patologista, a análise imunoistoquímica associada à identificação morfológica celular.

3.4.3.3. PAI-1

O PAI-1, apesar de ser primariamente fundamental no processo de coagulação agindo como principal regulador da atividade de plasminogênio *in vivo,* é também um importante componente da reparação tecidual(128-131). Recentes estudos mostraram elevados níveis de PAI-1 na obesidade (atribuindo a sua principal secreção pelos adipócitos) e nas pacientes com câncer de mama, estando envolvidos na diminuição da atividade apoptótica e degradação da matriz extracelular durante o crescimento tumoral, estimulando a sua invasão e disseminação metastática(77, 94, 128-130, 132, 133). Sendo assim, procuramos não apenas mensurar a concentração tecidual do PAI-1 nas amostras analisadas (realizado pelo PCR-RT) como também avaliar o padrão tecidual de sua distribuição e a sua relação com os adipócitos (por meio de análise imunoistoquímica).

3.4.3.4. Receptor de Estrógeno (1D5)

É um anticorpo de receptor alpha de estrógeno utilizado para se pesquisar a positividade das células ductais por meio de análise imunoistoquímica. A sua utilização no estudo foi feita para se analisar o comportamento das células ductais à maior concentração de adipócitos (com consequente elevação na concentração de estrógeno), para evitar vieses relacionados à diminuição da expressão do ki67 pelas células ductais resultantes da positividade destas para o 1d5, e, ainda, para se estudar o equilíbrio entre a expressão destes dois marcadores nas células ductais, que poderiam sugerir um *status* pré-canceroso(87, 117, 118, 134-136).

3.4.4. Imunoistoquímica (Experimentos 2 e 3):

Cortes de tecidos parafinados foram colocados em lâminas histológicas com o adesivo organosilano. Após os tecidos serem desparafinados e banhados em três álcoois para hidratação progressiva, as lâminas foram lavadas em água corrente por três minutos. Para inibição da peroxidase endógena, as lâminas passaram por três banhos de três minutos cada, em solução de peróxido de hidrogênio a 3% e novamente lavadas em água corrente por três minutos. A recuperação antigênica ocorreu pela imersão das lâminas em tampão específico segundo orientações do fabricante de cada *kit*, durante 30 minutos, a cerca de 98 $^{o.}$C, usando panela Pascal. Após o esfriamento das lâminas, foi feita a lavagem em água corrente e água destilada, após o que foram colocadas em solução tampão fosfato (PBS) durante cinco minutos. As lâminas foram incubadas com os anticorpos primários por 30 minutos em estufa a 37$^{o.}$C e, após, incubadas por toda a noite (16-20 horas) a 4$^{o.}$C em câmara úmida. Na manhã seguinte, após três lavagens em PBS de cinco minutos cada à temperatura ambiente, os cortes foram incubados com um polímero marcado com peroxidase por uma hora a 37ºC. A coloração foi realizada com solução de 60 mg de DAB (3,3 tetra-hidrocloreto de diamino-benzidina, Novocastra, código 7169) dissolvidos em 100 ml de PBS com 1 ml de dimetilssulfóxido e 0,5 ml de peróxido de hidrogênio a 10%, com lavagem posterior em água corrente. Seguiu-se contra-coloração com hematoxilina de Harris e nova lavagem em água corrente. Em seguida, as lâminas foram desidratadas com banhos em álcool absoluto e passadas em xilol para posterior montagem com resina Entellan (Merck, cód. 7961).

A positividade da reação foi evidenciada pela marcação da membrana, citoplasma ou núcleo de acordo com a marcação do anticorpo. A leitura das lâminas foi feita em microscópio ótico com objetiva de 400 vezes. Foram avaliados os adipócitos maduros, com citoplasma claro, e comparados com os “imaturos”, não tumorais, que apresentam aspecto vacuolado, e neste caso, contam com diâmetro reduzido. Para avaliação do marcador Ki67 (nuclear, diluído a 1:50; Milipore Darmstadt, Alemanha) foram contadas as células ductais que apresentaram positividade, sendo considerado o valor percentual encontrado em cada amostra(115). O valor percentual das células positivas para o receptor de Estrógeno (ER, clone 1D5, diluído a 1:300; Dako, Glostrup, Dinamarca) também foi realizado para cada amostra. Para a avaliação do CD68 (detecção de macrófagos, clone KP-1, diluído a 1:1000; Cell Marque, Rocklin, CA, EUA)(137) e PAI-1 (diluído a 1:100; Abcam, Cambridge, MA, EUA)(94, 138), foi comparada a concentração absoluta de células marcadas com estes anticorpos em cada tecido analisado.

3.4.5. Expressão RNAm em tecido adiposo por Real Time-PCR (Experimentos 2 e 3):

Para determinação da expressão gênica da proteína PAI-1 (“Plasminogen Activator Inhibitor-1”) e Ki67 envolvida na função dos adipócitos e relacionada à obesidade e câncer de mama, foram realizadas a extração de RNA e análise por PCR-RT. O RNA total foi extraído de aproximadamente 50mg de tecido congelado em nitrogênio líquido com o reagente Trizol (Gibco-Life Technology). A integridade do RNA foi confirmada por eletroforese em gel de agarose a 1,2%, com tampão Tris-borato 89 mmol/L, EDTA 2 mmol/L, e revelado com brometo de etídio. A pureza e quantificação foram determinadas em espectrofotômetro Gene Quant (Pharmacia Biotech). Para verificação de contaminação com DNA genômico, foi realizado PCR com 1 ug de RNA total, o que não produziu qualquer produto de PCR. As amostras que apresentaram contaminação foram re-extraídas até completa descontaminação. Das amostras não contaminadas foi, então, obtido cDNA a partir de 2 ug de RNA total por transcrição reversa usando amplímetros aleatórios (150 ng), dNTPs 10 mmol e 200 U de transcriptase reversa II (Super Script II, invitrogen) em volume final de 20ul, incubação a 42°C por 60 min., seguidos de 15 min. a 70°C para inativar a

enzima, conforme descrito pelo fabricante. A quantificação do RNAm dos genes de interesse foi feita pela metodologia de PCR em tempo real utilizando-se SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), o sistema de detecção Step one Real-time PCR System (Applied Biosystems, Foster City, CA) e pares específicos de *primers* (direto e reverso).

3.5. Processamento e análise estatística dos dados

Os dados adquiridos e anotados na ficha de coleta foram transferidos para planilhas eletrônicas tipo Excel®. Os dados foram analisados através do pacote estatístico SPSS versão 20 para Mac (IBM, Armank, NY – EUA). O nível de significância assumido foi de 5% ($p<=0,05$ e intervalos de confiança de 95%). Variáveis quantitativas foram inicialmente avaliadas quanto à sua distribuição, normal ou não-normal, através dos testes de Shapiro-Wilk e Kolmogorov-Smirnov. Variáveis com distribuição normal foram avaliadas com o teste Anova e as de distribuição não normal foram analisadas com o teste de Kruskal-Wallis.

3.6. Revisão Sistemática

Dados da literatura (70, 86, 107-113), juntamente com nossos estudos translacionais desenvolvidos em retalho de omento para o estudo de um modelo experimental de lipoenxertia, descrevem um comportamento semelhante do podencial adaptativo do omento pediculado com a lipoenxertia, assim como a interação destes com o sítio receptor. Por ser uma estrutura delgada composta principalmente de células de gordura, mergulhadas constantemente em líquido peritoneal, a sua translocação (mesmo que pediculada) para um meio extraperitoneal é capaz de estimular quase a totalidade do seu tecido adiposo. Esta característica faz com o retalho de omento apresente, assim como a lipoenxertia, um grande potencial de neovascularização e regeneração tecidual de seu sítio receptor. Desta forma, o retalho pediculado de omento aparenta exercer os mesmos

estímulos desencadeados pela lipoenxertia em uma área receptora extraperitoneal. Com base nestas informações, a utilização do omento na topografia mamária tem os mesmos estímulos locais observados na lipoxentia das mamas (como nos relatos de reversão de sequelas de radioterapia). Uma revisão sistemática destes dados clínicos representa, desta forma, um aumento na casuística da utilização do tecido adiposo nas mamas, com a possibilidade de se aumentar a observação clínica de sua segurança oncológica.

Foi realizada uma revisão sistemática sobre a utilização do tecido adiposo do omento em topografia de mama feminina a partir da base de dados da ”U.S. National Library of Medicine” (MEDLINE), EMBASE, “Scientific Electronic Library Online” (SciELO), e Google Acadêmico, de janeiro de 1984 a dezembro de 2013 (30 anos), utilizando os termos-chave: “Breast” AND “Omentum” OR “Epiploon”, com adaptação de sintaxe adequada para cada base de dados. Não houve restrição de língua, desde que os títulos dos artigos estivessem em inglês. Acreditamos que com estes termos conseguimos abranger parte relevante da literatura mundial sobre o assunto.

Por meio do protocolo estabelecido pelo Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) (139, 140), o estudo foi registrado no PROSPERO sob o número CRD42013005493(141). Foram incluídos neste estudo apenas os artigos com casos originais. Inicialmente, os artigos foram filtrados pelo título, estes foram também utilizados para a realização de busca manual através de suas referências bibliográficas. Em seguida, os resumos foram filtrados segundo os critérios de inclusão e, por fim, os artigos elegíveis para leitura na íntegra foram selecionados e incluídos. Foi ainda realizado contato, via *e-mail,* com autores de alguns artigos para esclarecimentos, e solicitação do estudo na íntegra quando não disponíveis eletronicamente ou em bibliotecas locais.

Excluíram-se os estudos que não eram casos originais e os que não descreviam a aplicabilidade clínica da técnica ou o seu resultado, assim como os estudos repetidos e/ou com casuística repetida. A seleção final dos artigos incluídos nesta revisão e a análise na íntegra de seus conteúdos foram definidas por três revisores. A qualidade metodológica dos estudos foi avaliada de acordo com critérios previamente definidos para o tipo de estudo, segundo a “Grading Recommendations Assessment, Development, and Evaluation” (GRADE)(142).

Para análise estatística foi calculada a prevalência das técnicas de extração do tecido adiposo do omento, a aplicação clínica da cirurgia (indicação), a incidência de complicações clínicas e a análise do ponto de vista oncológico da evolução das pacientes tratadas. A análise de homogeneidade dos estudos foi realizada com base na qualidade metodológica e, considerando a elevada heterogeneidade encontrada, não foi realizada análise complementar por meta-análise (140).

4. RESULTADOS

Artigo 1. Claro Jr. F, Moreira L, Stocchero G, Pinto G, Pinto-Neto A. The autologous greater omental flap as a structure for extraperitoneal surgical repair: A comparative, paired, and controlled experimental study of its adaptive properties. Rev Bras Cir Plást. 2014;29(1):128-35.

Artigo 2. Claro Jr. F, Morari J, Moreira LR, Sarian LO, Pinto GA, Velloso LA, Pinto-Neto AM. Unmanipulated native fat exposed to high-energy diet, but not autologous grafted fat by itself, may lead to overexpression of Ki67 and PAI-1. *Springerplus.* 2015;4:279

Artigo 3. Claro Jr. F, Moreira LR, Morari J, Sarian LO, Pinto GA, Velloso LA, Pinto-Neto AM. Why is the oncogenic potential of adipose tissue in the laboratory field of breast cancer not observed for lipofilling in clinical practice? (enviado para publicação no *Journal of Mammary Gland Biology and Neoplasia,* anexo 2*)*

Artigo 4. Claro Jr. F, Sarian LO, Pinto-Neto AM. Omentum for Mammary Disorders: A 30-Year Systematic Review. *Ann Surg Oncol.* 2015;22(8):2540-50 (Licença junto à editor: anexo 3).

Artigo 1.

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Original Article ●●●●

The autologous greater omental flap as a structure for extraperitoneal surgical repair: A comparative, paired, and controlled experimental study of its adaptive properties

Retalho autólogo de grande omento como estrutura cirúrgica de reparo extraperitoneal. Estudo experimental comparativo, pareado e controlado de suas propriedades adaptativas.

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ABSTRACT

Introduction: The greater omentum was initially used in the repair of gastrointestinal defects in the 19th century; during the 20th century, it has been used extraperitoneally in the treatment of various disorders, in several surgical specialties. Despite the fact that the greater omentum was studies in detail in the 1960s, there are no reported comparative studies concerning the use of omental flaps extraperitoneally. The present study analyzed the adaptive features of the greater omentum in the extraperitoneal space, with the aim of identifying its surgical applicability. **Methods:** A paired, controlled comparative study was conducted using 20 tissue samples from 5 obese female Sprague-Dawley rats (Rattus norvegicus). The following specimens from each animal were analyzed and compared, macroscopically and microscopically, using the hematoxylin-eosin (HE) technique: (1) omentum without manipulation; (2) intraperitoneally manipulated omentum; (3) extraperitoneally manipulated omentum; and (4) subcutaneous adipose tissue. **Results:** Macroscopically, the extraperitoneal omentum exhibited a more intense yellowish color and a higher degree of contraction than the control (intraperitoneal) omentum. The extraperitoneal omentum was similar in color to the adjacent subcutaneous adipose tissue. HE staining revealed a high degree of fibrosis and an average adipocyte size, similar to that in the control omentum, but lower than that in subcutaneous adipose tissue ($p < 0.001$). **Conclusion:** The results of this study indicate that the extraperitoneal omentum was not able to promote tissue regeneration, as metaplasia of the translocated flap was not observed in the histological analysis. However, this structure may be used to correct small deformities, in the treatment of ischemic areas, as a carrier structure for surgical reconstruction and as a germination platform for the development of new organs.

Keywords: Omentum; Breast; Reconstruction; Epiploon; Metaplasia; Fat

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areas repaired with this structure. Knowledge of the adaptive properties of the extraperitoneal greater omentum may also serve as a basis for future studies, in which omental tissue could be used as a structural framework and means of transportation for tissue regeneration, and even the reconstruction of new organs.

On the basis of these features of extraperitoneal omentum, and its great potential for the future, we undertook a paired comparative experimental study in rats, designed to investigate the structural, anatomical, and histological changes that occur when an omental flap is translocated into the subcutaneous tissues, and assess its potential for metaplasia. The aim of this study was therefore to analyze the adaptive features of extraperitoneal greater omental tissue, and to identify the surgical applications of this structure.

METHODS

Study design and sample size

This was a comparative experimental study, in which subjects were matched and controlled. Tissue samples of female obese Sprague-Dawley rats (n=5, Rattus norvegicus) were used. The rats were obtained at 56 days of age, from the Central Breeding Center of the State University of Campinas (UNICAMP). Obesity was induced to provide greater amount of omental tissue, and enhance the immunohistochemical identification of different adipose tissues, using anti-CD68 labeled macrophages. Obesity was achieved through the administration of the cafeteria diet for an 8-week period. This diet replaces standard rodent food with calorific foods consumed by the human population, such as chocolate, peanuts, bacon, condensed milk, cake, cream, soda, as recommended by Vanzela in 2010[23].

This study was approved by the Ethics Committee on Animal Experimentation (ECAE) of the Institute of Biology (IB), UNICAMP, and all rules relating to the ethical aspects established for the use of animals in research were followed. From the beginning of the fattening period until harvest for analysis materials, rats remained in the animal house of the IB UNICAMP, isolated in boxes with daily cleaning care and illumination (12hours/day) and a constant temperature (20–22°C), and submitted to the cafeteria diet ad libitum.

Surgical procedure

All procedures for translocation of the greater omentum were performed by two researchers. When the rats were 112 days old (weight, 323.8± 4.2g), general anesthesia was induced with an intraperitoneal injection of 0.5ml ketamine (Vetaset® Fort Dodge, 1 g/10 ml) and xylazine (Rompun® Bayer, 2g/10ml) in a 3:2 ratio, as per UNICAMP IB protocol. A maintenance dose was not required, since the total procedure time was 18±5 minutes.

The procedure for translocation of the omentum was performed after trichotomy, antisepsis with polyvinyl pyrrolidone iodine (1% active iodine) and asepsis. The following procedures were conducted as shown in Figure 1: (1) a skin incision of approximately 4cm was made in the midline; (2) dissection

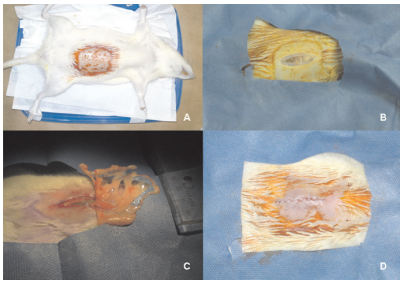

Figure 1. *The translocation process. A – Rat positioned during general anesthesia; B – After antisepsis and positioning of fenestrated sterile field, an incision is made in the midline of the abdomen to access the cavity. C – The omentum is exposed and 2 flaps (2 cm each) were made. One of the flaps was transposed into the subcutaneously; the other remained in the abdominal cavity, and served as a control. The remaining omental tissue was resected and used as a sample of "unhandled omentum." D – After translocation, the incision was closed in the muscular plane; a 1-cm opening at the upper area allowed maintenance of pedicle blood flow. The skin was closed completely with continuous inverted sutures.*

of the subcutaneous tissues of the right hemi-abdomen; (3) laparotomy incision through the abdominal muscles, 4 cm in length; (4) identification and exteriorization of the greater omentum of the abdominal cavity; (5) division of the greater omentum into 3 parts; a left pedicle flap (20mm^2) on the left gastroepiploic branch, remained in the abdominal cavity (control); a right flap (20mm^2) on the right gastroepiploic branch, translocated into the subcutaneous area through the right hemi-abdomen, and attached at the distal portion to the abdominal wall with a nylon 5.0 wire, for future identification. The remainder of the omentum was photographed and collected for analysis; (6) the muscle was closed with continuous stitches using nylon 5.0 wire, with the exception of the upper 0.3 cm, which was left open for the passage of the translocated omentum pedicle; (7) the skin was closed with continuous reversed nylon 5.0 wire stitches, and covered with micropore paper tape. Following the procedure, the rats were placed in the lateral position under thermal lamps for anesthetic recovery.

Preparation and macroscopic anatomical analysis

Eight weeks after surgery (168 days old; mean weight, 351 ± 26.92g), the rats were euthanized. At this time, 3 tissue samples were identified, photographed, and resected from each rat: the control omental flap, the translocated omental flap, and a sample of subcutaneous adipose from the left iliac fossa. The left iliac fossa was chosen as it is an area with abundant adipose tissue, distant from the site of omental translocation, and without local influence on inflammatory processes.

A macroscopic comparison of tissues, with reference to contraction and extent of staining, was performed by the 2 researchers who had conducted the translocation procedure, and removed the tissue samples after euthanasia. As both the translocated and control flap had a pedicle, it was not possible

to accurately measure the volume during the translocation process. Quantitative measurement of macroscopic contraction of the omental flaps, and volumetric loss of these structures, could not be carried out, although all flaps were of the same initial size (20mm^2).

Preparation and histological analysis

A microscopic analysis of the omental flaps was made by a pathologist, blinded to tissue origins; the obtained samples were identified only by numbers and letters. Only the researchers who performed the procedures of preparation and extraction knew the origin of the analyzed parts. Each collected sample was immediately identified and introduced in a bottle with 10% buffered formalin.

In the pathology laboratory, the tissue samples were processed; a slide was made from each sample and stained using the hematoxylin-eosin method (HE) to evaluate the following: (1) fibrosis, (2) adipocyte diameter, and (3) the presence of different cell populations to those usually present in the omentum, i.e. metaplasia.

Fibrosis was analyzed by a pathologist, using a simple optical microscope. To analyze the diameter of adipocytes, 100× images were captured using a digital camera (Leica DFC360 FX, Solms, Germany) connected to a clear field microscope (Leica DM5000 B). These images were evaluated by using image analysis software (Leica Qwin Standard V3 Imaging Microsystems), to measure the diameter of 100 random adipocytes, counted by a semi-automated procedure in the program.

Statistical analysis

Data from each rat and from each tissue sample collected were analyzed statistically. Changes in the rat's weight, and the average diameter of adipocytes, were analyzed using the Student t-test, paired in independent pairs. The significance level was 5% and the software used for analysis was SAS version 9.2.

RESULTS

Macroscopic anatomical analysis

At 8 weeks after the translocation the average weight of the rats increased from 323.8 ± 34.16 g to 351.0 ± 26.92 g (p = 0.012, 95% CI -44.68 to -9.72; Figure 2). During this period, complete healing of the surgical wound was observed, with full hair growth on the site (Figure 3A).

Macroscopically, omental tissue translocated into the subcutaneous environment was a more intense yellowish color, similar to adjacent subcutaneous adipose tissue (Figures 3B and C); this was in contrast to the lighter shade observed at the time of translocation, and still present in the intra-abdominal control omental flaps (Figure 3C).

Regarding the degree of contraction, the translocated omentum showed severe contraction, compared with the intra-abdominal omental control; the contracted omentum was thin, with an elastic-fibrous consistency (Figure 3C). The postoperative pedicle integrity was confirmed (Figure 3D).

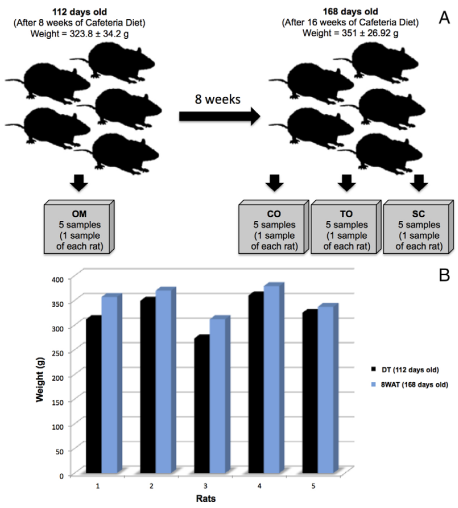

Figure 2. A – *After 8 weeks of cafeteria diet, rats (112 days of age; mean weight, 323.8 ± 34.2 g) underwent surgery for omental translocation to a subcutaneous site. At this time, a sample of omentum (OM) from each mouse was identified, extracted, and photographed. At 8 weeks post-surgery, the rats were euthanized (168 days of age; mean weight, 351 ± 26.92 g). Three tissue samples were identified, photographed, and extracted: (i) postoperative omental control flaps (CO); translocated omentum (TO); and a fragment of subcutaneous adipose tissue (SC) from the left iliac fossa. B – Graph depicting weight progression of each mouse from translocation (DT) until 8 weeks post-translocation (8WAT). The average weight increased significantly during the 8-week postoperative period.*

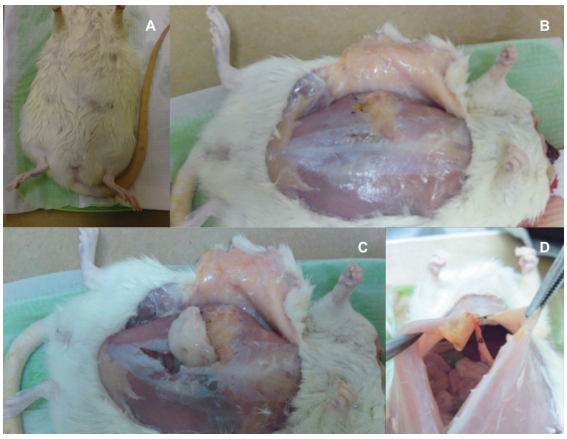

Figure 3 *(A) – A wound, showing complete healing 8 weeks following surgery. B – Translocated omentum set in the right upper quadrant of the abdomen. C – Coloration of the translocated omentum, similar to that of subcutaneous adipose tissue, but different to that of control omentum. D – Confirmation of pedicle integrity.*
a região abdominal.

to accurately measure the volume during the translocation process. Quantitative measurement of macroscopic contraction of the omental flaps, and volumetric loss of these structures, could not be carried out, although all flaps were of the same initial size (20mm²).

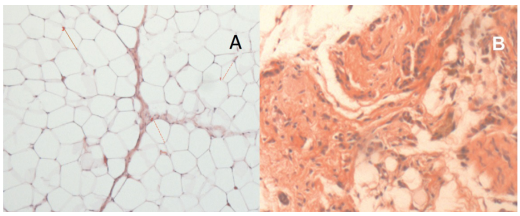

Figure 4 *(A) - Microscopic analysis to measure the diameter of 100 random adipocytes, and the histological appearance of the unhandled omentum (OM) (HE, 100×). (B) – Histological appearance of the translocated omentum (TO), showing a high degree of fibrosis (HE, 100×).*

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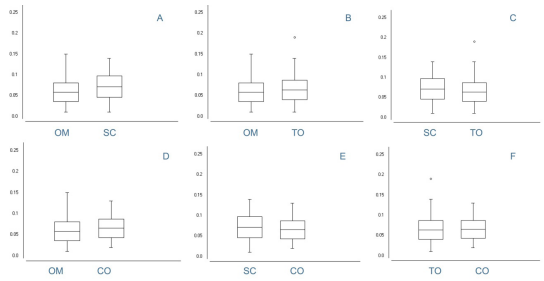

Figure 5. *Average diameter of adipocytes (Student t-test for independent variables). A – Unhandled omentum vs. subcutaneous adipose tissue, p < 0.0001 B – Translocated omentum vs. unhandled omentum, p < 0.0001. C – Translocated omentum vs. subcutaneous adipose tissue, p < 0.0001. D – Unhandled omentum vs. control omentum, p < 0.0001. E – Control omentum vs. subcutaneous adipose tissue, p < 0.0001. F – Control omentum vs. translocated omentum, p = 0.27.*

Table 1. Features of the omentum (before and after manipulation in the abdominal cavity (control), and after translocation into the subcutaneous tissue), and subcutaneous adipose tissue

	Omentum	SC	Translocated Omentum	Control Omentum
Time of Tissue Extraction	During Translocation	Eight Weeks after Translocation		
Rats*				
Body Weight (g) Mean ± SD	323.88 ± 34.16	351 ± 26.92**		
Number	5	5	5	5
Anatomical Analysis				
C.T.	Smooth	Elastic	Fibrous-elastic	Smooth
Colour	White	Light Yellow	Light Yellow	White
D.C.	N/A	N/A	Intense	Apparently Absent
Histological Analysis with Haematoxylin-Eosin (HE)				
V.T.	N/A	N/A	Yes	Yes
D.F.	N/A	N/A	High	Low
A.C.R.	N/A	N/A	No	No
M.A.D. (μm)	0.059	0.071	0.064***	0.065

N/A, Not applicable; C.T., Consistency of the tissue; D.C., Degree of contraction; V.T., Viable tissue; D.F., Degree of fibrosis; A.C.R., Atypia or cell replacement; M.A.D., Median adipocyte diameter in μm (interquartile range). *Each of the five rats provided the four different adipose tissue samples, and the sample of omentum without manipulation of was extracted eight weeks before the other three samples. **Rats' weights were significantly greater eight weeks after translocation. *** Adipocytes of the TO significantly differ of those of the OM and SC.

Fibrosis was analyzed by a pathologist, using a simple optical microscope. To analyze the diameter of adipocytes, 100× images were captured using a digital camera (Leica DFC360 FX, Solms, Germany) connected to a clear field microscope (Leica DM5000 B). These images were evaluated by using image analysis software (Leica Qwin Standard V3 Imaging Microsystems), to measure the diameter of 100 random adipocytes, counted by a semi-automated procedure in the program.

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Histological analysis

Under the microscope, it was apparent that all samples were from viable flaps (no necrosis). In the translocated omentum, there was a higher degree of fibrosis, compared with all other samples. No tissue types, besides those customarily present in the omentum, were identified in flaps samples (Figure 4).

Morphological assessment was used to ascertain the average diameter of omental adipocytes (0.059 µm), subcutaneous adipocytes (0.071 µm), translocated omental adipocytes (0.064 µm), and adipocytes from intra-abdominal omentum / control (0.065 µm) (Table 1). Unhandled omental adipocytes had a smaller diameter than those of subcutaneous adipose tissue (p < 0.001; 95% CI, -0.016 to -0.010). There was a statistically significant difference between the diameter of translocated omental adipocytes and the unhandled omental adipocytes (p = 0.0014; 95% CI, -0.008 to -0.003), and those from subcutaneous adipose tissue (p < 0.001; 95% CI, 0.004 to 0.010). Adipocytes from the translocated omental flap did not differ significantly from the control (p = 0.27) . (Figure 5).

DISCUSSION

Despite our detailed knowledge of the greater omentum and its widespread clinical applicability, in various medical specialties, there is a lack of comparative studies focusing on the adaptive properties of extraperitoneal omentum. The majority of studies that have examined the surgical and therapeutic potential of extraperitoneal flaps of the greater omentum have focused either on the recipient tissue or on the treated anatomic structures and the mechanisms involved[6-11,14,16].

The present study focused on evaluating the adaptive properties of translocated extraperitoneal omentum. It was therefore possible to compare important tissue changes, not previously reported in the literature. Regarding the postoperative time before collection of material for analysis i.e. 8 weeks after the omental translocation, the skin scar showed complete healing, with the regrowth of hair in the abdominal region (Figure 3A). This indicates that complete regeneration had occurred during the 8-week postoperative period, which is more than twice the duration of rat gestation (3 weeks), and is considered sufficient for subsidence of any inflammatory response to the procedure (including confirmed low concentration of macrophages by immunohistochemistry) and complete adaptation or metaplasia of the transposed flap.

Considering the anatomical evaluation, the translocated omentum showed the same characteristics as adjacent subcutaneous adipose tissue, with a light yellow color, a dense appearance, and an elastic-fibrous consistency; this is distinct from the control omentum, which retained the same features as the unhandled omentum. These findings suggest contraction of the greater omentum, possibly resulting in lack of peritoneal fluid, may be a contraindication to the use of this structure as a fill tissue or for volumetric gain. However, the high degree of fibrosis and the elastic-fiber consistency resulting from the extraperitoneal translocation may be useful for the treatment of deformities and repairs that require structural support or coverage without volume. The omentum can also be effectively used as a tissue or cell carrier, for tissues or organs in almost all body regions[12,16].

With regard to the HE stained histological analysis, the translocated omentum showed the same tissue pattern and the same cell morphology as control adipocytes, differing only in terms of the presence of moderate fibrosis. The absence of peritoneal fluid is apparently the main factor responsible for these adaptive changes to the omentum in the subcutaneous space. The contraction of the translocated omentum, and the absence of contraction in the control omentum may be due to dehydration and greater fibrosis[18-21,24].

As the subcutaneous adipose tissue is known to be different from the greater omentum[17-21], it was expected that the mean diameter of adipocytes in these two tissues would differ; however, the influence of translocation on an omental flap had previously been described. Data regarding differences between adipocytes of the greater omentum and the subcutaneous adipose tissue were statistically confirmed in the present study.

Regarding the comparison between the translocated flap and the other tissue samples, it was identified that the average diameter of adipocytes in the translocated omentum was similar to that of the control omental flap, but significantly different to that of the unhandled omentum and subcutaneous adipose tissue. These results suggest that the peritoneal fluid may predominantly influence the extracellular matrix of the omentum and its population of floating cells, with little effect on adipocytes. The two handled flaps had the same average adipocyte diameter, which was significantly smaller than that of adipocytes found in the subcutaneous adipose tissue. This suggests that the observed anatomical transformation stems mainly from changes in the extracellular matrix of the omental adipose tissue, and that there is no indication of replacement in the cell population by subcutaneous adipocytes. The statistically significant difference between the diameter of omental adipocytes without manipulation that was smaller than the diameter of the adipocytes in the omental flaps (control omentum flaps and translocated flaps) may have resulted in the significant fattening of animals, which occurred during the 8 weeks between the initial procedure and euthanasia of the rats (Table 1).

The greater omentum is an omnipotent source of stem cells (cells capable of differentiating into other cell types of the same embryonic origin) and has great angiogenic potential,

restoring ischemic tissues and actinic lesions [3,6,8,10,14,16]. Translocated flaps are in a different location to the site of origin, and may therefore be exposed to metaplasia, by the same principles observed in Barrett's esophagus or in metaplasia of intraoral skin flaps; hence, it was important to investigate whether there is substitution of the transposed tissue cell type. The results of our histological assessment did not show the presence of cell metaplasia in the translocated omental flap; no atypical tissue type was observed, and there was no change in adipocyte diameter between paired samples, when compared with the control omentum.

In mammals, the adipose tissue of the subcutaneous tissues, bone marrow, and intra-abdominal cavity have structural and functional differences, and distinct characteristics, despite being mainly formed of adipocytes[25,26]. In the present study, morphological assessment indicated that the extraperitoneal omental adipocyte was smaller than the adipocytes of other samples. Consequently, it can be inferred that these adipocytes partially lost their functional capacity or energy reserve, and started to serve a fill tissue function, probably similar to adipose tissue of the bone marrow. This is likely due to tissue adaptation. Therefore, the extraperitoneal omental flap does not seem to be able to transform the recipient tissue, despite its high concentration of omnipotent stem cells and its great angiogenic potential.

The findings of this study demonstrate that the omentum, similar to subcutaneous adipose tissue, is a rich source of material that can be used in the reconstruction of various organs and body structures, as a tissue filler and support, without any apparent ability to regenerate organ or tissue function[27,28]. In addition, the angiogenic potential of the omentum allows the revascularization of ischemic areas, such that the translocated omentum could serve as a carrier structure for surgical reconstruction, and a germinating platform for the development of new organs[16,29,30]. However, omental tissue appears inappropriate, when used alone, for large reconstructions, tissue regeneration, or volume replacement.

CONCLUSION

In conclusion, in the subcutaneous environment, the translocated omentum undergoes contraction and volume loss. Although translocated omentum appears macroscopically similar to subcutaneous adipose tissue, microscopically it maintains the same structural and morphological pattern of adipocytes present in the intra-abdominal environment, with no signs of metaplasia. Therefore, the extraperitoneal omentum appears incapable of promoting tissue regeneration, although it could be used in the correction of small deformities, for the treatment of ischemic areas, as a carrier structure for surgical reconstruction and as a germinating platform for the development of new organs.

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Artigo 2.

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Unmanipulated native fat exposed to high-energy diet, but not autologous grafted fat by itself, may lead to overexpression of Ki67 and PAI-1

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Abstract

Background: Although its unclear oncological risk, which led to more than 20 years of prohibition of its use, fat grafting to the breast is widely used nowadays even for aesthetic purposes. Thus, we proposed an experimental model in rats to analyze the inflammatory activity, cellular proliferation and levels of Plasminogen Activator Inhibitor (PAI-1) in grafted fat, and in native fat exposed to high-energy diet in order to study the oncological potential of fat tissue.

Methods: Samples of grafted fat of rats on regular-energy diet were compared with paired samples of native fat from the same rat on regular-energy diet and on high-energy diet in a different time. Analysis involved microscopic comparisons using hematoxylin-eosin staining, immunohistochemistry with anti-CD68-labelled macrophages, and gene expression of Ki-67 and PAI-1.

Results: Hematoxylin-eosin staining analyses did not find any atypical cellular infiltration or unusual tissue types in the samples of grafted fat. The inflammatory status, assessed through immunohistochemical identification of CD68-labelled macrophages, was similar among samples of native fat and grafted fat of rat on regular-energy diet and of native fat of rats on high-energy diet. Real-time PCR revealed that high-energy diet, but not fat grafting, leads to proliferative status on adipose tissue (overexpression of ki-67, $p = 0.046$) and raised its PAI-1 levels, $p < 0.001$.

Conclusion: While the native adipose tissue overexpressed PAI-1 and KI67 when exposed to high-energy diet, the grafted fat by itself was unable to induce cellular proliferation, chronic inflammatory activity and/or elevation of PAI-1 levels.

Keywords: Fat grafting, Breast cancer, Lipofilling, PAI-1, Ki67, CD68

Background

In 1895, Czerny described the first breast reconstruction, which was performed with adipose tissue, using a large lipoma from the dorsal flank to fill defects resulted from the excision of a breast benign lesion (Claro et al. 2012). Since then, the adipose tissue (as fat grafting or pedicle flaps from great omentum or subcutaneous tissue) became often used for breast reconstruction (Claro et al. 2012, 2015; Abbott and White 1986; Calderoli and Keiling 1985; Góes 2010; Illouz 1988; Kiricuta 1963). Its popularity though, appeared after the advent of liposuction in the 1970s, when the aspirated fat harvested from many body areas could be reinjected to the breast (Claro et al. 2012). So, the autologous fat grafting began to be used for aesthetic purposes as well, once it is performed using a non-immunogenic substitute/filler, through a versatile and inexpensive procedure obtained usually without donor site morbidity (Claro et al. 2012).

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However, it was suggested that adipose tissue might represent an oncological risk for breast cancer, what led the American Society of Plastic Surgeons to prohibit its use to the female breast in 1987 (Claro et al. 2012; ASPRS 1987; Chalmers and Newing 1986). Most of this theory was raised after some studies have correlated the higher potential of breast cancer in obese people (Carter and Church 2009, 2012). Long since great focus to adipose tissue in obesity has been given, such as its comparisons between the omentum and subcutaneous tissue in many disorders like diabetes and cancer (Harman-Boehm et al. 2007; Rodbell 1964; Tam et al. 2012; Weisberg et al. 2003). The most notable hypothesis related to adipocytes and breast cancer is based on the inflammatory potential of these cells, which release adipokines that may lead to chronic inflammation and cell proliferation (once the risk for metabolic disorders and breast cancer seems to be higher in obese people) (Claro et al. 2012, 2015; Chalmers and Newing 1986; Carter and Church 2009, 2012). However, this hypothesis has not been sustained for the procedure of lipofilling to the breast in clinical practice.

Thereupon, the lack of evidence for lipofilling and breast cancer almost 20 years after its prohibition lead to the publication of some case series reporting breast reconstruction with fat grafting in early 2000's, with good results and without report of higher cancer recurrence than other well established reconstruction procedures (Claro et al. 2012; Góes and Macedo 2006). This demanded in 2009, a review of the prohibition imposed by the American Society of Plastic Surgeons that, owing to lack of evidence, failed to prohibit the use of autologous adipose tissue to the breast, although they do not recommend it (Gutowski and Force 2009).

Since then, many cases series and reviews remain failing to demonstrate higher recurrence rate of breast cancer among women treated with fat grafting (Claro et al. 2012, 2015). So, great effort in experimental field has been made in order to analyze the oncological potential of fat cells to the breast. Some laboratorial studies have focused on adipocytes in vitro, while others have analyzed fat cells from people due to the great difficulty in established an effective experimental model in vivo (Carter and Church 2009, 2012; Baglioni et al. 2009; Baumert et al. 2007; Lin et al. 2001; Wyckoff et al. 2004). Most of these studies even compared fat cells from different fat compartments and/or from people in different conditions, such as obesity and non-obesity. Regarding these issues, great focus to Plasminogen Activator Inhibitor-1 (PAI-1) complex has been taken, because it is a protein found in high concentration in adipose tissue of obese people and/or of breasts with cancer and so, can be used as a marker for them (Carter and Church 2009, 2012; Harman-Boehm et al. 2007; Baglioni et al. 2009; Andreasen et al. 2000; Bianchi et al. 1995; Binder et al. 2002; Cojocaru et al. 2012; Condeelis and Pollard 2006; Dhanasekaran et al. 2012; Di Gregorio et al. 2005; Gomes-Giacoia et al. 2013; Goswami et al. 2005; Gutierrez et al. 2000; Lin et al. 2002; Sumiyoshi et al. 1991). Some of these studies were able to demonstrate the oncological potential of the adipocytes, primary in obese subjects. However, this potential remains uncertain for the procedure of lipofilling to the breast by itself, and was not observed in clinical practice. One believed reason for this, raised by us, is that the breast bed, even after a complete resection of mammary tissue, remains with a large amount of native adipose tissue. So, for locally oncological potential, the adipokines released from transposed/grafted fat cells would be the same of those released from native adipocytes that are already surrounding any remaining mammary glandular cell. And systemically, considering that the donor fat cells are from the same patient that will receive them, the serum concentration of any pro-oncological factor resulting from these adipocytes will remain the same after the procedure.

Moreover, beyond the adipocyte, some theories raised doubts regarding the procedure by itself, due to the great angiogenic potential of the adipose tissue observed in some clinical and experimental studies (Dhanasekaran et al. 2012; Figueiredo et al. 2010; Goldsmith et al. 1967; Liebermann-Meffert 2000; Morison 1903; O'Shaughnessy 1937; Oloumi et al. 2006; Williams and White 1991), that may lead to chronic inflammation and cell proliferation to the microenvironment of lipofilling host site and its surroundings. Thus, there are some issues that must be considered when studying fat grafting: (1) the unpredictable amount of grafted fat present in the sample extracted from the host site, because the long-term graft retention is inaccurate and its absorption can vary from less than 10% to more than 90%; (2) as fat graft is composed not only by adipocytes but also by many other cells and components present in fat tissue, the whole elements of the adipose tissue though, must be analyzed after fat grafting; (3) the grafted tissue must be analyzed in vivo in order to study its behavior in the host site and of its surroundings and; (4) the subjects must be genetically similar and, as controlled as possible, in relation to its diet intake and lifestyle.

Thus, an experimental model was proposed taken into account all the challenges reported above in order to analyze the carcinogenic potential of autologous fat grafting procedure focusing on its main suggested threats to the host site (higher concentration of PAI-1, chronic inflammation activity and higher proliferation rate). We also aimed to compare the findings for this potential resulted from the procedure by itself to those represented by the

unmanipulated native fat tissue when exposed to high-energy diet.

Methods

Study design and sample size

A comparative, paired and controlled experimental study was conducted using eight female Sprague–Dawley rats (*Rattus norvegicus*) from the same dam; obtained at 56-day-old from the Central Reproduction Centre at the State University of Campinas (UNICAMP), Campinas-SP, Brazil. The Sprague–Dawley rat was chosen because it is a breed prone to develop breast cancer when exposed to some risk factors (Russo and Russo 1996; Russo et al. 1983; Shan et al. 2004). Considering that a variable (PAI-1 levels) was used as positive and a negative control for the same tissue of the same rat in different times, a sample size of three rats was defined as enough. However, considering possible exclusions during the evolution of the study, the sample size was set to eight rats.

The study followed the ARRIVE guidelines (Kilkenny et al. 2010) and was approved by the UNICAMP Committee for Ethics in Animal Research (protocol no. 2210-1). The rats remained in the animal-breeding center at the Institute of Biology, UNICAMP from the beginning of the fattening period to the collection of the material for analysis. The rats were housed in isolated boxes that were cleaned daily under a 12-h/day light cycle at a constant temperature of 22 ± 2°C. An overview of the study is summarized in Figure 1.

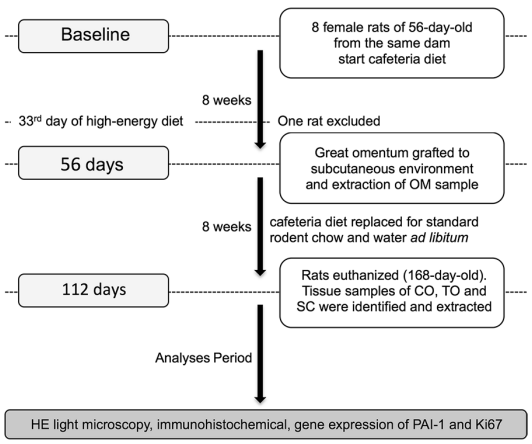

Figure 1 Flowchart overview of the study. *OM* unmanipulated native fat tissue of omentum of rats on cafeteria diet regimen (high-energy diet), *CO* native fat tissue of omentum of rats in regular-energy diet, used as post-operative control, *TO* grafted fat tissue of omentum of rats in regular-energy diet, *SC* fragment of unmanipulated native subcutaneous adipose tissue from the left iliac fossa used as control for fat tissue similarity, *HE* hematoxylin-eosin.

Experimental model proposed and adipose tissue chosen

In order to avoid bias related to the unpredictable amount of grafted fat present in the sample extracted from the host site, an experimental model was proposed using a pedicle fat graft of great omentum, instead of free fat graft from subcutaneous tissue, based on deep study of the literature, and discussions among researchers from some departments of biological institute and medical school at UNICAMP. Although they are from different embryological origin, the adipose tissue from omentum and subcutaneous tissue have the same cellular composition, with similar regenerative and physiological function, and even the same amount of stem cells able to differentiate into any mesenchymal tissue (Cancello et al. 2006; Dicker et al. 2009; Toyoda et al. 2009). A difference identified between these two adipose tissues is the greater metabolic and inflammatory potential of the great omentum if compared to the subcutaneous tissue. These issues are stated as oncogenic risk for breast cancer, what became this model of fat grafting using fat tissue of omentum, instead of subcutaneous, even more sensitive. Thus, in order to avoid bias related to this and false-positive results, a control of native subcutaneous fat of the same rat was used as control for fat tissue similarity.

Dietary regimen and composition of diet

Cafeteria diet was used for 56 days, until the time of great omentum grafting (rats of 112-day-old), so we were able to analyze the carcinogenic potential of high-energy foods consumed by humans in adipose tissue. After the great omentum translocation, the cafeteria diet was replaced for standard rodent chow and water ad libitum for more 56 days (until rats' age of 168-day-old).

The cafeteria diet consists of replacement of water for soft drinks ad libitum (Coca-cola®) and of standard rodent chow for a pellet made of 37.5% standard rodent chow, 25% peanuts, 25% chocolate, and 12.5% cookies, offered together with wafer, snacks, cakes, and biscuits (4.41 kcal/g, 43.1% from carbohydrates; 12.1% from proteins, and 46.9% from fats). The standard rodent chow diet Nuvilab CR-1 (Nuvital, Brazil) has 2.63 kcal/g (Vanzela et al. 2010). The cafeteria diet was used at the beginning of the study when the rats were younger and so, less prone to bias due to other metabolic disorders related to age. With the cafeteria diet we were able to confirm that the changes in results were only due to the replacement of the diet on the same rat (from high-energy to regular-energy) and so, analyze the influence of diet over the fat tissue and its oncological potential.

Surgical procedure

The rats underwent general anesthesia (intraperitoneally) at 112-day-old. The omentum grafting process was

performed through a midline skin incision of approximately 4 cm. Subcutaneous dissection of the entire right hemi-abdomen followed by laparotomy with a 4-cm incision at abdominal midline. The great omentum was divided into three parts (three samples for the study): (1) one was made with 20 mm^2 of the left pedicle flap in the left gastroepiploic branch and remained in the abdominal cavity (fat tissue control—CO); (2) a portion of the right flap (also 20 mm^2) based on the right gastroepiploic branch was grafted into the subcutaneous layer in the upper right abdomen and fixed to the abdominal wall at its ends with 5.0 nylon (transposed omentum—TO); (3) the remaining central portion of omentum was used to analyze the influence of high-energy diet over fat tissue (omentum on high-energy diet—OM). The abdominal wall was closed with running sutures of 5.0 nylon except the upper 0.3 cm, which was left open for the passage of the translocated omental pedicle. Finally, the skin was closed with running sutures of 5.0 nylon.

The rats were euthanized at 8 weeks post-operatively (168-day-old). Three tissue samples were identified and extracted: native fat tissue of omentum (CO), the grated fat tissue (TO); and a fragment of unmanipulated native subcutaneous adipose tissue (SC) from the left iliac fossa (an area with abundant adipose tissue distant from the manipulated surgical site, thus free from any postoperative inflammatory process).

Histological preparation and analysis

Each sample was immediately identified and introduced into a vial containing 10% buffered formalin. The tissue samples were processed in the pathology laboratory. Sections of 4 μm of each sample were placed on glass slides, dehydrated and stained with hematoxylin-eosin (HE). The evaluation was performed by a single pathologist blinded to the origin of the tissues (with the samples identified only by numbers and letters) with light microscopy, in order to identify the presence of cell populations that differ from the normal cells in the adipose tissue.

Immunohistochemistry

Immunohistochemistry using monoclonal anti-mouse anti-CD68 antibodies (clone Kp-1, Advance, Dako, Glostrup, Denmark) was used to identify the macrophage concentrations in each tissue type (Weisberg, Harman-Boehm) according to the manufacturer's instructions. A single pathologist, blinded to the origin of each sample, performed the immunohistochemical analysis. The number of anti-CD68 stained macrophages was counted in ten different randomly chosen areas in each processed slide at 40× magnification for each tissue sample. A cell was considered positive when the morphological aspects of a macrophage were observed with a marked cytoplasm outside the vascular lumen (Cancello et al. 2006; Aron-Wisnewsky et al. 2009).

Real-time PCR

Total mRNA extraction was performed using TRIzol® Reagent protocol (Life Technologies, #15596018). Ki67 and PAI-1 mRNA expression were measured in all groups by Real Time PCR (ABI Prism 7500—Applied Biosystems). The primer Ki67 (Rn.PT.58.8428180.g) was obtained from Integrated DNA Technology (IDT), and PAI-1 (Rn01481341_m1) were purchased from Applied Biosystems. GAPDH (#4352339E—Applied Biosystems) was used as endogenous control. Each PCR contained 40 ng of reverse-transcribed RNA, 0.25 μl of each specific primer, Taqman Universal master mix (#4369016—Applied Biosystems), and RNase free water to a 10 μl final volume. Real-time data were analyzed using the Sequence Detector System 7500 (Applied Biosystems).

Statistical analyses

The normal distribution of the data was assessed using the Kolmogorov–Smirnov and Shapiro–Wilk tests. As the data were normally distributed, there were used paired t-test and analysis of variance (ANOVA, one way, and repeated measures) and Tukey test (post hoc) paired t-test for statistical comparisons. The values are given as mean ± standard deviation (SD) and the significance level was 5%. The software used for the analysis was SPSS version 20 for MAC (IBM; Armonk, NY—EUA).

Results

One of the rats died on the 33rd day of high-energy diet, at 89-day-old, before the procedure of adipose tissue grafting. Then, seven rats remained and were used for analyses in this study. A complete healing of the surgical wound with full hair growth at the site was observed at time of samples extraction (rats' age of 168-day-old). All samples revealed viable flaps under microscopic analysis (without necrosis). No atypical cellular infiltration or unusual tissue types were observed in the samples of grafted fat.

High-energy diet or fat grafting did not influence the inflammatory activity involving CD68 macrophages in fat tissue

Immunohistochemical analyses of the average concentration of CD68-labelled macrophages (Figure 2) in unmanipulated native fat tissue samples of rats fed with high-energy food (OM) was 10.00 ± 4.02 macrophages/field in 10 fields per animal; the average among samples of those fed with standard rodent chow was 10.17 ± 4.17 in control native fat (CO); 6.60 ± 1.75 in grafted fat (TO), and 19.33 ± 5.60 in the subcutaneous fat used as control

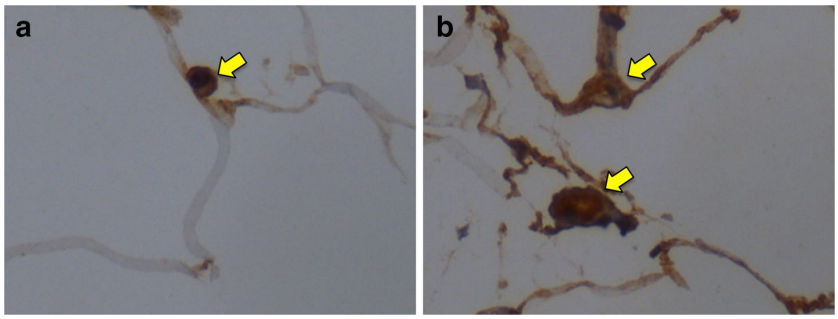

Figure 2 CD68-labelled macrophages (*yellow arrow*) in **a** fat tissue of rats fed with high-energy food (OM) and in **b** grafted fat of rats in regular-energy diet (TO) (magnification ×400).

for fat tissue similarity (SC). What represents that the inflammatory activity involving CD68 macrophages among all the adipose tissue samples were similar $p = 0.246$ (CI 95% 7.03–16.17), Figure 3.

The high-energy diet represents higher proliferation rate in the adipose tissue environment

The gene expression level of Ki67 (Figure 4a) in the CO samples was 0.52 ± 0.03. A similar pattern was observed in the SC (0.51 ± 0.11) however; the level of 1.20 ± 0.00 was significantly higher in the OM (ANOVA OM vs. CO and SC: $p = 0.046$, CI 95% 0.41–1.03).

The high-energy diet, but not fat grafting procedure, leads to elevation of PAI-1 levels in adipose tissue

Gene expression of PAI-1 (Figure 4b) revealed that its level in the OM of 12.63 ± 3.07 is significantly higher than those identified in CO (1.13 ± 0.32), SC (0.29 ± 0.12) and TO (3.44 ± 1.58), $p < 0.001$, CI 95% 0.01–6.88. The ANOVA test shows similar low levels of PAI-1 gene expression ($p = 0.70$) among samples of rats fed with standard rodent chow (CO, TO and SC).

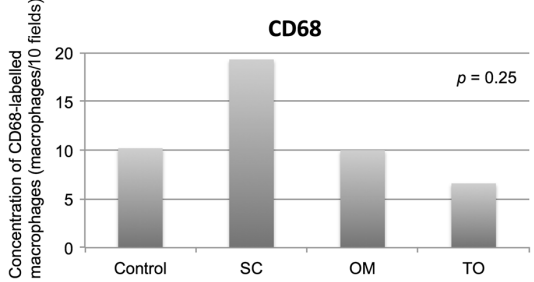

Figure 3 Immunohistochemical analyses of the concentration of CD68-labelled macrophages (macrophages/10 fields) per animal in unmanipulated native fat tissue of omentum of rats fed with high-energy food (OM), in native fat tissue of omentum of rats in regular-energy diet (CO), in fat of omentum of rats in regular-energy diet grafted to subcutaneous environment (TO) and in the fat tissue of unmanipulated native subcutaneous adipose tissue from the left iliac fossa (SC).

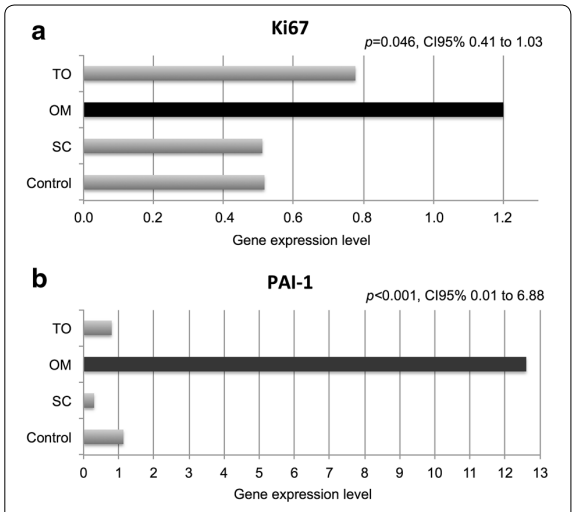

Figure 4 Gene expression level of **a** Ki67 and **b** PAI-1. *OM* unmanipulated native fat tissue of omentum of rats on cafeteria diet regimen (high-energy diet), *CO* native fat tissue of omentum of rats in regular-energy diet, used as post-operative control, *TO* grafted fat tissue of omentum of rats in regular-energy diet, *SC* fragment of unmanipulated native subcutaneous adipose tissue from the left iliac fossa used as control for fat tissue similarity.

Discussion

Our results showed that the grafted adipose tissue and its surroundings did not expressed inflammatory activity mediated by macrophages, higher cellular proliferation rate nor higher levels of PAI-1. However, high-energy diet leads to higher cellular proliferative rate in the unmanipulated native adipose tissue (analyzed through Ki67) and higher levels of PAI-1.

It is known that the adipose tissue provides a source of stem cells (i.e., cells that are capable of differentiating into other cell types of the same embryonic origin) (Baglioni et al. 2009; Baumert et al. 2007; Dhanasekaran et al. 2012; Oloumi et al. 2006; Kobayashi et al. 2006) and exhibits great angiogenic potential (Figueiredo et al. 2010; Oloumi et al. 2006) for restoring ischemic tissues and actinic lesions (Claro et al. 2012; Illouz 1988; Goldsmith et al. 1967; Liebermann-Meffert 2000; Morison 1903; Williams and White 1991). The movement of this tissue from its primary site to a different environment might expose it to metaplasia. In the present study this theory was not proved, once the histological results using HE did not reveal the presence of cell metaplasia in the TO.

The CD68 is a membrane glycoprotein type 1, strongly expressed by tissue macrophages, present in inflammatory events of vascular and adipose tissues, usually related to fat metabolism. These macrophages have been cited as present in high concentration in adipose tissue of patients with plurimetabolic syndrome and/or insulin-resistance (Di Gregorio et al. 2005), in atherosclerotic plaques (Cojocaru et al. 2012), and in breasts with cancer (with unfavorable impact on disease invasion and progression) (Lin et al. 2001; Wyckoff et al. 2004; Condeelis and Pollard 2006; Goswami et al. 2005; Lin et al. 2002; Piras et al. 2005; Soeda et al. 2008; Offersen et al. 2003). However, according to our findings, no connection was identified in literature between the level of CD68-labelled macrophages and obesity in patients without any metabolic disorder (Tam et al. 2012; Di Gregorio et al. 2005). What represents that those macrophages levels in a tissue depend of inflammatory events and pathological chemotaxis from sick adipocyte mediators but not from healthy adipose tissue. In this study, the CD68 antigen was used to identify the chemotaxis potential of adipose tissue to attract CD68-labelled macrophages, which may represent a risk for breast cancer and/or its unfavorable evolution (Lin et al. 2001; Wyckoff et al. 2004; Condeelis and Pollard 2006; Goswami et al. 2005; Lin et al. 2002; Piras et al. 2005; Soeda et al. 2008).

Di Gregorio et al. (2005) have shown that the CD68 expression is also higher at the stromal vascular fraction of the adipose tissue than its adipocyte fraction. Considering that the angiogenesis is intense just after adipose tissue grafting or translocation (Figueiredo et al. 2010), what represents one among many theories for its potential for breast cancer, a chronic higher concentration of CD68-labelled macrophage was expected. However, this data was not found in this study and the CD68 expression was similar in all adipose tissue samples. Therefore, adipose tissue grafting does not seem to generate chronic inflammatory activity involving CD68 macrophages that may lead to a carcinogenic potential in its host microenvironment.

Ki67 is a nuclear protein present in proliferating cells, but absent in resting cells. What means that ki67 is associated with cell proliferation (Gerdes et al. 1984; Pathmanathan and Balleine 2013; Rahmanzadeh et al. 2007). Nowadays, Ki67 scoring is used as a prognostic factor for early breast cancer and even as a predictor of its treatment efficacy (Pathmanathan and Balleine 2013; Urruticoechea et al. 2005; Bullwinkel et al. 2006; Luporsi et al. 2012; Inwald et al. 2013; Nishimura et al. 2010). The gene expression of Ki67 was used in order to evaluate the proliferation rate of adipose tissue from different body compartments, as well as its behavior on high-energy diet and after its translocation to a different environment. The possibility of transposed fat creating a proliferative status in the host microenvironment is considered mostly due to the knowledge that the adipose tissue promotes angiogenesis, deeply explored in the great omentum experiments (Baumert et al. 2007; Figueiredo et al. 2010; Goldsmith et al. 1967; O'Shaughnessy 1937; Oloumi et al. 2006), what theoretically may represent another threat for breast cancer. In this study, the adipose tissue per se, did not show any change in its proliferative rate according to its origin site or after its translocation to a different environment. However, a proliferative status was observed on samples of unmanipulated native fat exposed to a high-energy dietary regimen.

Plasminogen Activator Inhibitor 1 is a single-chain glycoprotein that acts as the primary regulator of plasminogen activation in vivo. It is secreted by several cells and participates in tissue repair processes (Binder et al. 2002; Gomes-Giacoia et al. 2013; Sumiyoshi et al. 1991; Jankun et al. 1993). High PAI-1 level correlates with obesity, hyperinsulinemia, hyperglycemia, and hypertriglyceridemia (Carter and Church 2009; Binder et al. 2002). Some oncological conditions show high level of PAI-1 as well. Its role in breast cancer has been widely studied, where it is involved in the decrease of apoptotic activity, degradation of extracellular matrix during tumor growth, invasion, and metastasis. High levels of PAI-1 in breast cancer thus, is linked to the poor prognosis of disease progression (Carter and Church 2009, 2012; Andreasen et al. 2000; Bianchi et al. 1995; Gomes-Giacoia et al. 2013; Gutierrez et al. 2000; Sumiyoshi et al. 1991; Offersen et al. 2003; Jankun et al. 1993; Foekens et al. 2000). Our study found high levels of PAI-1 only on unmanipulated adipose tissue samples of rats fed with high-energy food, which posteriorly became low in the same rats after change of their diet regimen. Carter and Church (2012) demonstrated that the native mature fat cells of breast seems to represent a greater threat to breast cancer than fat cells from other regions and, even higher

than immature adipocytes or stem cells. In addition, fat cells are widely present in our body and there is no evidence that a fat grafting procedure by itself would bring additional oncological risk to a region that is already surrounded by adipose tissue.

Conclusion

We found in this study in rats that, while the unmanipulated native adipose tissue overexpressed PAI-1 and KI67 when exposed to high-energy diet, the grafted fat by itself was unable to induce cellular proliferation, chronic inflammatory activity and/or elevation of PAI-1 levels. This findings highlight that fat grafting procedure alone does not seem to change the oncological potential of its host microenvironment, however more experimental studies focusing on the fat grafting behavior in vivo must be made in order to confirm this issue.

Authors' contributions

FCJR conceived the study and its design, participated in analysis and interpretation of data, performed the statistical analysis and drafted of manuscript. LRM carried out the immunoassays and helped to draft the manuscript. JM carried out the molecular genetic studies, participated in the sequence alignment and helped to draft the manuscript. LOZS participated in analysis and interpretation of data, helped with the statistical analysis and to draft of manuscript, GAP carried out the immunoassays and interpretation of data. LAV carried out the molecular genetic studies and participated in interpretation of data, AMPN participated in the study conception, design, interpretation of data and coordination. All authors read and approved the final manuscript.

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Compliance with ethical guidelines

Competing interests

The authors declare that they have no competing interests.

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Artigo 3.

Why is the oncogenic potential of adipose tissue in the laboratory field of breast cancer not observed for lipofilling in clinical practice?

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Why is the oncogenic potential of adipose tissue in the laboratory field of breast cancer not observed for lipofilling in clinical practice?

ABSTRACT

Fat grafting to the breast has a questionable oncologic risk according to laboratory reports on adipose tissue. However, the oncogenic potential of this practice has not been observed in the clinic. Thus, to explain this discrepancy, a translational study was designed to analyze the behavior of mammary tissue when hosting autologous grafted fat.

METHOD: Fifty-four paired-breasts of female rats were divided into three groups (control, grafted with autologous fat of subcutaneous, and omentum). The following factors were analyzed: histology, immunohistochemical staining for CD68-expressing macrophages, Plasminogen activator inhibitor-1 (PAI-1) expression, numbers of Ki67+ and estrogen receptor-positive (ER+) cells, and gene quantification of Ki67 and PAI-1 (via real-time PCR).

RESULTS: No alterations in lobular structures or atypia were observed in lipofilled breasts compared with controls. Additionally, no increases in numbers of ductal cell layers or terminal ductal units were found in the lipofilled breasts ($p = 0.15$ and $p = 0.62$, respectively). The mean macrophage concentration in the three groups was 5,6 ($p = 0.53$). PAI-1+ reactivity showed similar pattern in both groups. The percentages of Ki67+ and ER+ cells were, respectively, 2.54% ($p = 0.34$) and 27.92% ($p = 0.22$) among the groups, and the ER/Ki67 balance did not significantly differ between groups ($p = 0.26$). Real-time PCR revealed that the expression levels of Ki67 and PAI-1 were similar among all groups ($p = 0.71$ and $p = 0.94$, respectively).

CONCLUSIONS: No evidence was found that lipofilling stimulates inflammation, enhances tissue proliferation or increases PAI-1 expression in the mammary gland.

Keywords: Fat grafting; Breast Cancer; ER; PAI-1; Ki67; CD68

INTRODUCTION

Autologous fat grafting to the breast is one of the most performed procedures worldwide for reconstructive and aesthetic purposes[1-3]. The low rate of clinical complications observed with the most recent techniques and the versatility afforded by the ability to obtain non-immunogenic filler from donor sites without causing morbidity have increased the popularity of breast lipofilling over conventional procedures[4, 1, 5, 3, 6-9, 2]. Moreover, this procedure is effective for the treatment of radiation sequelae and other issues that have remained untreated thus far[1, 10].

Nevertheless, fat grafting to the breast has a questionable oncological risk; thus, its use has not been recommended by the American Society of Plastic Surgeons[11-15]. This theoretical risk was made known when obesity was identified as a risk factor for breast cancer[14, 16-22]. Regarding this issue, in the laboratory, some *in vitro* and preclinical studies have shown the oncogenic potential of adipocytes and adipose stromal cells (ASC) in mammary tissue[23, 18, 24, 19, 22]. However, many studies have failed to show that autologous breast lipofilling is a risk factor for breast cancer in clinical practice. Thus, this issue remains controversial[25, 1, 10, 3].

Laboratory analyses have reported three main factors in white adipose tissue (WAT) that are associated with cancer risk: chronic inflammation induced by adipokines (with the recruitment of macrophages), the induction of proliferation by perivascular macrophages and ASCs, and the increased plasminogen activator inhibitor-1 (PAI-1) concentration present in adipocytes. PAI-1 has been reported to decrease apoptotic activity and increase extracellular matrix degradation, facilitating tumor development, growth and invasion[26-28, 22, 19, 29-31].

The clinical evidence relating obesity to breast cancer in combination with laboratory findings using isolated cells from WAT suggests that breast lipofilling potentially increases the risk for breast cancer. However, there is a lack of *in vivo* experiments that have focused on this issue using whole autologous WAT cells (as applied in human beings). Analysis of this risk in clinical practice is challenging due to the difficulty in establishing an ideal prospective trial with well-controlled subjects. In addition, the collection of lipofilled breast tissues from women for analysis is a complex undertaking and therefore preclinical studies are needed.

WAT possesses a known oncological risk for many types of cancer, primarily under certain conditions, such as obesity. However, the discrepancy between laboratory reports on the oncogenic potential of adipose tissue and the lack of oncogenesis observed in clinical practice in association with breast lipofilling has not been addressed. In the last ten years, fat grafting to the breast has become one of the most commonly performed procedures for breast reconstruction, despite its unclear theoretical oncological risk due to the lack of clinical evidence[3]. Although WAT represents a cancer risk, it appears that the use of autologous fat transplants to fill an environment naturally composed of fat tissue does not. In a comparative preclinical model, it was demonstrated that native unmanipulated WAT may lead to a precancerous status in its microenvironment following the consumption of a high-energy diet, while the lipofilling procedure by itself does not[32]. The latter finding suggests that lipofilling cannot increase the existing paracrine activity of native WAT in its microenvironment.

Thus, to gather more evidence concerning the safety of fat grafting in the breast and to determine why previous laboratory results are not echoed in clinical practice, we conducted an experimental study in rats to comparatively analyze the behavior of mammary tissue when hosting healthy autologous grafted WAT. The model used here reproduces the fat lipofilling procedure used in humans with high fidelity.

METHODS

Experimental model for white adipose tissue collection

Nine female, 8-week-old Sprague-Dawley rats (*Rattus norvegicus*) born of the same dam (corresponding to a teenager in breast development) and virgin for pregnancy were used. The breed and age of the rats were chosen according to previous descriptions of their susceptibility toward developing breast cancer when exposed to any risk factor.[33, 34] This study followed the ARRIVE[35] guidelines and was approved by the Ethics Committee on Animal Experimentation of the University where the study was developed. The rats were housed in isolated boxes with daily cleaning, controlled illumination (12 hours/day) and a constant temperature (20–22 ℃) after fat grafting procedures and until the harvesting of samples for analysis.

Paired and controlled breasts with and without autologous fat grafting were compared. Fifty-four breasts were divided into three groups as follows: (1) control breasts without any manipulation; (2) breasts with autologous fat grafting of subcutaneous tissue; and (3) breasts with autologous fat grafting of the great omentum.

Fat harvesting procedure

The rats underwent general anesthesia (intraperitoneally) at 8 weeks old. Omentum fat was harvested through a midline laparotomy of approximately 4 cm in length. Subcutaneous fat was harvested through skin incisions of approximately 2 cm in length over the left and right iliac fossa. The abdominal wall was closed with running sutures using 5.0 nylon. The skin incisions were also closed with running sutures using 5.0 nylon.

WAT preparation and lipofilling procedure

The harvested fat tissue was manually chopped with a scalpel into very small pieces and then placed into a 10-ml syringe. The fat was then transferred into another 10-ml syringe and passed through an emulsifier (Tulip Medical Products, San Diego, CA, USA); in this way, the samples were homogeneously fragmented. Next, the samples were centrifuged at 3,000 rpm for 3 minutes. The lower and uppermost levels were discharged, such that only the middle layer (containing viable adipocytes) was transferred into a 1-ml syringe. Finally, 0.2 ml of prepared autologous fat (from the omentum or subcutaneous tissue) was grafted through a 0.9-mm diameter cannula with a blunt tip into the thoracic breasts of each rat[36, 1, 37, 38].

The rats were euthanized at 8 weeks post-operatively (112 days old), and their breasts were harvested for analysis. This period of time was defined according to pervious studies that established a preclinical model for breast cancer in rats[39, 33].

Histological and immunohistochemical preparation

Tissue samples were fixed in 10% buffered formalin and routinely embedded in paraffin at the Laboratory of Pathology. Four-micrometer sections were made for hematoxylin-eosin (HE) staining and immunohistochemical reactions. Before incubating the tissue sections with antibodies, they were submitted to heat-induced antigen retrieval with Tris-EDTA buffer at pH 9.0 (except for Ki67, for which citrate buffer at pH 6.0 was used). The primary antibodies used were anti-CD68 (detection of macrophages; clone KP-1, diluted 1:1000; Cell Marque, Rocklin, CA, USA); anti-plasminogen activator inhibitor-1 (PAI-1; diluted 1:100; Abcam, Cambridge, MA, USA); anti-Ki67 (diluted 1:50; Millipore Darmstadt, Germany), and anti-estrogen receptor (ER, clone 1D5, diluted 1:300; Dako, Glostrup, Denmark). For antigen-antibody detection, a biotin-free polymer was used (Advance, Dako). Immunostaining was visualized using diaminobenzidine, and counterstaining was

achieved with hematoxylin. Positive and negative controls were run for each batch. For CD68 and PAI1, brownish cytoplasm staining was considered positive. For Ki67 and ER, brown staining was localized in the nuclei.

Histological analysis

A hotspot field on each slide was analyzed at 400× magnification to determine the absolute number of breast terminal duct lobular units, number of epithelial cell layers in the lobular ducts, ductal cell morphology (flat epithelium, cuboidal epithelium, modifications in columnar cells), ductal ectasia, papillary lesions (projections) and nuclear atypia.

Immunohistochemical analysis

A single pathologist, blinded to the origin of each sample, performed the immunohistochemical analysis for each marker along with cell morphological identification in ten different randomly chosen areas for each slide at 400× magnification. For Ki67, 1d5 and PAI-1, the percentage of positive cells was analyzed; for CD68, the number of positive cells outside the vascular lumen was counted[40, 41]. PAI-1 immunohistochemical analysis was used to identify positive cells and their distribution in the mammary gland, as well as to establish patterns in each of the three analyzed groups[42, 43].

Assessing proliferative and precancerous status

The number of positive cells for Ki67 and 1d5 in each of the lobular terminal ducts was assessed via immunohistochemistry, and the relationship between them was compared and analyzed in each sample. The percentage of coexisting positive Ki67/1D5 cells was then compared among the three samples studied. A high percentage of these cells in the lobular ducts may lead to a precancerous status[44, 29, 45].

Real-time PCR

An RNeasy Lipid Tissue Mini Kit was used for mRNA extraction (Qiagen, Cat. No. 74804). Ki67 and PAI-1 mRNA levels were assessed in all groups by real-time PCR (ABI Prism 7500, Applied Biosystems) using

the following primers: Ki67 - Rn.PT.58.8428180.g (Integrated DNA Technology, IDT); PAI-1 - Rn01481341_m1 (Applied Biosystems) and GAPDH (#4352339E; Applied Biosystems) as a reference gene. cDNA was reverse transcribed using a High Capacity cDNA Reverse Transcription Kit (#4368813; Applied Biosystems). The PCR reaction consisted of 40 ng of cDNA, 0,25 µl of each primer, 3 µl of TaqMan Universal master mix (#4369016; Applied Biosystems) and RNase-free water to a final volume of 10 µl. Data were analyzed using a Sequence Detector System 7500 (Applied Biosystems).

Statistical analyses

SPSS version 20 for MAC was used for statistical analysis (IBM; Armonk, NY—EUA). The normality distribution of the data was assessed using the Kolmogorov–Smirnov and Shapiro–Wilk tests. Normally distributed data were analyzed using paired t-tests, analysis of variance (ANOVA, one way and repeated measures) and Tukey's test (post hoc), and the values were expressed as the means ± standard deviation (SD). Non-normally distributed data were analyzed using the Kruskal-Wallis test, and the values were expressed as medians and quartiles. The significance level was 5%.

RESULTS

Histological findings

The analyzed ductal structures did not show epithelial hyperplasia, and the number of intraductal epithelial layers was similar among the three groups, with a mean of 1.56 ± 0.58 layers (range 1 to 3 layers, $p = 0.15$). No cystic alterations, papillary lesions (projection) or nuclear atypia were observed. The numbers of breast terminal duct lobular units were similar among the three groups, with a mean of 8.20 ± 2.26 ($p = 0.62$) (Figure 1).

Analysis of chronic inflammatory activity mediated by CD68-positive macrophages

The immunohistochemical reactivity of CD68-labeled macrophages in the breasts did not change after grafting of subcutaneous or omental fat. The mean macrophage concentration observed among the three groups analyzed was 5,6 macrophages/10 fields (SD 1,4), $p = 0.53$ (Tukey's post-hoc test; $p = 0.24$; Figure 2a).

Therefore, cytokines from the grafted fat did not seem to induce inflammatory activity in the host microenvironment, as the number of macrophages remained similar among all studied samples.

Immunohistochemical analysis of the reactivity of Ki67-positive and ER-positive (1D5) cells in mammary tissue

Ki67+ cells represented a mean percentage of 2.54% among all of the samples analyzed (median of 2% with Q1 = 1% and Q3 = 3%; $p = 0.34$; Figure 2b). Our results showed that the concentration of ER-positive cells (1D5) in the control breasts was similar to those in the breasts grafted with subcutaneous or omental fat, with a mean general percentage among the groups of 27.92% (from 23.26% to 32.57%; $p = 0.22$; Figure 2c).

Assessment of precancerous status based on the balance of ER/Ki67-positive cells among ductal cells

The balance between ER+ and Ki67+, cited as predictive of pre-cancerous status[29, 44, 45], did not show any changes among any of the samples. The control breasts had a mean value of 20.19 (SD 14.67), while the breasts grafted with subcutaneous and omentum fat showed means of 12.50 (SD 5.69) and 11.50 (SD 9.20), respectively (p=0.26; Figure 3).

Cellular distribution of PAI-1 reactivity in breasts

A high level of PAI-1 has been widely associated with an unfavorable status for many types of cancer. High PAI-1 levels are usually found in obese individuals and in cancerous breast tissues. Although this marker is expressed in many cell types, adipocytes are especially prone to dangerously high PAI-1 levels. Our immunohistochemical results showed moderate PAI-1 reactivity that was similarly distributed among ductal cells, adipocytes, fibroblasts and macrophages, with similar patterns among the three analyzed groups (Figure 4).

Assessment of proliferation activity and PAI-1 levels via RNA quantification

The use of RT-PCR to detect Ki67 gene expression has been described as a highly sensitive method of assessing cell proliferative activity [46]. Thus, in this study, RT-PCR was used to assess cell proliferation rate in whole breast tissue (including fat cells, stromal cells, and ductal cells, which were previously assessed via

immunohistochemical analysis). Similar values of Ki67 expression were found among the three analyzed groups (p= 0.71; Table 1 and Figure 5a).

PAI-1 levels did not differ between lipofilled breasts and control breasts (p = 0.94), as shown in Table 1 and Figure 5b. These results illustrate that the grafting of healthy adipose tissue from the omentum or subcutaneous tissue cannot influence PAI-1 levels or proliferative status in mammary tissue (Table 1).

DISCUSSION

Our findings demonstrated that the procedure of grafting healthy adipose tissue to the breast did not lead to chronic inflammatory activity or induce proliferation in the mammary gland. Patterns of Ki67+, ER+ and PAI-1+ expression did not change in the breast after fat grafting. The levels of Ki67 and PAI-1 mRNA expression were the same between breasts with and without fat grafting.

Histological findings showed that autologous grafting of WAT did not change mammary ductal morphology and did not lead to nuclear atypia or proliferative activity. As observed in clinical practice and our previous study[32], autologous-grafted WAT seems to incorporate into existing adipose tissue without changing existing paracrine activity in native cells.

Immunohistochemical staining of CD68 in combination with morphological identification and analysis of mammary cells by a pathologist was used as a means of identifying macrophages. Macrophages infiltrate tissues in response to inflammation, and they are present in adipose tissues in patients with plurimetabolic syndrome and/or insulin resistance[47] and in cancerous breast tissue (with an unfavorable impact on disease invasion and progression)[48, 27, 49-53, 31].

The CD68 antigen was used to identify the chemotactic potential of the grafted fat to attract CD68-expressing macrophages, which may lead to oncologic potential[48, 27, 49, 50, 52, 53, 31]. Considering that the CD68 reactivity in the analyzed lipofilled breasts was similar to that in the control breasts, our findings suggest that fat grafting to the breast cannot trigger chronic inflammation involving macrophages.

Ki67 is associated with cell proliferation and may be used as a prognostic score in early breast cancer[54-61]. In this study, this marker was used to identify the absolute rate of cellular proliferation in each group. Its levels in ductal cells were assessed via immunohistochemical analysis. The proliferation rate in whole mammary tissue (including adipose and stromal cells) was analyzed using RT-PCR. Grafted fat is highly

expected to possess a proliferative status within the host microenvironment given that adipose tissue promotes angiogenesis immediately after grafting[62-66]; theoretically, this represents a breast cancer risk. In this study, the fat grafting procedure did not chronically increase the proliferative rate in lipofilled breasts compared with control breasts.

Moreover, it has been hypothesized that an increased quantity of fat tissue in the breast, such as that resulting from fat grafting, might increase the proportion of ER-positive luminal epithelial cells. In normal breasts, the higher the density of ER-positive cells, the lower the reactivity of Ki67. Thus, it is expected that younger women will have lower ER levels and higher Ki67 levels than older women (who have fatty breasts and are therefore expected to have higher ER and lower Ki67 levels). When the balance between ER-positive cells and cell proliferation is disrupted (high ER and high Ki67), a precancerous status is established[29, 45, 44]. Our analyses demonstrated that fat grafting to the breast does not increase ER positivity in ductal cells and does not disrupt the balance between estrogen expression and cellular proliferation in breast lobules.

Plasminogen activator inhibitor 1 (PAI-1), although secreted by several cell types, seems to be released by adipocytes at dangerous levels under some pathological conditions, such as obesity, hyperinsulinemia, hyperglycemia, and hypertriglyceridemia[67, 20]. PAI-1 participates in tissue repair processes and has been reported to be involved in decreasing apoptotic activity and degrading the extracellular matrix during tumor growth, invasion, and metastasis [67-71]. As such PAI-1 poses a breast cancer risk and [72] has been associated with unfavorable breast cancer progression. [73, 42, 20, 26, 68, 30, 69, 51, 70] It has been suggested that this protein is mainly expressed in adipocytes; thus, fat grafting may increase its concentration in the host. However, via immunohistochemistry, we identified moderate PAI-1 reactivity that had similar levels among ductal cells, fibroblasts, adipocytes and macrophages. In addition, this study did not find any changes in gene expression or immunogenic reactivity between breast lobules grafted with fat and those that were not.

In our previous *in vivo*[32] study, we observed that grafted fat by itself cannot induce an oncogenic state within its microenvironment, while native, unmanipulated fat exposed to a high-energy diet, which was used as a control, led to tissue proliferation and increased levels of PAI-1[32]. Thus, we hypothesized that fat grafting by itself does not pose a breast cancer risk, despite studies showing that adipocytes are associated with such a risk. We believe that adipocytes do not increase adipokine levels in the host because native, unmanipulated adipocytes in the breast possess their own paracrine activity[18, 32]. Thus, the risk for cancer

would only exist in body compartments without native fat cells and not in environments with a high proportion of adipose tissue, such as the breast.

The data collected in the current study led us to believe that the discrepancy in the oncogenic potential of WAT observed between preclinical and clinical studies occurs because of different focuses during analysis. While laboratory studies have identified the oncogenic potential of WAT cells (primarily when exposed to certain cancer risk factors or in contact with cancer cells), clinical studies have reported that autologous grafting of WAT in bodily compartments already composed of adipose tissue (such as the breast) does not change the existing paracrine activities of native cells. In the present study, we analyzed the influence of autologous fat grafting to the breast (as is performed in clinical practice) in a rat breed prone toward developing breast cancer. We found that the lipofilling procedure itself does not increase the oncogenic potential of the native subcutaneous tissue that surrounds the breast relative to the mammary tissue.

CONCLUSION

We demonstrated that the procedure of grafting healthy adipocytes to the breast does not change mammary ductal morphology, increase the proliferative rate of mammary lobules, disrupt the balance of Ki67/ER-positive cells, induce macrophage-mediated inflammation, increase PAI-1 levels or alter PAI-1 distribution in breast lobules. Thus, according to our findings, breast lipofilling does not seem to be a risk factor for breast cancer. However, considering the lack of *in vivo* studies concerning this issue, we suggest that additional experimental studies focusing on the behavior of grafted fat within the breast should be performed to confirm our results.

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CONFLICT OF INTEREST STATEMENT

The authors of this manuscript have no conflicts of interest to disclose.

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FIGURES LEGENDS:

Table 1: Summary of analyzed variables

Figure 1: Panoramic view (at 100x magnification) of HE staining slides of control breast, breast grafted with subcutaneous fat (SC) and breast grafted with omentum (OM), illustrating the similar pattern among them and absence of tissue abnormality. At 400x magnification, analyzed ductal structures did not show epithelial hyperplasia and the number of intraductal epithelial layer was similar among the three groups with a mean of 1.56 ± 0.58 layers (range from 1 to 3 layers, p=0.15). No cystic alteration, papillary lesion (projection) or nuclear atypia were observed. The numbers of breast terminal duct lobular units were similar among the three groups with a mean of 8.20 ± 2.26 (p=0.62)

Figure 2: Column A. Graphic showing the average number of anti-CD68 stained macrophages counted in 10 different randomly chosen areas in each processed slide and pictures (400x magnification) from control breast, breast grafted with subcutaneous fat (SC) and breast grafted with omentum (OM), illustrating the immunohistochemical reactivity of CD68+ (yellow arrow). **Column B.** The percentage of Ki67 positive cells in each group was similar, the pictures show samples (x400 magnification) from control breast, breast grafted with subcutaneous fat (SC) and breast grafted with omentum (OM), illustrating the immunohistochemical reactivity of Ki67+ cells. Both pictures illustrate similar expression of ki67 marker, with an example of a positive cell (yellow arrow) and negative one (white arrow). **Column C.** Graphic depicting the similar percentage of Estrogen positive cells (ER+) in each group and pictures showing samples (at 400x magnification) from control breast, breast grafted with subcutaneous fat (SC) and breast grafted with omentum (OM), illustrating the immunohistochemical reactivity of a positive ER cell (yellow arrow) and a negative one (white arrow).

Figure 3: Boxplot illustrating the similar pattern in breast tissue of immunohistochemical reactivity of (A) Ki67+ cells, (B) Estrogen Receptor positive (ER+) cells, and (C) Ki67+/ER+ ratio among the control, breast grafted with autologous subcutaneous fat tissue (SC) and breast grafted with autologous fat tissue of omentum (OM).

Figure 4: Pattern of PAI-1 in breast lobules among the three groups analyzed (at x400 magnification).

Figure 5: Gene quantification of RNA expression by real-time PCR of (A) Ki67 (used to assess the proliferative rate of breast tissues) and (B) PAI-1, confirming the same pattern among samples of breasts of control, breast grafted with autologous subcutaneous fat tissue (SC) and breast grafted with autologous fat tissue of omentum (OM).

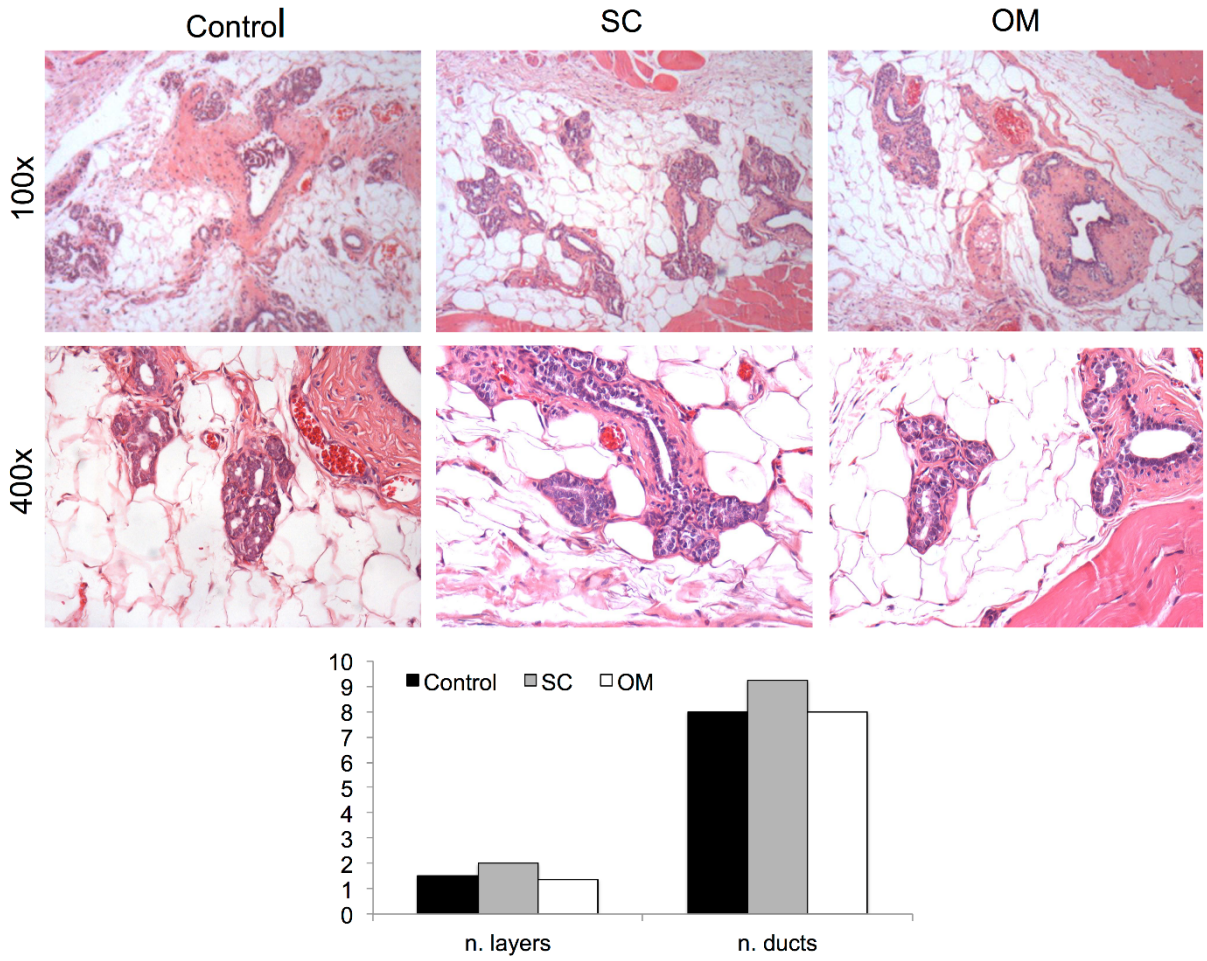
Table 1

Parameters		Control Mean (SD)	SC Mean (SD)	OM Mean (SD)	Total Mean (SD)	Total Range n	*p* value
Histology							
	n of BTDLU	8.00(2.44)	9.25(1.50)	8.00(2.39)	8.20(2.26)	4 to 12	0.622
	n of ECLLD	1.50(0.52)	2.00(0.70)	1.38(0.52)	1.56(0.58)	1 to 3	0.152
	DCM	normal	normal	normal	normal	NA	NA
	DE	no	no	no	no	NA	NA
	PL	no	no	no	no	NA	NA
	NCA	no	no	no	no	NA	NA
Immunohistochemical							
	CD68 (n)	5,15(1.46)	5.25(1.26)	6.56(1.01)	5.65 (1.41)	3 to 8	0.053
	Ki67 (%)	2(1.35)	2.75(0.5)	3.25(3.01)	2.54(2.00)	0 to 10	0.343*
	ER (%)	30.00(12.79)	32.50(9.57)	22.50(7.07)	27.92(11.03)	10 to 50	0.224
	PAI-1 (%)	MR and SDT	MR and SDT	MR and SDT	MR and SDT	NA	NA
ER+/Ki67+ balance							
	Ratio	20.20(14.68)	12.50(5.69)	11.50(9.20)	16.01(12.30)	3 to 50	0.257
PCR RT							
	PAI-1	1.27(0.94)	1.45(0.87)	1.22(1.47)	1.29(1.09)	0.11 to 4.48	0.737
	Ki67	1.24(0.93)	0.70(1.05)	1.34(1.96)	1.15(1.36)	0.03 to 5.17	0.709

BTDLU=breast terminal duct lobular units; ECLLD=epithelial cell layers in lobular ducts; DCM=epithelial cell layers in lobular ducts; DE=ductal ectasia; PL=papillary lesions; NCA=nuclear cell atypia; MR=moderate reactivity; SDT=similar distribution among breast tissue; NA=not applicable

* Kruskal-Wallis test was used because data were not normally distribuited (median among the three groups was 2, Q1=1 and Q3=3)

Figure 1


Control
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n. layers
n. ducts

Figure 2

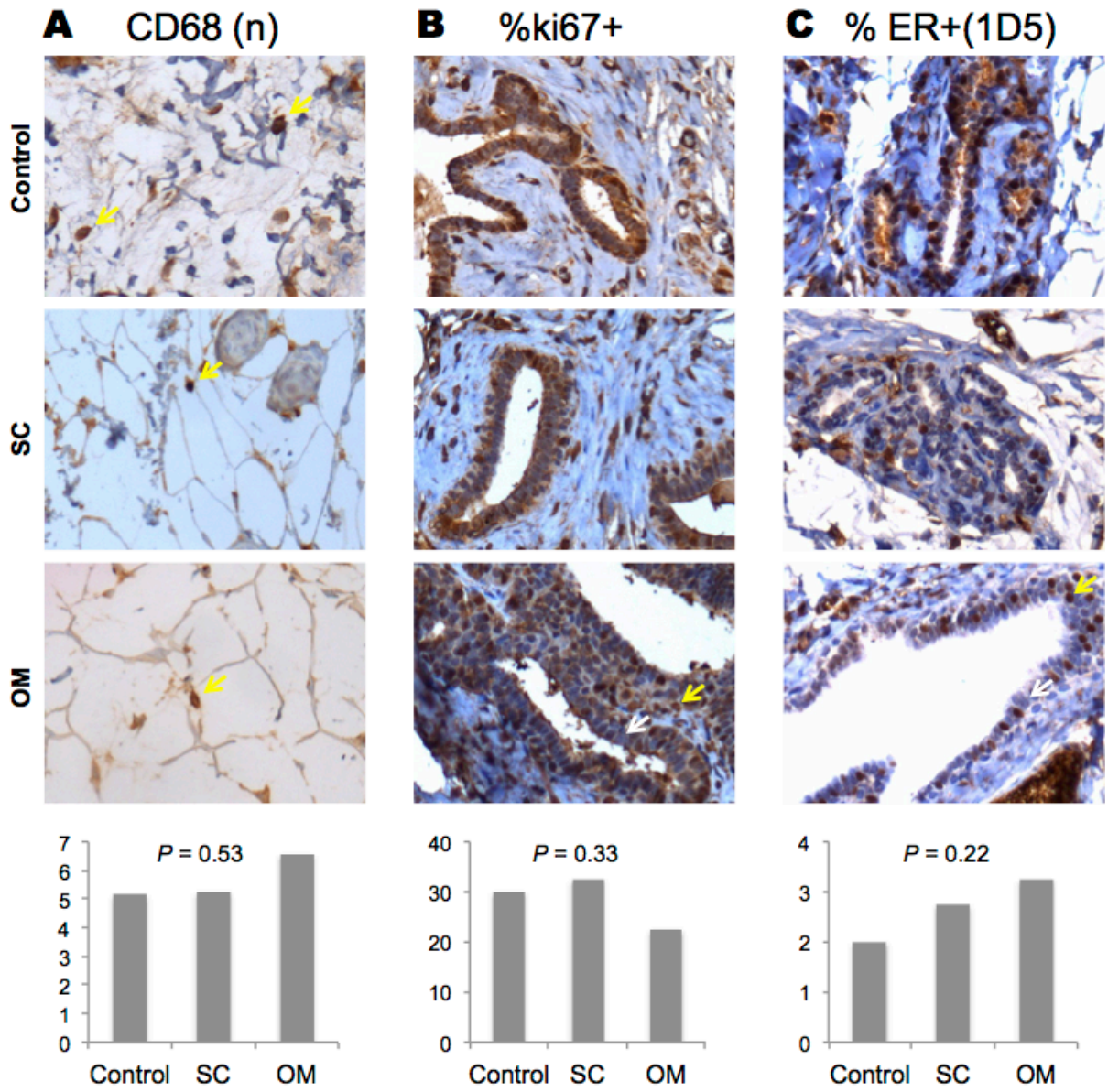

A CD68 (n)
B %ki67+
C % ER+(1D5)
Control
SC
OM
P = 0.53
P = 0.33
P = 0.22
Control SC OM
Control SC OM
Control SC OM

Figure 3

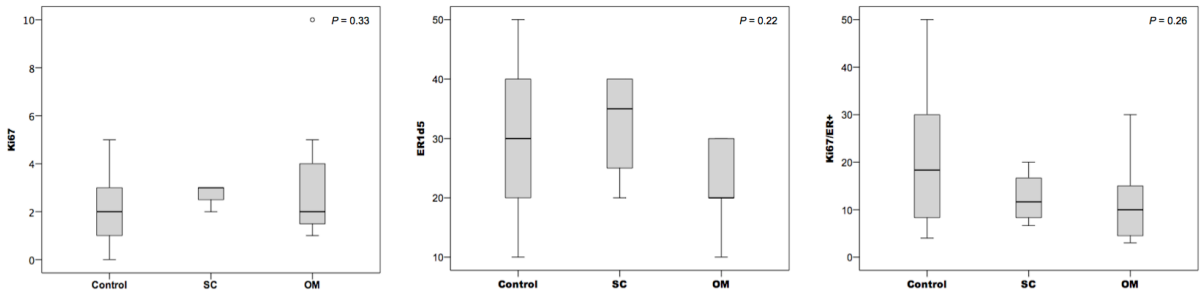
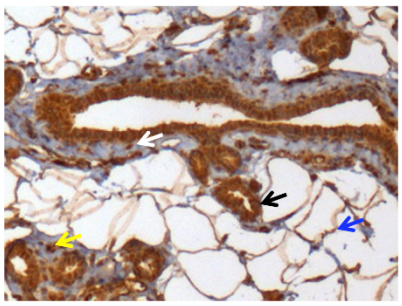


Figure 4

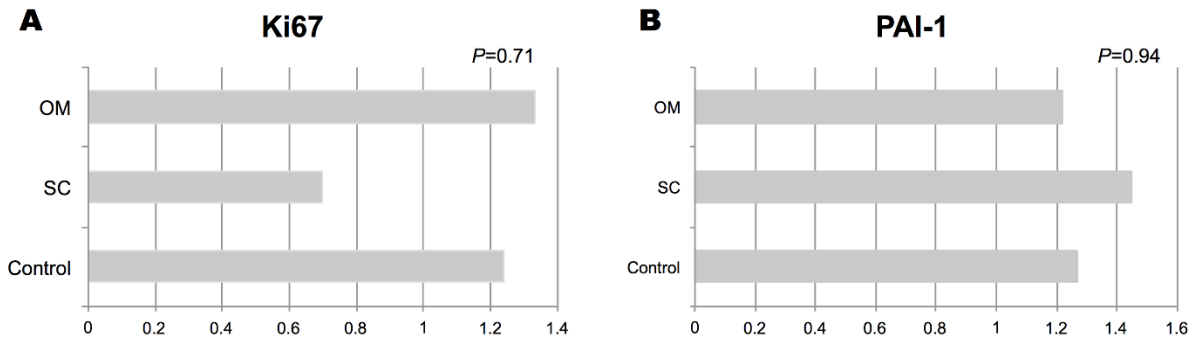
	Ductal Cells			Adipocytes			Fibroblasts			Macrophages		
	Negative	Slight	Moderate	Negative	Slight	Moderate	Negative	Slight	Moderate	Negative	Slight	Moderate
Control	0,00	22,22	77,78	0,00	33,33	66,67	0,00	0,00	100,00	0,00	0,00	100,00
SC	0,00	25,00	75,00	0,00	11,11	88,89	0,00	0,00	100,00	0,00	0,00	100,00
OM	0,00	0,00	100,00	0,00	0,00	100,00	0,00	0,00	100,00	0,00	0,00	100,00



Above: Table showing the cellular distribution of PAI-1 positive cells in the breast of each group.

Beside: illustration of PAI-1 reactivity in a sample of breast grafted with subcutaneous fat, it is possible to observe moderate reactively of PAI-1 in ductal cells (black arrow), adipocytes (blue arrow), fibroblasts (white arrow) and macrophages (yellow arrow).

Figure 5



Artigo 4.

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REVIEW ARTICLE – BREAST ONCOLOGY

Omentum for Mammary Disorders: A 30-Year Systematic Review

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ABSTRACT

Purpose. Although the safety of applying omentum to the female breast for total breast reconstruction is controversial, it has recently been used to treat certain mammary disorders as well. A systematic review was therefore conducted to analyze and establish the suitability and safety of applying omentum to the breast.

Methods. Covereing the interval from January 1984 to December 2013, we performed searches in MEDLINE, Embase, SciELO, and Google-Scholar for original articles describing the applicability of greater omentum to the breast and its clinical complications.

Results. Sixty observational articles with 985 women were chosen. The main clinical indications were total breast reconstruction after mastectomy due to breast cancer (45 studies), radiation damage (23 studies), and congenital Poland syndrome (4 studies). Altogether, 273 complications were identified among the 985 women treated. The most frequent was flap necrosis (26.74 %). The most serious was injury to the digestive system (1.10 %). There was a 35.48 % incidence of local breast cancer recurrence in eight observational studies on oncological risk. Seven of the eight included only women with advanced cancer. One of these studies reported the incidence and relapse time predominantly according to the primary tumor size.

Conclusions. Although the oncological risk remains unclear, there was a high volume of complications that affected the digestive system. These findings suggest that omentum has well established applicability, but only for total breast reconstruction of huge defects, where muscular/myocutaneous or perforator flaps may be unsuitable.

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Kirikuta first described the use of greater omentum for breast reconstruction in 1963.[1] Since then, there have been controversies regarding the safety of applying omentum to the female breast. More recently, however, from a procedure used for more than half a century ago by some surgical specialties as the last choice for thoracic and total breast reconstruction, omentum has been used to treat congenital deformities of female breasts, for aesthetic mammary procedures, after radiation treatment, and for partial breast reconstruction.[2–5] Some large case series in which these disorders were treated with omentum were published during the last few years, renewing interest in the procedure, although its safety remains unclear.[4]

The articles published up to now concerning this issue were mostly case reports or case series, so they do not seem to sustain the suitability and safety of applying omentum to the breast. In addition, considering the potential risk of fat cells for breast cancer, as proposed in 1986[6] and 1987,[7] its use in the female breast might represent a theoretical contraindication. In addition to the many possible oncogenic factors associated with fat cells, the greater omentum has a well-known potential for neovascularization because of its high concentration of stem cells.[8]

Based on the aforementioned information, our objective was to gather enough data from the literature via a systematic review to analyze and establish the suitability and safety of applying omentum to the breast.

METHODS

Search Strategy

We conducted a systematic review of the use of autologous extraperitoneal greater omentum in the female breast. The study was performed according to the guidelines in the preferred reporting items for systematic reviews and meta-analyses (PRISMA)[9] and was registered on PROSPERO under number CRD42013005493.[10] The articles published during the last 30 years (January 1984–December 2013) were objects of

the search performed independently by two reviewers after accessing the electronic databases of the U.S. National Library of Medicine (MEDLINE), EMBASE, scientific electronic library online (SciELO), and Google Scholar. Appropriate keywords in the English language were combined by Boolean logical operators, as follows: Breast AND Omentum OR Epiploon, adapted for the proper syntax for each database. Titles in references were crossed in search of additional items of potential interest, without language restriction. The type of studies and/or publication media were analyzed for potentially relevant studies for inclusion criteria.[11]

Inclusion Criteria

Eligible for inclusion in this review were articles with original data concerning the omentum and human female breast published during the last 30 years.

Exclusion Criteria

Duplicate articles, different articles using duplicated data, and articles without original data (e.g., comments, reviews, technical descriptions) were considered ineligible. Articles without a description of the clinical indication or clinical results and complications were excluded as well.

Selection of Studies

Two reviewers independently reviewed the abstracts of studies initially selected. If considered eligible for full-text reading, the articles were retrieved for assessment, data extraction, and inclusion in the systematic review. Authors of selected studies that were not available in electronic media or local libraries were contacted by e-mail.

Data Extraction

Data were extracted independently by two reviewers and tabulated. Discrepancies were discussed and reviewed by all reviewers until agreement was reached. The data extracted included the following: authors, date of publication, number of subjects, indication for the procedure, type of study, technique used for omental flap harvesting, flap design, postoperative monitoring duration, treatment efficacy, clinical complications, and history of postoperative breast cancer (primary or recurrence).

ASSESSMENT OF STUDY QUALITY

Two independent reviewers assessed the methodological quality of the studies. The level of evidence and grade of recommendation were scored according to the criteria of the grading recommendations assessment, development, and evaluation (GRADE).[12] Observational studies and clinical trials that lack a detailed description of the randomization process were considered to have a high potential for bias.[11,13]

STATISTICAL ANALYSIS

The prevalence of indications and clinical complications were identified. The oncological risk was assessed. Statistical meta-analysis was not performed because of the low quality and great heterogeneity of the studies.[11]

RESULTS

We identified 355 articles (120 in Embase, 98 in MEDLINE, 2 in SciELO, 135 in Google Scholar). After inclusion of five articles identified by manual cross-referencing[14–18] and exclusion of duplicate articles, 282 articles were eligible for more detailed evaluation. Among them, 74 remained for full-text reading. We then excluded 3 of the 74 for failing to identify applicability,[19–21] 8 that had duplicated databases,[15,17,22–27] 1 that did not have original data,[28] and 2 that did not use omentum for reconstruction.[29,30] That left 60 articles that included more than 985 women to be used in this systematic review.[5,14,16,18,31–86] Twelve articles (692 women) describing a standard technique that involved at least 20 women who were adequately followed were used for more detailed analysis to access the incidence of clinical complications.[18,33,43,46,48,51,57,58,65,77,81,85] Among these 12 studies, 8 (403 women) analyzed breast cancer recurrence and so were used to evaluate oncological risk[33,43,46,48,58,65,77,81] (Fig. 1).

All 60 studies were observational. Among them, 22 were case reports, 32 were case series, and 6 were retrospective cohort studies. Therefore, according to the GRADE criteria, they were all considered to be of "low" or "very low" quality.[11,12] The methodologies and quality of each study are shown in Table 1.

CLINICAL APPLICABILITY

The number of publications describing the use of omentum in the breast was apparently constant during the last 30 years. The 60 studies included in this review were distributed during this period at a mean of two articles per year. Figure 2a shows the main applicability of greater omentum in the breast according to the year of publication. Figure 2a shows that although the procedure remained in a

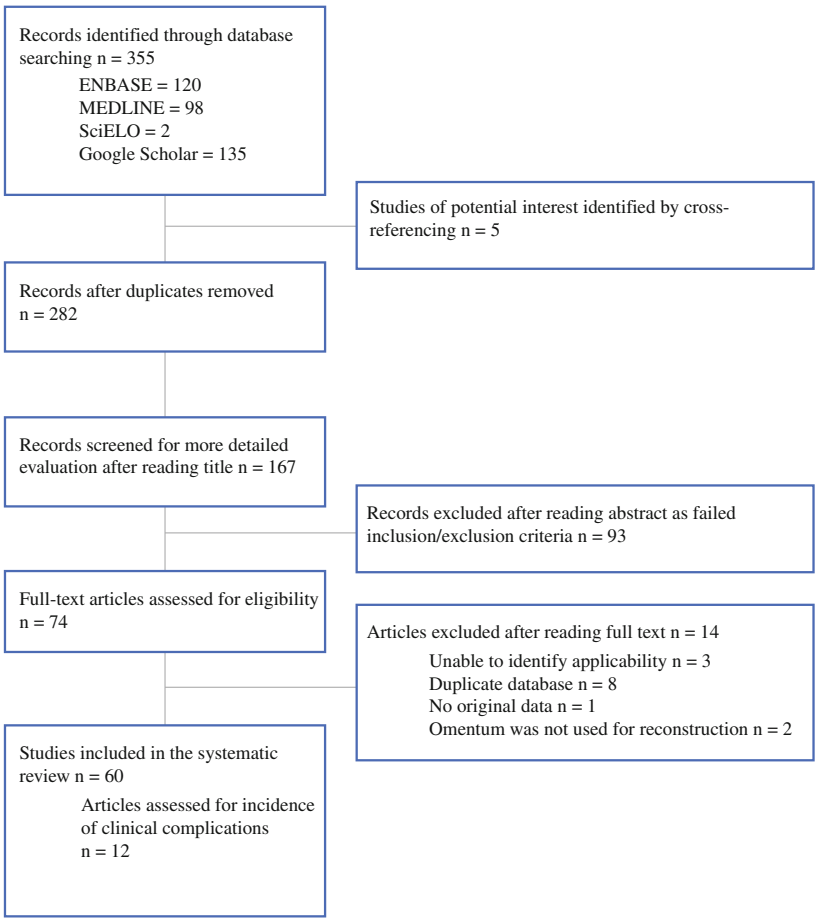

FIG. 1 Selection of articles for review

constant number of publications during the 30 years of this study its main applicability changed somewhat during the last 15 years. For the first half of that 30-year span, greater omentum was primarily used to reconstruct huge defects caused by breast resection and/or radiation damage. For the last 15 years, however, it has been used also to improve breast shape and to treat Poland syndrome and/or congenital deformities of female breasts.[5,18,63,80,81,83,85,86]

Figure 2b describes the applicability of greater omentum to the breast during the last 30 years using the technique of flap harvesting. The main clinical applications were breast reconstruction after mastectomy due to breast cancer, described in 45 studies (9 for partial reconstruction and 36 for total reconstruction), followed by treatment for radiation damage (23 studies) and congenital deformities of female breasts (4 studies). Laparoscopy was the preferred technique for harvesting the omental flap in women treated for breast congenital deformities (75 %) followed by breast reconstruction after mastectomy (26.19 %) and treatment for radiation damage (13.04 %).

CLINICAL COMPLICATIONS

We identified 273 clinical complications in 60 studies with more than 985 women. Systemic complications and deaths known to have resulted from advanced breast cancer disease and/or from extensive mastectomy were excluded. Partial omental flap necrosis represented about one-fourth of the complications identified (73 cases, 26.74 %) followed by infection (19.05 %) and abdominal hernia (16.12 %). More serious complications were hematoma or bleeding that required prompt surgical intervention (4.76 %) and colectomy due to damage of the colonic vascular pedicle during omental flap harvesting (3 patients, 1.10 %). There were 14 cases (5.13 % of all complications) of total omental flap necrosis that required additional procedures. Although omentum harvesting by laparoscopy was performed in only one-fourth of the studies, it was responsible for the two cases of injury to the omentum vascular pedicle, about half of the cases of hematoma or bleeding (46.15 %), and one of the two seromas (50.00 %).

TABLE 1 Characteristics of included studies and their main clinical applicability

Study	Year	Study design	Grade[a]	Applicability	Flap design	Harvesting technique	Efficacy	No. of patients (breasts)
McKenna[1]	1984	Case series	Very low	BR	Pedicled	Laparotomy	Satisfatory	8
Arnold[32]	1984	Case series	Very low	RT	Pedicled	Laparotomy	Satisfatory	10
Calderoli[33]	1985	Retrospective cohort	Low	BR + RT	Pedicled	Laparotomy	Satisfatory	35
Hoch[34]	1985	Case series	Very low	BR	Pedicled	Laparotomy	Satisfatory	8 (8)
Kuwabara[35]	1986	Case report	Very low	BR	Pedicled	Laparotomy	Satisfatory	1 (1)
Nakao[36]	1986	Case series	Very low	BR	Pedicled	Laparotomy	Satisfatory	8 (12)
Hollinger[37]	1986	Retrospective cohort	Very low	BR	Pedicled	Laparotomy	Satisfatory	12
Abbott[38]	1986	Case series	Very low	BR	Pedicled	Laparotomy	Satisfatory	17
Rostom[39]	1987	Case report	Very low	BR	Pedicled	Laparotomy	Satisfatory	1
Petit[40]	1987	Case report	Very low	RT	Pedicled	Laparotomy	Satisfatory	1
Zoetmulder[41]	1988	Case series	Very low	BR	Pedicled	Laparotomy	Satisfatory	19
Pittam[42]	1988	Case report	Very low	BR + RT	Pedicled	Laparotomy	Satisfatory	2
Williams[43]	1989	Retrospective cohort	Low	BR + RT	Pedicled	Laparotomy	Satisfatory	43
Milanov[44]	1989	Case report	Very low	RT	Pedicled	Laparotomy	Satisfatory	3
Nagadowska[45]	1989	Case report	Very low	RT	Pedicled	Laparotomy	Satisfatory	1 (1)
Erol[46]	1990	Case series	Low	BR	Pedicled	Laparotomy	Satisfatory	27 (27)
Lee[47]	1990	Case report	Very low	BR	Pedicled	Laparotomy	Satisfatory	2
Lopez[48]	1990	Case series	Low	BR + RT	Pedicled	Laparotomy	Satisfatory	50 (50)
Van Garderen[14]	1991	Case series	Very low	BR + RT	Pedicled	Laparotomy	Satisfatory	17
Abbes[49]	1991	Case series	Very low	BR + RT	Pedicled	Laparotomy	Satisfatory	10
Viiachki[50]	1991	Case report	Very low	BR	Pedicled	Laparotomy	NR	1 (1)
Calderoli[51]	1991	Case series	Low	BR	Pedicled	Laparotomy	Satisfatory	49
Papadia[52]	1992	Case series	Very low	RT	Pedicled	Laparotomy	Satisfatory	15
Samuels[53]	1993	Case report	Very low	RT	Pedicled	Laparotomy	Satisfatory	1 (1)
Corral[54]	1994	Case report	Very low	RT	Pedicled	Laparoscopy	Satisfatory	1 (1)
Zhang[55]	1993	Case series	Very low	RT	Pedicled	Laparotomy	Satisfatory	5 (5)
Ribuffo[56]	1994	Case series	Very low	BR	Free	NR	Satisfatory	4 (4)
Rouanet[57]	1995	Case series	Low	RT	Pedicled	Laparotomy	Efective[b]	20
Contant[58]	1996	Case series	Low	BR	Pedicled	Laparotomy	Satisfatory	34
Bufo et al.[59]	1997	Case report	Very low	BR	Pedicled	Laparotomy	Satisfatory	1 (1)
Cheung[60]	1997	Case series	Very low	BR	Pedicled	Laparotomy	Efective	11 (11)
Van Geel[16]	1998	Case series	Very low	BR + RT	Pedicled	Laparotomy	Satisfatory	18
Nemsadze[61]	1998	Case series	Very low	BR	Pedicled	Laparotomy	Satisfatory	9
Fui[62]	1998	Case series	Very low	BR	Pedicled	Laparotomy	Satisfatory	3
Hultman[63]	2001	Case series	Very low	BR + RT + PC	Pedicled	Laparotomy	Satisfatory	14
Cothier-Savey[64]	2001	Case series	Very low	BR	Pedicled	Laparoscopy	Satisfatory	10
Henderson[65]	2001	Case series	Low	BR	Pedicled	Laparotomy	Satisfatory	61
Peiper[66]	2002	Case report	Very low	BR	Pedicled	Laparotomy	Satisfatory	2
Ida[67]	2002	Case report	Very low	RT	Pedicled	Laparoscopy	Satisfatory	1
Jimenez[68]	2002	Case report	Very low	BR + PC	Free	Both	Satisfatory	4
Sato[69]	2002	Case report	Very low	RT	Pedicled	Laparotomy	Satisfatory	1 (1)
Kamei[70]	2003	Case report	Very low	BR	Pedicled	Laparoscopy	"Excelent"	1
Hogewind[71]	2004	Case report	Very low	BR	Pedicled	Laparoscopy	Satisfatory	1 (1)
Kulakowski[72]	2005	Case report	Very low	BR + RT	Pedicled	Laparotomy	Satisfatory	6 (6)
Novoa[73]	2005	Case report	Very low	BR	Pedicled	Laparoscopy	Satisfatory	1 (1)
Kolodziejski[74]	2005	Case report	Very low	BR	Pedicled	NR	Satisfatory	1

TABLE 1 continued

Study	Year	Study design	Grade[a]	Applicability	Flap design	Harvesting technique	Efficacy	No. of patients (breasts)
Ferron[75]	2007	Case series	Very low	BR + RT	Pedicled	Laparoscopy	Satisfatory	11
Han[76]	2008	Case report	Very low	BR	Pedicled	Laparotomy	Satisfatory	1
Aukema[77]	2009	Retrospective cohort	Low	BR	Pedicled	Laparotomy	Satisfatory	60
Lans[78]	2009	Retrospective cohort	Very low	BR	Pedicled	NR	Satisfatory	NE/58
Aquilina[79]	2009	Case report	Very low	RT	Pedicled	Laparotomy	Satisfatory	1
Costa[80]	2010	Case series	Very low	PC	Pedicled	Laparoscopy	Satisfatory	13
Zaha and Inamine[81c]	2010	Case series	Low	BR	Pedicled	Laparoscopy	Satisfatory	89
Bosc[82]	2011	Case series	Very low	RT	Pedicled	Laparotomy	Satisfatory	3
Song[83]	2011	Case series	Very low	BR	Pedicled	Laparoscopy	Satisfatory	5
Costa[84]	2011	Case series	Very low	BR	Pedicled	Laparoscopy	Satisfatory	5
Zaha et al.[5]	2012	Case series	Very low	BR	Free	Laparoscopy	Satisfatory	10
Goés[18]	2013	Retrospective cohort	Low	BR	Pedicled	Laparoscopy	Satisfatory	200
Khater[85]	2013	Case series	Low	BR	Pedicled	Laparotomy	Satisfatory	24
Romanini[86]	2013	Case series	Very low	PC	Pedicled	Laparoscopy	Satisfatory	13
Total								985 + NR

BR breast reconstruction, *RT* radiation treatment, *PC* treatment of Poland syndrome or congenital deformity, *N*R not referred, *NE* authors mentioned clinical applicability although the number of patients was not specified

[a] Methodological quality according to grade classification[12]

[b] Efective but produces an inferior aesthetic result if compared to other techniques

[c] Some cases are duplicated, having been described in other articles

Laparoscopy was performed in only 4.00 % (1/25) of bowel obstructions and 4.55 % (2/44) of incisional hernias (Fig. 2c).

The incidence of complications was assessed in the 12 studies with the better ("low") methodological quality involving 692 women (Table 2). Each of these studies described a standard technique and involved at least 20 women that were adequately followed. The overall incidence of complications was 169/692 (24.42 %). The pattern of complications in this particular subset of higher-quality studies was similar to that observed when all studies analyzed in this review were considered. One outstanding exception was the absence of pain, seroma, and breast asymmetry (Fig. 2d). The complication rate for studies that used the laparoscopic technique was 6.57 % (19 complications among 289 women). It was 37.22 % (150/403 women) in studies that used the open technique for omentum harvesting. Statistical analyses were not performed because of the low quality and the great heterogeneity of these studies.

ONCOLOGICAL SAFETY

The incidence of local breast cancer or sarcoma and their recurrence among 403 women described in eight studies with the better ("low") methodological quality that evaluated the oncological risk of the procedure was 35.48 % (143/403). Seven of these studies [33,43,46,48,58,65,79] used the omental flap for breast reconstruction in patients with locally advanced cancer or recurrent cancer, and one of the seven reported a higher incidence and shorter relapse time according to the primary tumor size.[43] The eighth study in this series[81] described the use of omentum for partial breast reconstruction in patients with nonadvanced disease and found no breast cancer recurrence during a mean follow-up of 63.4 months (Table 3).

DISCUSSION

This systematic review was performed using studies of different designs from all over the world with a wide range of techniques, depending on the surgeon. Although greater omentum has been used in the breast since the 1960s,[1] until now no studies of adequate quality have evaluated its suitability and safety. The majority of those studies had a "very low" grade of recommendation, and none had more than a "low grade."[9,11,13] The information we provide in this review, such as suitability and expected complications of the usage of greater omentum in the breast, are compiled into a single report.

During the 30 years covered by this review, the studies we selected during the first half demonstrated that laparotomy was almost the only technique used for harvesting greater omentum. During the second half, however,

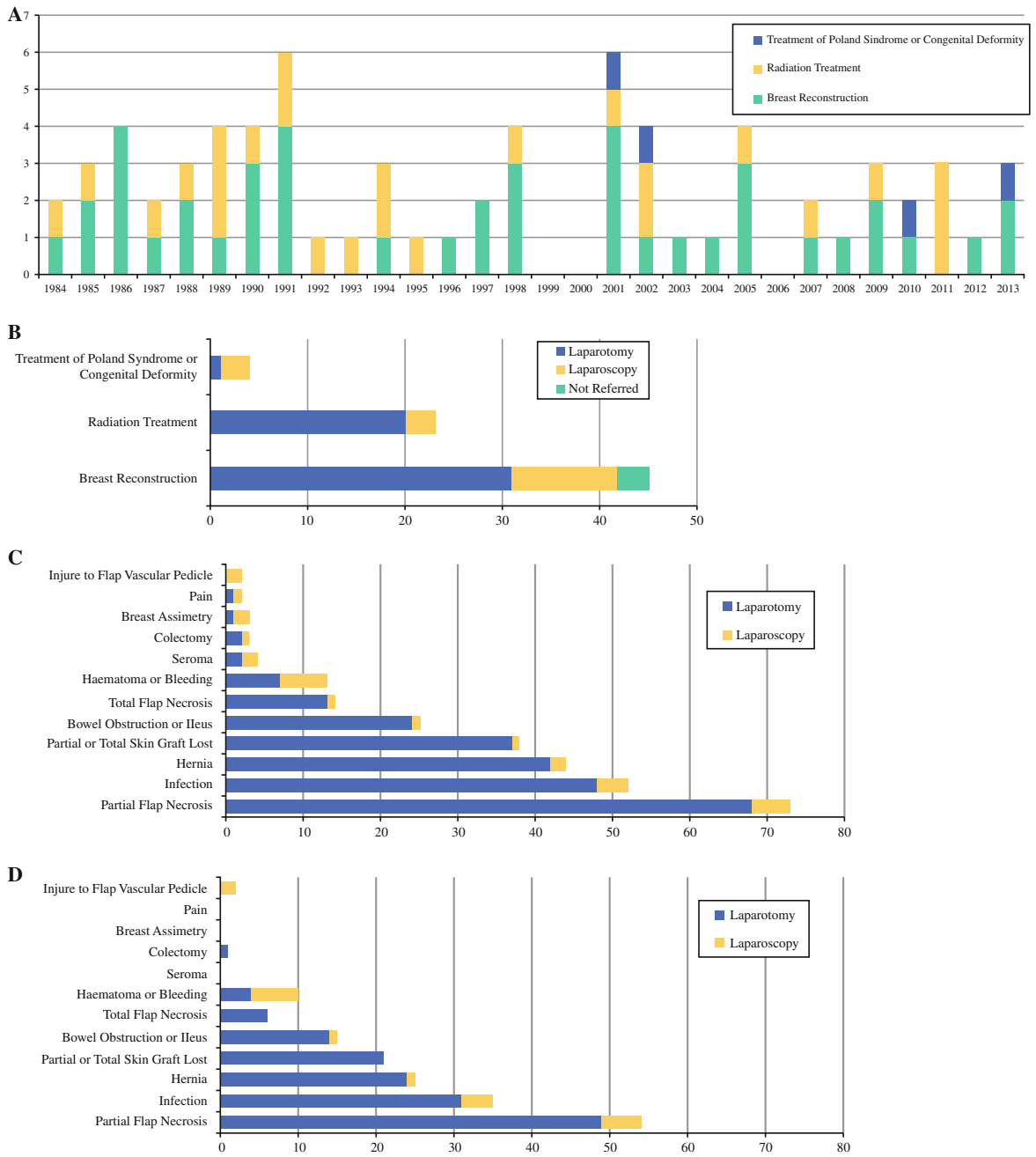

FIG. 2 **a** Evolution of publications for studies using omentum in the breast during the last 30 years and its applicability. **b** Indications for using an omental flap for the breast in relation to the harvesting technique described in 60 studies. **c** Distribution of 275 complications identified in 60 studies (985 women). **d** Distribution of 169 complications identified in the 12 studies with better ("low," not "very low") methodological quality

laparoscopy was used more often (Table 2). This information seems to be justified by the great evolution of surgery and more accessibility of laparoscopic techniques. The kind of breast reconstruction has also changed during the latter period. During the first 15 years, greater omentum (along with skin grafts) was used only as the last option for covering huge deformities resulting from mastectomy performed in women with advanced breast cancer.

TABLE 2 Incidence of clinical complications according to flap design and harvesting technique in studies with better methodological quality

Study	Year	No. of patients (breasts)	Harvesting technique	Follow-up (months)[a]	Clinical complications
Calderoli[33]	1985	35	Laparotomy	NR	2/35
Williams[43]	1989	43	Laparotomy	22	23/43
Erol[46]	1990	27 (27)	Laparotomy	12–96	6/27
Lopez[48]	1990	50 (50)	Laparotomy	47	10/50
Calderoli[51]	1991	49	Laparotomy	48	2/49
Rouanet[57]	1995	20	Laparotomy	NR	13/20
Contant[58]	1996	34	Laparotomy	55	26/34
Henderson[65b]	2001	61	Laparotomy	79	53/61
Aukema[77]	2009	60	Laparotomy	60	9/60
Zaha[81]	2010	89	Laparoscopy	63.4	13/89
Goés[18]	2013	200	Laparoscopy	NR	6/200
Khater[85]	2013	24	Laparotomy	28	6/24
Total		692			169/692 (24.42 %)

All of these studies had a pedicled flap design

[a] Expressed as the mean

[b] Seven cases were cited in the author's 2012 study and were not included in this data collection

TABLE 3 Assessment of oncological risk (local recurrence)

Study	Year	No. of patients (breasts)	Applicability	Follow-up (mean months)	Interval to relapse (mean months)	Local recurrence of breast cancer (no.)
Calderoli[33]	1985	35	BR (RC) + RT	NR	6	2/35
Williams[43]	1989	35[a]	BR (LA + RC)	36	12 (for tumors < 5 cm) 6 (for tumor 5–10 cm) <6 (for tumors > 10 cm)	31/5
Lopez[48]	1990	50 (50)	BR (LA + RC) + RT	47	NR	8//50
Calderoli[51]	1991	49	BR (RC)	>48	NR	26//49
Contant[58]	1996	24[b]	BR (LA + RC) + RT	55	8	15/24
Henderson[65]	2001	61	BR (LA + RC)	79	20	32/61
Aukema[77]	2009	60	BR (RC)	60	24	29/60
Zaha[81]	2010	89[c]	BR (PA)	63.4	NA	0/89
Total		403				143/403 (35.48 %)

LA locally advanced cancer, *RC* recurrent cancer, *PA* partial breast reconstruction or adenomastectomy after primary tumor resection, *NA* not applicable

[a] Eight cases from their study were not included in authors' analyses of recurrence

[b] Ten patients from the study underwent palliative treatment and were not included with this data collection

[c] Seven cases were cited in the authors' 2012 study and were not included in this data collection

During the last 15 years, however, it was used also for breast reconstruction in patients with early-stage breast cancer[5,18,85] and even for additional coverage of alloplastic breast implants or mesh.[18]

Being able to treat women with radiation-related tissue damage has produced stunning results, allowing regeneration and complete healing of these damaged tissues. More recently, greater omentum has been used to improve breast shape in patients with congenital deformities such as is seen with Poland syndrome. Laparoscopic harvesting of a greater omental flap, especially for these cases, is an efficient treatment with fewer co-morbidities and scars (Fig. 2). From the technical standpoint, great heterogeneity was observed among the studies and even among cases in a single study. The morbidity treated was not the primary indication for the technique chosen. It was the defect that had been identified that was the primary reason for the surgeon's choice and the best way to attain the ultimate

goal. As omentum with or without an implant has now been used for several indications, the technique cannot be standardized.

Flap failure was the most frequent complication, accounting for almost one-third of the complications (31.87 %, 87 women). Among the women with omental flap failure, 73 had partial flap necrosis, and 14 had total necrosis. Six cases of flap necrosis were reported (five partial, one total) in the laparoscopy group, which may indicate that laparoscopy for flap harvesting is safer for flap survival than laparotomy. Two cases of omental flap vascular pedicle injury (one was converted to a free flap, one was treated with hemostasis) that would have resulted in total flap loss were reported after laparoscopy, which seems to demonstrate better control of the vascular pedicle status during the harvesting procedure, allowing prompt treatment.

Regarding the vessel of choice for constructing the pedicle, although the right gastroepiploic vessel seems to have been used most often, pedicled flaps were generally described as based on the most convenient gastroepiploic vessel at the time. The omental flap was usually delivered to the breast bed subcutaneously, with the pedicle passing through a small incision at the upper abdominal midline, which can be performed using laparoscopy or laparotomy. Some reasons for partial loss (without damage to the pedicle) that have been identified are the following: (1) The entire omentum is used, and the most peripheral segments may not receive adequate blood supply from a single pedicle. (2) During the process of moving it from the abdominal cavity, trauma may injure some segmental vessels. (3) It may be compressed in the breast bed. (4) Adverse conditions (dehydration, hypothermia, trauma) may be present as the omentum is constantly exposed to the extraperitoneal environment.

The second most common complication reported was infection. It was described in 52 women (19.05 %), followed by abdominal wall hernia in 44 women (16.12 %) and bowel obstruction or ileus in 25 women (9.16 %). For the latter two complications, the rate was low for those who underwent laparoscopic omentum harvesting. However, laparoscopy was performed in about half of the cases in which there was a seroma, despite the fact that it had been performed in fewer than one-fourth of all cases included in this review. Although laparoscopy was associated with a lower complication rate than the open technique, the more serious cases were observed among women in this group. Half of the bleeding/hematoma episodes and one-third of colectomies were found in the studies that used laparoscopy to harvest the greater omentum.

Considering the 12 studies that described a standard technique and involved at least 20 women that were adequately followed, the overall incidence of complications was high. It is justified, however, by the high rate of complications observed in the studies that used the procedure to treat locally advanced breast cancer that required coverage of huge chest wall defects.[1,3,4,16,28,31–36,45,47,49,58,62,63,65,79,87,88] Laparoscopy, predominantly used to treat minor defects, is associated with a complication rate of 6.57 % (19/289 women). Considering the high incidence of partial/total flap loss and some complications involving the digestive system, muscular/myocutaneous and perforator flaps and/or structured fat grafting seem to be safer than using omentum for some mammary disorders as they show similar results.[89–94]

Concerning the potential risk of fat cells for breast cancer, as proposed in 1986[6] and 1987,[7] some experimental studies have reported that adipocytes may increase the local concentration of estrogen and release some adipocytokines that may interact with the growth. They may also cause apoptosis of normal epithelial breast cells. In addition, the adipocytokines seem to stimulate the behavior of breast carcinoma cell lines, enhancing their potential for migration, invasion, and cell adhesion while inhibiting apoptosis.[8,95–104] The greater omentum may also offer additional risk if it has a high concentration of stem cells with their great angiogenic potential.[8,84,101]

In this review, only eight studies with better ("low") methodological quality analyzed oncological risk. Seven of them described local relapse in patients with advanced breast cancer disease. There was a mean rate of 45.54 % among them. Williams et al.[43] compared the local recurrence in breasts reconstructed using greater omentum with the primary tumor size at resection and found a mean relapse time of less than 6 months in tumors >10 cm and a relapse time of 12 months for those <5 cm. Their study suggested that the breast cancer stage may have greater influence on its recurrence than the technique used for breast reconstruction, which may represent a high potential of bias for analyses of this subject in these studies.[11,105–108] In contrast, the eighth study of this series[81] used omentum for partial breast reconstruction in patients with early-stage disease and reported no recurrence at a mean postoperative follow-up of 63.4 months.

CONCLUSIONS

Considering the high volume of abdominal complications, with the possibility of bowel injury which (although relatively infrequent) is a more serious complication), the omentum has well-established applicability only for total breast reconstruction of huge defects, where muscular/myocutaneous or perforator flaps may be unsuitable. Concerning the oncological risk, the lack of evidence up to now did not show that using the omentum in the breast might have a potential for mammary cancer.

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5. DISCUSSÃO GERAL

As técnicas de reconstrução mamária evoluíram, desde seus primórdios, e a identificação dos problemas relacionados a cada tipo de reconstrução permitiram uma adequada individualização do procedimento, permitindo a escolha da técnica mais adequada para cada situação (1, 25). Somado à variedade de procedimentos disponíveis, a lipoenxertia de gordura, apesar de ser a primeira técnica de reconstrução mamária descrita (há mais de 100 anos), apenas no século XXI, passou a ter seu reconhecimento na prática clínica (61, 67). Isso porque, para sua efetividade e segurança, grande desenvolvimento nas técnicas de coleta, preparo e enxertia foram exigidos, principalmente no decorrer dos últimos 50 anos(61, 69, 143).

Apesar da segurança clínica comprovada com a técnica de lipoenxertia estruturada, inicialmente proposta em 1987(60) e padronizada em 2004(62), um teórico potencial oncogênico foi aventado, com base no conhecido risco do câncer de mama associado à obesidade, somado às evidências laboratoriais do potencial inflamatório e proliferativos das adipocinas produzidas pelos adipócitos(68, 75, 84, 94, 144).

Na área experimental, procuramos em um primeiro momento avaliar as características adaptativas do omento pediculado translocado (propusemos um modelo experimental de lipoenxertia em ratos). A utilização do tecido pediculado foi adotada neste estudo para garantir que o tecido enxertado não fosse absorvido (a lipoenxertia em humanos tem uma taxa de absorção imprevisível que pode variar de 10% a 90%), evitando, desta forma, viéses relacionados à ausência tecidual ou até mesmo o risco de se avaliar um produto resultante de necrose.

Muitos estudos já mostraram um grande potencial angiogênico do omento pediculado (assim como da lipoenxertia), sendo relatado o seu uso para o tratamento de epilepsia, revascularização miocárdica, tratamento de edemas, hipóxia tecidual, regeneração de sequelas de radioterapia, entre outros(36, 85, 86, 107, 109, 145, 146). Com base nestes dados foi questionado o potencial do tecido adiposo em regenerar outras estruturas de origem mesodérmica, como ossos, músculos e até outros tecidos adiposos por meio de suas células-tronco multipotentes com grande potencial angiogênico. Estes dados identificam que o tecido adiposo, independentemente se da região intra-abdominal ou subcutânea e

se enxertado de forma vascularizada ou livre, apresenta um grande potencial regenerativo que poderia estimular células tumorais(70, 72, 147). A análise deste estudo, entretanto, identificou que o tecido adiposo do omento, mesmo tendo sido transplantando em um meio de origem embriológica tecidual semelhante (subcutâneo) mantém as características morfológicas de seus adipócitos e não é capaz de sofrer degeneração ou evoluir para atipia celular, o estímulo à neovascularização e regeneração no sítio receptor, deve-se à população flutuante de células estromais, como células-tronco mesenquimais, macrófagos e fibroblastos.

Em nossos outros estudos experimentais, procurou-se identificar alguns riscos oncológicos possivelmente associados às funções biológicas dos adipócitos. Entre eles, destacam-se a inflamação crônica, a proliferação celular e o aumento nos níveis PAI-1.

O CD68 é uma glicoproteína que se expressa predominantemente em macrófagos e está presente em processos inflamatórios e em distúrbios metabólicos, como resistência insulínica(127), placas de aterosclerose(137) e no tecido tumoral(119-126). Di Gregorio et al.(127) identificaram também que a expressão do CD68 é maior na fração estromal vascular do tecido adiposo do que nos adipócitos, o que torna este marcador importante fonte de identificação de inflamação crônica e proliferação celular crônica.

O Ki67 é uma proteína celular presente em células em proliferação e ausente em células em repouso, o que torna este marcador importante para a identificação da proliferação celular(115-118). Atualmente, a sua utilização tem ganhado grande destaque como um fator prognóstico de câncer de mama em fase inicial, e até mesmo como um preditivo de eficácia terapêutica para esta doença (117, 148-152). Este marcador foi utilizado em nossos estudos, portanto, para se avaliar a taxa proliferativa dos adipócitos enxertados e do tecido glandular mamário exposto à lipoenxertia.

A glicoproteína Inibidora da Atividade de Plasminogênio-1 (“Plasminogen Activator Inhibitor 1" - PAI-1) é fundamental no processo de coagulação, agindo como principal regulador da atividade de plasminogênio *in vivo*. É secretada por várias células e recentemente vem ganhando destaque também como um importante componente da reparação tecidual(128-131). Elevados níveis de PAI-1, isoladamente estão correlacionados à obesidade, hiperinsulinemia, hiperglicemia e hipertrigliceridemia(94, 130). Os níveis de PAI-1 e uroquinase (uPA) também têm

sido descritos como elevados em condições tumorais. Entretanto, achados recentes identificaram o PAI-1 como o mais importante marcador tumoral, uma vez que o uPA (o primeiro marcador do ciclo de ativação/inibição do plasminogênio a ser considerado um marcador tumoral) tem se mostrado elevado em qualquer condição de reparação tecidual(77, 104, 131, 133, 153-156). Isso justifica a sua elevação em áreas traumatizadas, sem necessariamente representar uma condição oncológica ou potencialmente oncogênica(157-160). Desta forma, visamos pesquisar apenas os níveis de PAI-1 nos procedimentos experimentais realizados a fim de se evitar vieses decorrentes da resposta pós-traumática sobre o tecido analisado em detrimento de seu possível efeito oncogênico.

Desta forma, o papel do PAI-1 no câncer de mama tem sido exaustivamente estudado, sendo este envolvido na diminuição da atividade apoptótica e degradação da matriz extracelular durante o crescimento tumoral, estimulando a sua invasão e disseminação metastática. Isso caracteriza os altos níveis de PAI-1 como um fator de mau prognóstico para o câncer de mama(77, 94, 128, 129, 131, 132, 154, 156, 161, 162).

Com base nestes marcadores, portanto, em uma análise experimental procuramos identificar e comparar o potencial oncogênico do tecido adiposo e do procedimento de lipoenxertia. Para isso, também se utilizou a gordura do omento pediculada, como no primeiro experimento citado anteriormente, a fim de se evitar vieses decorrentes da absorção imprecisa da gordura livre enxertada. Com a finalidade de comprovar a similaridade da gordura intra-abdominal com a do subcutâneo para as variáveis analisadas (CD68, Ki67 e PAI-1), foi coletada, simultaneamente, uma amostra da gordura subcutânea do mesmo rato exposto às mesmas condições da gordura intra-abdominal, que foi utilizada como um controle tecidual(100, 103-106).

O presente estudo identificou que a dieta hipercalórica, como um fator isolado, foi capaz de estimular a proliferação celular e o aumento nos níveis da proteína PAI-1 na gordura nativa sem qualquer manipulação. O procedimento de enxerto de gordura, entretanto, não foi capaz de estimular qualquer resposta inflamatória, proliferação celular ou aumento nos níveis de PAI-1. Tal fato demonstra que a gordura nativa de cada parte do corpo já tem uma atividade parácrina local, e que através dela pode exercer uma atividade oncogênica quando exposta a fatores de risco como obesidade, dieta hipercalórica, entre outros. Em contrapartida, o

procedimento de lipoenxertia de tecido adiposo sadio (não exposto a um fator de risco) por si só, não parece ser capaz de aumentar a atividade parácrina já existente no local onde há tecido adiposo, como a mama. É importante destacar que por mais radical que seja uma mastectomia, a região vizinha, ou até mesmo a pele utilizada para cobrir a área de ressecção contém adipócitos. Isso não sustenta a teoria de que a lipoenxertia poderia aumentar o estímulo parácrino decorrente dos adipócitos enxertados sobre o tecido glandular mamário residual.

Em um terceiro estudo experimental, procurou-se identificar a resposta do tecido mamário à enxertia de gordura. Neste caso, como o foco da análise era o tecido glandular exposto ao enxerto de gordura, não houve a necessidade de se utilizar tecido adiposo pediculado, uma vez que a taxa de reabsorção do tecido enxertado não iria influenciar na análise das mamas. Entretanto, a possibilidade de se utilizar um tecido microfagmentado, que poderia ser injetado dentro do tecido glandular com maior exposição às células ductais, pareceu ser mais relevante para esta análise que a utilização de um tecido adiposo pediculado. E para reforçar os dados deste estudo com o modelo experimental utilizado nos estudos anteriores, também se optou por amostras teciduais do omento e do subcutâneo para observar e comparar a existência de estímulo oncogênico da lipoenxertia entre diferentes tipos teciduais (duas linhagens de tecido adiposo).

Em uma análise histológica inicial, nenhuma alteração estrutural ou morfológica foi identificada nos lóbulos mamários que pudesse identificar algum processo proliferativo(163). Como neste terceiro estudo experimental foi avaliado o comportamento da glândula mamária à exposição da lipoenxertia, procuramos analisar também a expressão do receptor de estrógeno neste tecido, a fim de se observar a resposta das células ductais a uma maior concentração de gordura em seu parênquima e evitar vieses decorrentes da interação entre Ki67 e ER(1D5). Procurou-se ainda avaliar a relação ER(1D5)/Ki67 entre as mamas de controle e as que foram enxertadas com tecido adiposo do omento e do subcutâneo, uma vez que uma alteração nesta relação com aumento simultâneo no níveis deste dois marcadores tem sido descrito como uma possível condição pré-cancerosa(87, 116-118, 134-136, 144, 151, 164).

Para reforçar os dados clínicos presentes até o momento, somado aos nossos dois primeiros experimentos, não se observou neste terceiro experimento, qualquer alteração nas mamas que receberam lipoenxertia de tecido adiposo sadio

(sem exposição a fatores de risco conhecidos), seja do omento ou do subcutâneo, em relação às mamas de controle. Vale ressaltar ainda que foi utilizada para esta análise uma raça de rato altamente susceptível ao câncer de mama, quando exposto a qualquer fator de risco(96, 97). Como estudos experimentais *in vivo* com foco no potencial oncogênico do tecido adiposo enxertado é praticamente inexistente, acreditamos ser necessários maiores estudos para comprovar nossos achados. Entretanto, nossos resultados nas três pesquisas vão de acordo com o que tem sido observado na prática clínica e pode vir a somar informações quanto à segurança do procedimento. Uma limitação deste terceiro estudo em tecido mamário foi o fato de termos utilizado apenas mamas sadias, o que demonstra a importância de análises futuras de novos experimentos utilizando mamas com câncer para complementar estes resultados.

Em busca de dados clínicos ainda não explorados, referentes ao uso de tecido adiposo na topografia mamária, realizamos também uma revisão sistemática das pacientes que receberam tecido adiposo da região intra-abdominal (omento) para o tratamento de desordens mamárias e reconstruções após câncer de mama. Com base em relatos da literatura, experiência pessoal clínica e nos experimentos desta tese, de que o tecido adiposo do omento pediculado sofre estímulo semelhante ao do enxerto livre de gordura quando translocado para o meio extraperitoneal, procuramos avaliar a segurança oncológica destes procedimentos para o câncer de mama.

Diferentemente dos retalhos miocutâneos ou cutâneos, em que a gordura é levada em bloco com preservação de sua arquitetura e vascularização (o que não causa estímulo e contato significativos de grande parte das células do retalho com o sítio receptor), no omento pediculado translocado para o meio extraperitoneal, a quase totalidade do retalho sofre estímulo semelhante ao que ocorre na lipoenxertia livre. Isto porque o omento é uma estrutura delgada, em que o tecido adiposo está distribuído difusamente, mantendo contato e recebendo nutrição do líquido peritoneal. Durante o processo de translocação do omento para o meio subcutâneo, apesar da manutenção de sua vascularização, todo o seu componente celular e extracelular sofre uma significativa mudança na sua arquitetura estrutural e nutricional, o que desencadeia o desenvolvimento de um processo adaptativo semelhante ao observado com a lipoenxertia livre, representados pela intensa neovascularização, aumento significativo de seus componentes intersticiais e poder

regenerativo das estruturas adjacentes(61, 66, 71, 72, 74, 85, 86, 107-110, 145, 165-170).

Nessa revisão sistemática, a exemplo do que vem sendo relatado na literatura para lipoenxertia, não foi identificada qualquer relação com eventos adversos relacionados ao câncer de mama, se comparada aos tratamentos convencionais. E, ao contrário, foram identificados dados que podem vir a somar ainda mais evidências sobre a segurança oncológica do uso do tecido adiposo na mama.

6. CONCLUSÃO

6.1. O tecido adiposo enxertado (omento) de forma pediculada apresentou uma resposta adaptativa em toda sua estrutura à translocação para o meio subcutâneo, com neovascularização, intensificação da matriz extracelular e fibrose sem atipias celulares, e os seus adipócitos mantiveram o mesmo padrão morfológico dos adipócitos do omento intra-abdominal.

6.2. O tecido adiposo nativo sem manipulação, quando exposto a uma dieta hipercalórica (fator de risco conhecido), apresenta um potencial oncogênico, representado por proliferação celular (aumento do marcador Ki67) e elevação dos níveis de PAI-1. Entretanto, o tecido adiposo do omento translocado (modelo experimental de lipoenxertia) manteve os mesmos padrões observados nas amostras de controle.

6.3. Nenhuma alteração morfológica ou estrutural foi comparativamente observada nos lóbulos mamários que receberam lipoenxertia. Assim como não foram identificados qualquer aumento na taxa de proliferação celular, resposta inflamatória crônica, alteração no padrão de positividade estrogênica ou de concentração e distribuição da proteína PAI-1.

6.4. Não foram identificadas evidências clínicas na revisão sistemática que sustente o risco oncológico da gordura translocada do omento para as mamas.

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8. ANEXOS

Anexo 1: Aprovação da Comissão de Ética no Uso de Animais – CEUA/UNICAMP

UNICAMP

CEUA/Unicamp

Comissão de Ética no Uso de Animais
CEUA/Unicamp

C E R T I F I C A D O

Certificamos que o Protocolo nº **2210-1**, sobre **"Omento para a reconstrução de mama: estudo experimental de suas propriedades adaptativas no subcutâneo"**, sob a responsabilidade de **Prof. Dr. Aarão Mendes Pinto Neto / Francisco Claro de Oliveira Junior**, está de acordo com os Princípios Éticos na Experimentação Animal adotados pelo Colégio Brasileiro de Experimentação Animal (COBEA), tendo sido aprovado pela Comissão de Ética no Uso de Animais – CEUA/Unicamp em **09 de agosto de 2010**.

C E R T I F I C A T E

We certify that the protocol nº **2210-1**, entitled **"Omentum for breast reconstruction: an experimental study of its adaptive properties into the subcutaneous space"**, is in agreement with the Ethical Principles for Animal Research established by the Brazilian College for Animal Experimentation (COBEA). This project was approved by the institutional Committee for Ethics in Animal Research (State University of Campinas - Unicamp) on **August 9, 2010**.

Campinas, 16 de abril de 2013.

2ª. VIA

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Anexo 2: Comprovante de Recebimento do Artigo pela Revista: Journal of Mammary Gland Biology and Neoplasia

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Acknowledgement of Receipt

1 mensagem

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Although for now you don't have to do anything, we would like to let you know about your upcoming options.

Anexo 3: Licença obtida junto à Editora para inclusão do artigo publicado:

Omentrum for Mammary Disorders: A 30-Year Systematic Review