Etiopatogénese da contratura capsular de próteses mamárias

Etiopathogenesis of capsular contracture in breast implants

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Contents

List of original publications

This thesis is based on the following publications which are referred in the text by their Roman numerals I-VI:

I- Adams, WP., Haydon, MS., Raniere, J., Trott S., Marques, M., Feliciano, M., Robinson, JB., Tang, L., Brown, SA. A rabbit model for capsular contracture: development and clinical implications. *Plast Reconstr Surg.* 2006; 117: 1214-9.
Plastic and Reconstructive Surgery journal is indexed by Thomson Reuters (ISI); the journal's impact factor is 2,647 the highest in Plastic Surgery. The publication`s Scopus times cited is 15.
The publication was presented in the XXXIV Reunião da Sociedade Portuguesa de Cirurgia Plástica, Reconstrutiva e Estética, Oporto, Portugal, 2004.

II- Marques, M., Brown, SA., Oliveira, I., Cordeiro, N., Morales-Helgera, A., Gonçalves-Rodrigues, A., Amarante, J. Long-term follow-up of breast capsule contracture rates in cosmetic and reconstructive cases. *Plast Reconstr Surg*. 2010; 126: 769-778.
Plastic and Reconstructive Surgery journal is indexed by Thomson Reuters (ISI); the journal's impact factor is 2,647 the highest in Plastic Surgery. The publication`s Scopus times cited is 4 and was the most popular publication in this journal in September 16, 2010.
The publication was presented in the XL Reunião Anual da Sociedade Portuguesa de Cirurgia Plástica Reconstrutiva e Estética, Coimbra, Portugal, 2010.

III-Marques, M., Brown, SA., Cordeiro, N., Rodrigues-Pereira, P., Cobrado, L., Morales-Helgera, A., Lima, N., Luís, A., Mendanha, M., Gonçalves-Rodrigues, A., Amarante, J. Effects of fibrin, thrombin and blood on breast capsule formation in a pre-clinical model. *Aesthet Surg J.* 2011; 31: 302-309.
Aesthet Surgery Journal is indexed with Thomson Reuters (ISI). The publication`s Scopus times cited is 2.
The publication was presented in the XL Reunião Anual da Sociedade Portuguesa de Cirurgia Plástica Reconstrutiva e Estética, Coimbra, Portugal, 2010 and received the prize of the "Best Aesthetic Surgery Communication" to represent Portugal in the "Voice of Europe", 4º European Association of the Societies of Aesthetic Plastic Surgery (EASAPS) Congress.

IV-Marques, M., Brown, SA., Cordeiro, N., Rodrigues-Pereira, P., Cobrado, L., Morales-Helgera, A., Queirós, L., Luís, A., Freitas, R., Gonçalves-Rodrigues, A., Amarante, J. Effects of coagulase negative staphylococci and fibrin on breast capsule formation in a rabbit model. *Aesthet Surg J.* 2011; 3: 420-428.
Aesthet Surgery Journal is indexed by Thomson Reuters (ISI). The publication`s Scopus times cited is 2.
The publication was presented in the XL Reunião Anual da Sociedade Portuguesa de Cirurgia Plástica Reconstrutiva e Estética, Coimbra, Portugal, 2010, and received the prize of the "Best Aesthetic Surgery Communication" simultaneously with communication above.
Both publications (III and IV) represented Portugal in the "Voice of Europe", EASAPS Congress, Milan, Italy, 2011.

V-Marques, M., Brown, SA., Rodrigues-Pereira, P., Cordeiro, N., Morales-Helgera, A., Cobrado, L., Queirós, L., Freitas, R., Fernandes, J., Correia-Sá, I., Gonçalves-Rodrigues, A., Amarante, J. Animal Model of Implant Capsule Contracture: effects of chitosan. *Aesthet Surg J.* 2011; 31: 540-550.

Aesthet Surgery Journal is indexed by Thomson Reuters (ISI). The publication`s Scopus times cited is 0.

The publication was presented in the XL Reunião Anual da Sociedade Portuguesa de Cirurgia Plástica Reconstrutiva e Estética, Coimbra, Portugal, 2011.

VI- Marques, M., Brown, SA., Cordeiro, N.D.S., Rodrigues-Pereira, P., Gonçalves-Rodrigues, A., Amarante, J. The impact of triamcinolone-acetonide in early breast capsule formation, in a rabbit model. *Aesthetic Plastic Surgery*. 2012; Apr.

Aesthetic Plastic Surgery journal is indexed by Thomson Reuters (ISI); the journal's impact factor is 1,252. The publication`s Scopus times cited is 0.

The publication was presented in the I Congresso Ibero-Escandinavo de Cirurgia Plástica Reconstrutiva e Estética / XLVII Congresso Nacional da Sociedade Espanhola de CPRE, Palma de Mallorca, Spain, 2012.

Abstract publication

Marques, M. Effects of fibrin (Tisseel/Tissucol®) on breast capsule formation in a rabbit model. *Aesthetic Plastic Surgery*. 2012; Jun.

The publication was presented in the Voice of Europe 2011 as the Voice of Portugal (from EASAPS Milan Congress).

Aesthetic Plastic Surgery journal is a publication of the International Society of Aesthetic Plastic Surgery and the official journal of the European Association of Societies of Aesthetic Plastic Surgery (EASAPS). Aesthetic Plastic Surgery journal is indexed by Thomson Reuters (ISI); the journal's impact factor is 1,252.

Abbreviations

CD: cluster of differentiation
CHAID: Chi−squared Automatic Interaction Detection
CI: confidence intervals
COS: chitooligosaccharide
CS: chitosan
CTGF: connective tissue growth factor

DCs: dendritic cells

ECM: collagenous extracellular matrix
EGF: epidermal growth factor
ET-1: endothelin 1

bFGF: basic fibroblast growth factor

HSP 60: heat shock proteins 60

IC: inhibitory concentration
ICAM-1: intercellular adhesion molecule 1
IL: interleukin
IFN-y: interferon y
IGF-1: insulin like growth factor-1

LMWC: low molecular weight chitosan
LPS: lipopolysaccharide

MCP-1 : monocyte chemotactic protein-1
f-MLP: formyl-methionyl-leucyl-phenylalanine
MALP-2: macrophage activating lipopeptide 2
MMP: macrophage-derivated matrix metalloproteinases
MPI-1α : macrophage inflammatory proteins 1α

NK: Natural killer
NO: oxide
iNOS: nitric oxide synthase

OPN: osteopontin
OSM: oncostatin M

PDGF: platelet-derived growth factor
PEGA: polyethylene glycol adipate
PF-4: plated factor 4
PMN: polymorphonuclear leukocytes

RANTES: regulated upon activation normal T-cell expressed presumed secreted
ROS: reactive oxygen species
RR: relative risks

α-SMA: α-smooth muscle cell actine
SPSS: Statistical Package for Social Sciences

TA: triamcinolone acetonide
TDA: toluenediamine
TDI: toluene diisocyanate
TGF-β1: transforming growth factor beta 1
Th: distinct types of T-helper cells
TNF-α: Tumor necrosis factor alpha

VCAM-1: vascular cell adhesion molecule 1
VEGF: vascular endothelial growth factor

WHI: Women´s Health Initiative

1. Introduction

Silicone gel breast implants have been implanted world-wide, for cosmetic augmentation and breast reconstruction, since 1962[1]. Research studies have focused on the potential adverse health effects of silicone implants, particularly, possible links with cancer or connective tissue disorders, but none have yet shown an increased risk of other diseases associated with those implants[2],[3],[4],[5],[6],[7],[8],[9],[10],[11]. Additional reports have focused on postoperative local complications, and patient safety issues in women receiving silicone breast implants[12],[13],[14],[15],[16],[17],[18],[19].

Capsular contracture is the formation of fibrous scar tissue investing a foreign body or surgically implanted device and is the most common severe chronic complication associated with silicone breast implants[12],[13],[14],[15],[16],[17],[18],[19], with a clinical realistic incidence ranging from 8 to 45%[20],[21],[22],[23],[24],[25].

Silicone breast implants have been certified according to European Union's safety and efficacy requirements as class III medical devices and reclassified by the European Union into the strictest category of medical devices for sale to the public. Silicone breast implants are the most widely applied medical implants in European countries. Saline-based implants, on the other hand, have been almost exclusively used in North America and are related to much more complications than silicone-based implants, such us rippling, firmer consistency, leaking and complete deflation[26]. So far, there is a lack of current prospective data comparing capsule contracture with saline *versus* silicone breast implants[27]. Recently McCarthy *et al.* [28] concluded in the setting of postmastectomy reconstruction, patients who received silicone breast implants (n = 176) reported significantly higher satisfaction with the results of reconstruction than those who received saline implants (n = 306). The first-generation of silicone implants had a thick shell and viscous gel, while the second-generation possessed a much thinner

gel and shell[29]. However, with these implants, excessive silicone gel bleed and rupture rates occurred[30]. The development of the third-generation gave birth to the "low bleed" implant containing a barrier-coated shell[29]. Ever since polyurethane-coated implants were reported to be associated with lower rates of capsular contracture[31],[32],[33],[34],[35],[36], the use of breast implants with a textured surface have been the subject of recent reviews[37].

Several studies have shown that textured implants have a lower tendency to develop capsular contracture than smooth-surface implants[22],[38],[39],[40],[41],[42], although others have reported the opposite[43],[44],[45]. In addition, there is substantial evidence that placement in the subglandular plane is associated with a higher incidence of capsular contracture[46] and is less satisfactory for mammography[47]. On the contrary, other studies show a lower proportion of capsular contracture, which was not however statistically significant[22],[48]. That observation may be attributed to a greater difficulty in appreciating capsular contracture in a deeper submuscular plane or to the fact that textured surface implants have no impact on capsular contracture when placed submuscularly.

The polyurethane foam-covered breast implant, a silicone gel-filled device surrounded by a 1- to 2mm-thick layer of polyurethane foam, is associated with a lower incidence of capsular contracture[49],[50]. In the late 1980s it was reported that in vitro degradation of polyurethane could lead to formation of substances known to be carcinogenic in animals[51]. In a retrospective study comprised of individuals receiving either polyurethane breast implants (n = 568) or other types of silicone gel-filled breast implants (n = 963), between 1981 and 2004 (23 years), Handel[52] concluded that the incidence of capsular contracture was dramatically lower with polyurethane foam-covered implants compared to smooth or textured implants. This beneficial effect

persisted at least 10 years after implantation. Aside from skin rash, the polyurethane foam-covered implants appear to have a safety profile similar to other silicone gel-filled devices. The polyurethane used in the manufacture of breast implants is a polyesterurethane made from polyethylene glycol adipate (PEGA) and toluene diisocyanate (TDI). The TDI is unstable in an aqueous environment and converts slowly into toluenediamine (TDA). The carcinogenic effect of toluenediamine (TDA) has never been established in humans[53]. To quantify in vivo release of TDA, Hester *et al.*[54] collected urine and serum samples from 61 patients with polyurethane foam-covered implants and 61 controls on two occasions separated by 10 +/- 3 days. No patients or controls had detectable free 2,4-TDA in their sera. Eighteen patients with polyurethane foam-covered implants had detectable levels in their urine, compared to 7 control subjects. The biodegradative half-life of the polyurethane foam was estimated to be 2 years. The risk assessment of approximately one in one million derived from this study strengthens earlier conclusions by the Health Protection Branch (Canada) that there is no significant risk of cancer from exposure to the 2,4-TDA formed from this biodegradation. In a review of the literature, McGrath and Burkhardt[55] concluded there was no evidence to link breast implants of any kind with an increased risk of breast cancer. So far, the polyurethane foam-covered breast implants are not FDA-approved in the USA. One reason is the fact that the polyurethane disappears over time; the lack of a surgical plane of dissection, high rate of intraoperative bleeding, and difficult postexplantation reconstruction make this operation very demanding[56]. Where polyurethane goes and what effects it might cause will take many years of study to answer. The fact that we are again noticing an interest in using polyurethane implants should remind us of the real problem of capsule contracture with silicone gel breast implants.

In a consecutive, population-based study consisting of 1529 patients receiving 3495 implants at a multidisciplinary breast center between 1979 and 2004 (25 years), by Handel *et al.*[57], the authors concluded: 1) the longer implants were in place, the greater the cumulative risk of developing contracture, consistent with other studies[9],[20],[58],[59]; 2) hematoma significantly increased the risk of contracture, consistent with other studies[16],[60]; 3) smooth and textured implants had similar contracture rates, controversial in many studies[22],[38],[39],[40],[41],[42],[43],[44],[45]; 4) polyurethane foam-covered implants had a reduced risk of contracture persisting for at least 10 years after implantation, consistent with other studies[31],[32],[33],[34],[35],[36],[52]. On a systemic review of the literature by Shaub, Ahmad and Rohrich[27], the authors were unable to conclude which implants, silicone *versus* saline, have a higher incidence of CC.

1.1 Etiology of capsular contracture

The true etiology and subsequent treatment of capsular contracture remains yet elusive. Two prevailing theories have emerged[21],[39],[61],[62],[63],[64],[65],[66],[67],[68],[69],[70],[71],[72],[73],[74],[75],[76]: the infectious hypothesis and the hypertrophic scar hypothesis. The infectious hypothesis, which has been championed by Burkhardt[61],[63] , supported by others[71],[72],[73],[74],[76],[77],[78],[79] and more recently studied by Rohrich and Adams *et al* [21],[62],[64], implicated subclinical infection in the development of capsular contracture. The hypertrophic scar hypothesis[61],[68],[69],[70],[75],[76],[80],[81],[82],[83], implicated non-infectious stimuli, namely hematoma, granuloma, or hereditary factors, which may confer a foreign body reaction and result in formation of a hypertrophic scar around an implanted device.

In our opinion the cause of capsular contracture is multifactorial. We purpose this point of view in the publication I[84], which was refuted by Burkhardt in the

discussion of this paper. The purpose of this thesis is to clarify its etiology as an extension of this first publication.

1.2 Classification of capsular contracture

The tables I[25] and II[78] describe the two classifications of capsular contracture. Because the Baker classification is widely used, it will be the one reported in this thesis.

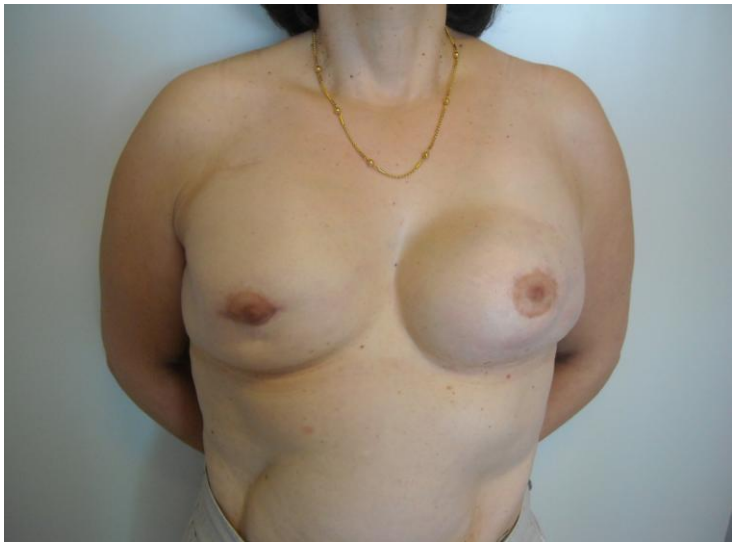
Table I. Baker Classification

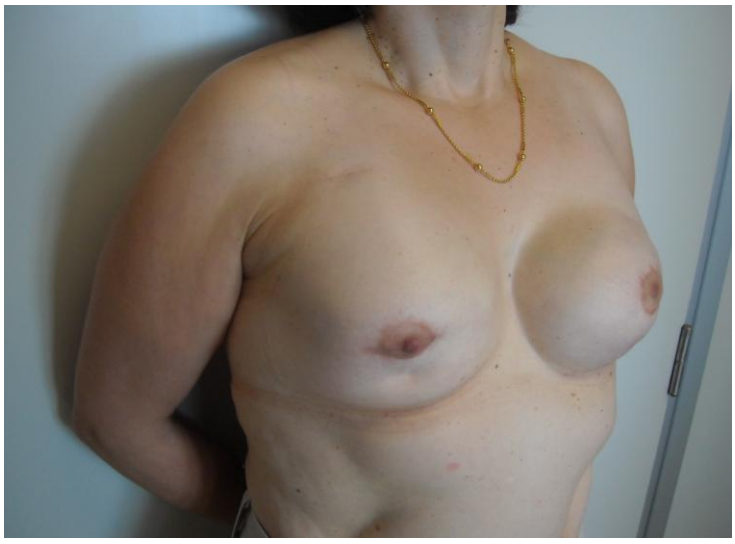
Grade	Description
I	Breast absolutely natural, no one could tell breast was augmented
II	Minimal contracture; surgeon can tell surgery was performed but patient has no complaint
III	Moderate contracture; patient feels some firmness
IV	Severe contracture; obvious just from observation

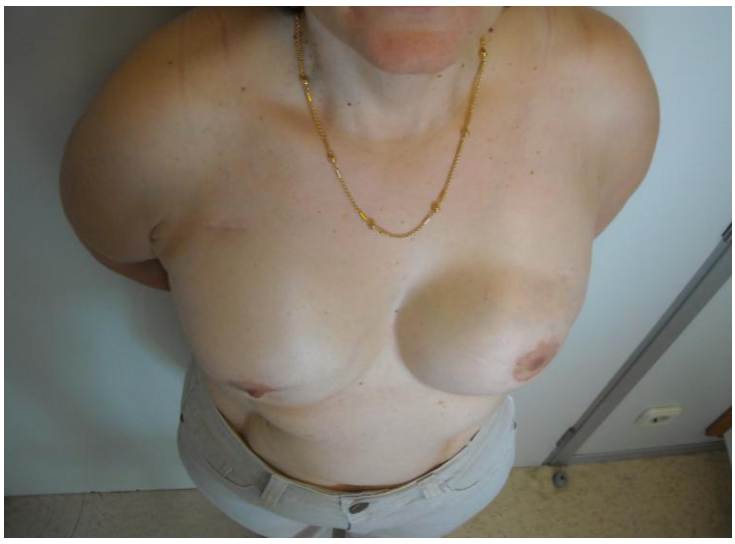
Table II. Breast Augmentation Classification

Grade	Description
I	Soft; no deformation
II	Slightly thickened consistency; none to slight deformation
III	Firm to hard; none to slight deformation
IV	Hard; severe deformation

Figure 1 (a, b, c). Right breast: reconstruction with implant; Baker grade II. Left breast: reconstruction with *latissimus dorsi* muscle flap and implant; Baker grade IV (with patient authorization)







The Baker classification system defines stages of breast capsule clinical presentation into distinct grades[25]. Grade II (Figure 1 a/b/c) is the first stage of capsular contracture and clinical interpretation of grade II may be highly dependent on individual surgeons' opinions. Although the clinical impact of grade II is relevant to the continuum of breast capsule formation, the majority of retrospective and prospective reports do not include grade II subjects as breast capsule cases[85],[86],[87],[88]. The exclusion of grade II subjects in these reports may result in under-reporting of capsular contracture rates. The purpose of this thesis (Study 1) was to report the incidence of complications with breast implants in a Portuguese population, in aesthetic and reconstructive groups and to perform a comprehensive evaluation of the importance of grade II and the follow-up time period. In this study we analyse the possible associations among surgical route, implant placement, body index mass, smoking habits, alcohol consumption, and capsular contracture.

1.2.1 Baker classification rates and follow-up

Table III[12],[13],[14],[18],[19],[57] ,[85],[86],[87],[88],[89],[90],[91],[92],[93],[94] demonstrates that reported capsular contracture rates vary widely due to authors' reporting Baker classification and follow-up time periods. These data showed incidence complications were elevated in reconstruction patients compared to cosmetic augmentation patients[13],[95].

Table III. Studies average follow-up *versus* capsular contracture

Studies	Type of study	Number of patients	Average follow-up	Capsular contracture
Spear *et al*., 2003	Prospective	85 cosmetic revisions	11.5 months	2% Baker II No Baker III-IV
Adams *et al*., 2006	Prospective	235 (172 cosmetic primary augmentation; 63 reconstructive)	14 months	Baker III-IV: 1.8% cosmetic primary augmentation 9.5% reconstructive
Henriksen *et al*., 2005	Retrospective	2277	19.5 months	4.3% (Baker II-IV)
Brown *et al*., 2005	Retrospective	150 (118 cosmetic; 32 reconstructive)	21 months	Cosm: 2 cases Reconst: 3 cases Just Baker II; no cases Baker III-IV
Fruhstorfer *et al*., 2004	Prospective	35	23 months	0%
Henriksen *et al*., 2003	Prospective	1090	2 years	4.1% (Baker II-IV)
Cunningham *et al*., 2007	Prospective	955 (572 primary augmentation ; 123 revisions-augmentation ; 191 reconstruction ; 69 revisions-reconstruction)	2 years	Baker III-IV: 0.8% primary augmentation 5.4 revisions-augmentation 2.2% primary reconstruction 6% revisions-reconstruction
Camirand *et al*., 1999	Prospective	830	2.39 years	0%
Seify *et al*., 2005	Retrospective	44	34 months	20% (Baker II-IV)
Cunningham *et al*., 2007	Prospective	1007 (551 primary augmentation ; 146 revisions-augmentation ; 251 reconstruction ; 59 revisions-reconstruction)	3 years	Baker III-IV: 8.1% primary augmentation 18.9 revisions-augmentation 8.3% primary reconstruction 16.3% revisions-reconstruction
Bengtson *et al*., 2007	Prospective	941 (492 cosmetic primary augmentation; 225 reconstructive; 224 revisions)	3 years	Baker III-IV: 5.9%
Spear *et al*., 2007	Prospective	940 (455 cosmetic primary augmentation; 98 reconstructive; 162 revisions)	6 years	Baker III-IV: 14.8% primary augmentation 20.5% revisions-augmentation 15.9% primary reconstruction
Kjoller *et al*., 2002	Retrospective	754	7 years	11.4% of implantation
Kulmala *et al*., 2004	Retrospective	685	10.9 years	17.7% (15.4% of implantation) Baker II-IV
Holmich *et al*., 2007	Retrospective	190	19 years	62%
Handel *et al*., 2006	Retrospective	1529 (825 cosmetic; 264 reconstructive)	23.3 years	Baker III-IV per 1000 patient-month: 1.99 cosmetic 5.37 reconstructive 4.36 revision

Capsular contracture may be apparent within the first year after implantation[13],[14],[20],[58]. Breiting *et al.*[9] reported 18% of severe breast pain, indicative of severe capsular contracture and in a previous study, involving a subgroup of this population they had diagnosed 45% of capsular contracture (Baker II to IV) of the breast after a 5-year period following breast implantation[96]. Capsular contracture may also be symptomatic several years after surgery[9],[20],[58],[59]. The follow-up time period remains, until now, unclear.

1.3 Estrogens

It is well known the protective role of estrogens in the progression of liver fibrosis[97],[98] and the fact that estrogen deprivation has been associated with declining dermal collagen content and impaired wound healing[99]. Nevertheless there are no studies reporting menopause or estrogens *versus* capsular contracture. In this thesis (Study 1) we purpose to analyse the association between capsular contracture and menopause or estrogen *status*.

1.3.1 Estrogens review of the literature

By 1990s, there were estimates that up to 50% of postmenopausal women in western Europe[100] and about 35% in United States[101] were on hormone replacement therapy because of numerous beneficial effects attributed to such therapy[102],[103].

In 2002 the Women´s Health Initiative (WHI)[104] trial reported that combined use of an estrogen and progestogen regimen increased the risk of breast cancer and cardiovascular events and decreased the risks of fracture and colorectal cancer. Since the publication of results from the WHI[104], many women have either stopped or become reluctant to use hormone replacement therapy[104],[105] and clinicians have had to

revise their treatment algorithms. The relative risk (RR) to benefit ratio of hormone replacement therapy was shifted toward excess risk by this study, and the use of hormone replacement therapy after publication of this trial dramatically declined. A significant minority of postmenopausal women remains on hormone replacement therapy for treatment of menopausal symptoms, osteopenia, or personal preference.

The three most commonly used hormonal replacement therapy regimens are: estrogen-only, continuous combined (estrogen and progesterone) and sequential combined (estrogen followed by progesterone). In postmenopausal breast, the number of estrogen receptors-positive cells within lobules is increased to about 50% in the absence of hormone therapy[106]. In animal studies, long-term estrogen or combination estrogen/progesterone therapy increases cell proliferation and the percentage of glandular tissue in the breast[107],[108],[109] but the pathology of the breast with hormone replacement therapy has not been well established[104],[110],[111],[112],[113],[114].

Staa *et al.*[115] reported that hormone therapy used for 5 years initiated at age 45 increased the absolute risk of myocardial infarction by 0.04% and breast cancer by 0.3% and reduced the risk of hip fracture by 0.03%. In most of the younger hormone therapy users, the frequency of risks exceeds that of the benefits, although the absolute excess risks are small.

Even though estrogen therapy can significantly improve vasomotor symptoms, postmenopausal Portuguese have a low rate of estrogen replacement therapy use, just as surgically menopausal women in Taiwan[116].

1.4 The subclinical infection in the development of capsular contracture

Investigation of capsular contracture associated with breast implants has focused on microorganisms found in the periprosthetic capsule or outer implant surface[61],[62],[74],[76],[87],[117],[118],[119],[120],[121],[122],[123], inflammatory responses[81],[82],[124] and histological characteristics of the capsule[44],[60],[84],[125],[126],[127],[128],[129].

There is evidence that bacterial colonization of mammary implants is partially responsible for capsule contracture, and coagulase-negative *Staphylococci*, particularly *S. epidermidis*, have been largely implicated [71],[72],[130],[131],[132],[133],[134],[135],[136],[137],[138],[139],[140],[141],[142]. Adams *et al.*[62],[64] results explained that *S. epidermidis* colonization of mammary implants is more likely to occur because of bacterial contamination at the time of implantation than because of ongoing contamination from the adjacent ductal system. Because of the low pathogenicity of coagulase-negative *Staphylococci* and the existence of organisms in a dormant phase within the biofilm around the implant, capsular contracture does not usually clinically manifestate until some remote time after placement of mammary implants.

The authors perform microbial analysis of rabbits' skin, operation air, capsules, tissue expander and breast implants, to clarify the contamination surrounding the procedure (Studies 2, 3, 4 and 5). In study 3, infection surrounding breast implants in the presence of coagulase-negative *Staphylococci* was performed.

1.5 Histology and capsular pressure

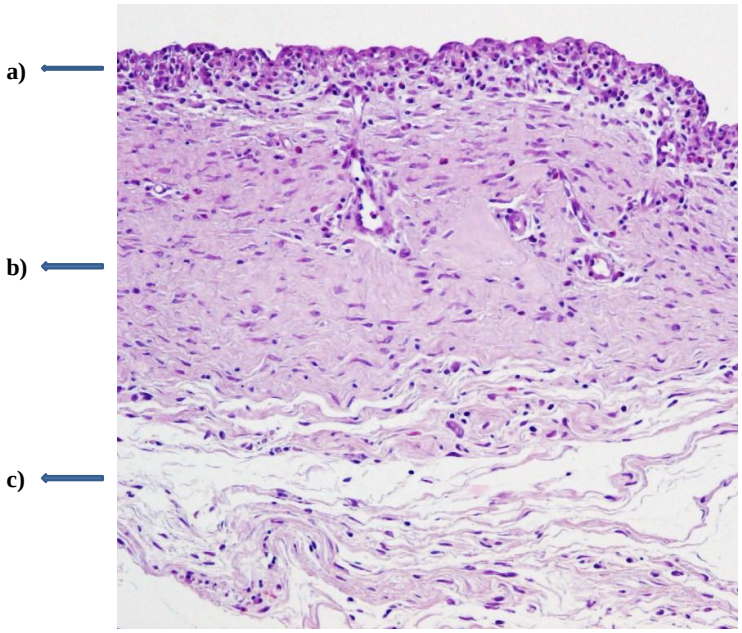
Histologically, fibrous capsules showed three-layered composition (Figure 2):

- A inner layer abutting the silicone surface: single or multilayered formed basically by macrophages (histiocytes) not with abundant fibroblast[124] .

- A thicker layer of collagen bundles arranged in a parallel array[59],[143] or haphazard[144].

- A outer layer comprised loose or dense connective tissue with vascular supply[124] .

Figure 2. a) Inner layer; **b)** Middle layer **c)** Outer layer (hematoxylin-eosin stain magnification 100x; from the Control group in Study 4)



From a clinical perspective, most authors consider the degree of capsule thicknesses to be commensurate with the severity of capsular contracture; this has never been definitively proven as some reports found no correlation between microbiological contamination, thickness and clinical contracture[66].

In publication I[84], on gross examination of the capsules, the Control group capsule appeared more transparent and had less vessel predominance on the capsular surface. The Fibrin group had a more opacified capsule and in many cases appeared thicker. The average capsular thickness (histologically measured) was 0.6 mm in the rabbit Control group, 1.0 mm in the rabbit Fibrin group and in human capsules, and 2.5 mm in human capsule contractures. There was a non–statistically significant increase in capsular thickness in the Fibrin group. Hematoxylin and eosin sections of rabbit Control capsules at 8 weeks, rabbit Fibrin capsules at 8 weeks, human capsules, and human contractures were compared. Synovial-like reaction of fibrohistiocytic cells (synovial metaplasia) was most pronounced in the rabbit Control capsule at 8 weeks, focal in the rabbit Fibrin capsules at 8 weeks, and absent in the human contractures and control capsules. The differences in synovial metaplasia in the specimens constitute a histological detail that carries no clinicopathological significance; however, they were reported for the sake of completeness. Inflammation (consisting of lymphocytes, histiocytes, and eosinophils) was moderate in the 8-week rabbit Control capsule and mild in the 8-week rabbit Fibrin capsule. The human capsule demonstrated minimal inflammation, whereas the human contracture showed mild inflammation. The degree of fibrosis was greater in the 8-week rabbit Fibrin capsules and human contracture than in their counterparts (the 8-week rabbit Control and human capsules, respectively).

In the revision article from Broughton *et al.*[145] about wound healing, it is known that early in the wound healing process the matrix is thin and compliant and allows fibroblasts, neutrophils, lymphocytes and macrophages to easily maneuver through it; as the matrix becomes denser with thicker, stronger collagen fibrils, it becomes stiff and less compliant. Isometric tension is defined as a situation in which internal and external

mechanical forces are balanced such that cell contraction occurs without cell shortening or lengthening which explains the higher pressure in capsule with contracture.

In Adams and Marisa *et al.*[84] (Publication I) the pressure-volume curve was generated at 2 and 8 weeks. There was no significant difference between the Fibrin and the Control groups at 2 weeks; however, at 8 weeks there was a significant increase in intracapsular pressure in the Fibrin group. The limitation of this study was the measurement of intracapsular pressure, given that it was not recorded directly but through a small capsular window. The purpose of this study was to record directly the pressure and to realize how fibrin modulates the capsule formation.

The underlying mechanism behind this process involves the activation of the myofibroblast cells within the capsule, which supposes that contractile elements exert the force necessary to produce capsular contracture. Myofibroblasts contain the contractile elements actin and myosin and have been identified inconsistently within the capsules of implanted devices; however, they have proven difficult to culture and study in detail and, when found in the capsule, are found in exceedingly small quantities, are located sporadically throughout the capsule, and are not found to attach to each other. This scenario poses an inconsistent model for the development of contractile forces necessary to produce contracture.

To study capsule firmness and the contracture development, we measured the capsule pressure directly[146], reason why studies 2, 3 and 5 were performed with tissue expanders.

Histological analysis of the capsule was performed in all studies.

1.6 The immunology of fibrosis

Fibrosis is an excessive extracellular matrix (ECM) due to the formation and production cells and the occurrence of mononuclear inflammatory infiltrates, with proliferation and activation of myofibroblasts. In this context, macrophages and mast cells have been implicated as important participants in the inflammatory process involving fibrosis.

Fibrosis is a major global health problem, but its etiology, pathogenesis, diagnosis and therapy have yet not been addressed. Fibrosis can occur as a consequence of many pathological conditions: 1) spontaneous (keloids, Dupuytren´s contracture); 2) from tissue damage (post-operative adhesions, burns, alcoholic and post-infection liver fibrosis, silica dust, asbestos, antibiotic bleomycin); 3) inflammatory disease (infections, scleroderma); 4) in response to foreign implants (breast implants, cardiac pacemakers, heart valves, artificial joints, central venous catheter ports); and 5) from tumors (fibromas, neurofibromatosis).

Several mutually non-exclusive hypotheses have been proposed: 1) infection; 2) reaction to altered self; 3) overproduction of reactive oxygen species (ROS) and nitric oxide (NO); and 4) mechanical stress.

In all cases studied, the early stages of fibrotic conditions are characterized by a perivascular infiltration of mononuclear cells and the subsequent imbalance of anti and profibrotic cytokine profiles. One of the most prominent activators of mononuclear cells and fibroblasts are hyaluron fragments that not only induce the expression of various cytokines (IL-1, IL-12 and TNF-α), chemokines (MPI-1A, MCP-1, IL-8) and inducible nitric oxide synthase (iNOS), but also trigger the expression and secretion of macrophage-derivated matrix metalloproteinases (MMP), enzymes essential for ECM cleavage[147].

Mast cells can play a role in fibrosis by their secretion of tryptases, contributing to connective tissue breakdown. As a consequence of activation of procollagenase and induction of a cascade of MMPs, the connective tissue becomes more penetrable for infiltrating leucocytes during inflammation. Mast cell-derived tryptase indirectly induces fibroblasts proliferation by stimulating the synthesis of cyclooxygenase and prostaglandins[148],[149]. Natural killer (NK) cells display predominantly anti-fibrotic properties in several fibrosis model systems[150]. Furthermore, NKT-derived interferon (IFN)-y inhibits the production of the profibrotic cytokine transforming growth factor beta (TGF-β1)[151].

Cells and cytokines play a prominent role in the initiation and progression to fibrosis and Th1 and Th2 cytokines play opposing roles in fibrosis[152]:

- Th1 cytokines (IFN-y and IL-12) suppress the development of tissue fibrosis.
- Th2 cytokines (IL-4 and IL-13) are strongly pro-fibrotic.

Fibroblasts can be derived from local quiescent connective tissue fibroblasts by proliferation, but there is also ample evidence that at least some of them originate from myeloid precursors in the blood or bone marrow that then migrate to sites of injury[153]. Once in an active state, fibroblasts are designated as myofibroblasts which express α-smooth muscle cell actin (α-SMA), produce increased amounts of ECM proteins, such as collagen type I and fibronectin, proliferate and show contractile properties. Their usual activators are IL-6 and TGF-β1, although they can also be activated by a variety of other cytokines, chemokines, growth factors, components of microbial cells walls and members of oxidative burns cascade[152]. Fibroblasts also receive stimuli from lymphocytes via the CD40-CD40 ligand (CD40L or CD154); CD40 ligation results in the synthesis of IL-6, IL-8, hyaluronan and the adhesion molecules ICAM-1 and VCAM-1[154]. Among the various pro-and anti-fibrotic cytokines, TGF-β isoforms seem

to play a key role in the development of fibrosis[155],[156]. TGF-β1 has a fibrogenic role while TGF-β3 has anti-fibrotic properties. Studies on the role of TGF-β2 are rare and the results contradictory. TGF-β1 is a central mediator of fibrosis, but alone it is insufficient to cause a persistent fibrotic response; only in synergy with other pro-fibrotic cytokines, such as connective tissue growth factor (CTGF), results in chronic fibrosis[157].

In summary TGF-β1, CTGF, osteopontin (OPN), IL-4, IL-6, IL-10, IL-13, IL-21, basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), insulin like growth factor-1 (IGF-1), platelet-derived growth factor (PDGF), oncostatin M and endothelin 1 (ET-1)[158] all promote fibrosis , whereas IFN-y, TGF-β3, IL-10 and IL-12 are anti-fibrotic. IL-5[152], TGF-β2 and TNF-α[159], exerting either pro-or anti-fibrotic activities depending on the disease, animal model and experimental settings.

1.6.1 Pathophysiological hallmarks of breast implant capsule formation

- Fibroblasts and macrophages (by its location in connective tissue namely histiocytes), formed a palisade-like multilayered cell wall toward the silicone implant, and represents the major cell population[124] .

- Ample presence of T cells (CD4+/CD8+), macrophages, dendritic cells (DCs), CD25 and CD45RO expressing cells; Langerhans-cell like denditric cells are found at the frontier layer zone abutting the silicone implant[59],[124],[125] .

- No accumulation of B-cells[59],[124],[125].

- Cells at the frontier layer, endothelial cells and smooth muscle cells showed massive HSP60 expression (reflecting the mechanical effect or other forms of stress exerted on implant and capsule); HSP60 positively predominantly in fibroblast, followed by macrophages and T-cells[124].

- The layers in closest proximity to the silicone showed massive expression of adhesion molecules namely intercellular adhesion molecule (ICAM-1) but not to E-selectine or VCAM-1; the endothelial cells of the neovasculative vessels in the fibrous capsules were P-selectine positive[124].

- Actin+ smooth muscle cells found in vascular walls but also in interstitium, occasionally formed dense bands[124].

- Collagenous extracellular matrix (ECM) proteins: high procollagen (type I and III) expression correlated with high fibrotic activity; the proportion of procollagen to collagen, showed a decreased in procollagen expression and an increase of mature collagen deposition with longer implant duration[124].

- Non-collagenous extracellular matrix (ECM) proteins: fibronectin shows a high affinity for silicone and for cellular components such as macrophages, fibroblasts and T-cells; tenascin, mainly synthesized for fibroblasts, mediate adhesion of mononuclear cells in on the frontier zone[124].

- Serum proteins from many protein families adhere to silicone surface and mediate adhesion of fibroblasts, macrophages and ECM proteins[160].

- Macrophages are activated by cryptic or altered protein domains exposed on silicone surfaces or by silicone degradation products[161].

- Activated intracapsular lymphoid cells stimulate transdifferentiation of fibroblasts to myofibroblasts by CTGF, IL-1 and TNF-α. Macrophages contribute to this process by the production of TGF-β1 and IL-6[162].

- Soluble ICAM-1, procollagen III, circulating immune complexes and anti-polymer antibodies are elevated in sera of women with strong fibrotic reactions to silicone[163] .

- A special ELISA-based system (SILISA®) demonstrating the "signature" of serum protein adhesion to different silicone types can be used to determine the potential risk of fibrosis development around silicone breast implants[164].

Summary:

- The immune response comprises primarily T-cells.
- The preferential distribution of dendritic cells in the frontier layer zone underlines that this immunological process is not identical or comparable with an unspecific local immune reaction or so called foreign body granuloma formation.
- The constant presence of CD1a+ cells in the frontier zone adjacent to the silicone implant as well as next accumulation of CD4+ cells support the hypothesis that silicone is not inert, as postulated by the manufacturers, but induces directly or indirectly a T-cell immune response. The peri-implant connective tissue capsule may represent a possible site of antigen processing and presentation[163].
- The massive deposition of tenascin in the frontier layer zone supports the theory of mechanical stress depending of tenascin expression[163]. T-lymphocytes significantly increase the synthesis rate of tenascin via certain cytokines such as IL-4 and TNF-α[165].
- The mechanical stress to which breast implant is exposed is associated with HSP60 expression, a family of highly conserved proteins produced by all cells in response to various physiological and non-physiological stress-situations to protect the cells from potential lethal assaults[163]; HSP70 was associated with structural changes of the implant capsule, in terms of capsular thickness and the Baker score[166].

1.6.2 Microdialysis, IL-8 and TNF-α

Microdialysis enables measurement of the chemistry of the capsule extracellular fluid. Although initially developed over 30 years ago[167], microdialysis studies in humans have been mainly limited to head injury[168],[169],[170],[171], subarachnoid haemorrahage[172], epilepsy[173] and cerebral tumors[174],[175].

The chemotatic cytokine (chemokin) IL-8 (CXCL8) is an important mediator in pathogenesis of many acute and chronic inflammatory disorders[176]. IL-8 mainly targets polymorphonuclear cells (PMN), the major phagocyte cell, but also mediates attraction of basophils, eosinophils and T-cells to the inflammatory site[177].

Interleukin-8 (IL-8) is induced by a wide range of stimuli, including: TNF-α, IL-1[178], bacterial agents[179], formyl-methionyl-leucyl-phenylalanine (f-MLP)[180], zymosan[181], plated factor 4 (PF-4)[182], and P-selectin together with RANTES (regulated upon activation normal T-cell expressed presumed secreted)[183]. The many cell types thus responding are: monocytes[184], PMN[185], endothelial cells[186], fibroblasts[187], T-lymphocytes[188], natural killer cells (NK)[182] and human mast cell line [189]. In the study by Lund *et al.*[190], lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria, potentially induced IL-8 release in monocytes, while TNF-α was a good inducter of IL-8 in PMN. Furthermore, a relatively high level of IL-8 was associated with PMN cells. Lund *et al.*[190] concluded that under pathophysiological condition-associated exposure of blood to LPS, one may anticipate that IL-8 is generated as a direct effect of LPS acting on monocytes and that it is further amplified due to TNF-α endogenously produced by monocytes.

IL-8 is an important chemotactic regulator of neutrophil in vivo[177], and its concentration increases during different infections, such as bacteraemia[191] and meningococcal infection[192]. IL-8 concentrations have also been demonstrated to play

an important role in the immunological response to inflammatory disorders characterized by neuthrophilic infiltration including psoriasis[193], rheumatoid arthritis and asthma.

To monitor levels of interleukin-8 (IL-8) and tumor necrosis factor-α (TNF-α), the authors utilized microdialysis, and to our knowledge, this had never been previously studied in capsule extracellular fluid by this technically demanding method.

1.7 Prevention and treatment of capsular contracture

Despite innovations in shell surface textures, implant shapes, inner gel composition, surgical implantation techniques and pocket irrigation[20],[38],[39],[55],[62],[63],[64],[194],[195],[196],[197],[198],[199],[200],[201],[202],[203],[204],[205] to prevent capsular contracture, this major complication remains a serious problem.

In a pre-clinical study by Tamboto *et al.*[206], the authors concluded that *Staphylococcus epidermidis* biofilm formation was associated with a fourfold increased risk of developing CC. To prevent CC, many plastic surgeons follow the general principles of the "Betadine Era"[62] and the "Post-Betadine Era"[64],[87]. However, it also known that other factors related to wound healing influence the development of this clinical condition[124]. In preclinical studies, the treatment with mesna[126], mitomicina C[207], zafirlukast[208],[209], pirfenidone[210] or halofuginone[211] reduced capsule thickness, fibroblast cell proliferation and collagen deposition. Nevertheless, these drugs are not commonly used in clinical practice, with the exception of the the antileukotriene drugs (zafirlukast, montelukast and pranlukast). Scuderi *et al.*[212],[213],[214] reported clinical experience with zafirlukast and suggests that this drug may be effective in reducing pain and breast capsule distortion in patients with longstanding contracture who are either not surgical candidates or who do not wish to

undergo surgery. The antileukotriene drugs are currently used in asthma and lung diseases, however, the experience is limited to severe CC because of the severity of possible side effects such as liver failure[215] or Churg-Strauss syndrome[216]. Research concerning cause and prevention has moved forward; however, in clinical practice is still a difficult issue, especially when comparing decreased CC rates achieved with polyurethane implants.

Some reports correlate clinical contracture and hematoma[16],[60]; to clarify this implication, the authors perform study 2 with tissue expanders surrounded by rabbit´s blood to simulate a hematoma, and tissue expanders in the presence of thrombin (FloSeal®), an absorbable hemostatic agent and in the presence of a fibrin wound healing agent (Tisseel/Tissucol®).

Recent evidence investigating the chitosan and the chitooligosaccharide, have revealed that they have intrinsic antibacterial and antifungal activity[217],[218],[219],[220] and ability to bind growth factors[221]. In study 4, breast implants impregnated with chitooligosaccharide mixture (COS) and low molecular weight chitosan (LMWC) were introduced in the rabbit model.

Steroids have shown to be effective in treatment of others pathologic disorders characterized for an unorganized scar tissue in dermal structures[222],[223],[224],[225], as keloids , hypertrophic scars and burn scar contractures[226]. Corticosteroids administered during wound healing showed to stop the growth of granulation completely, the proliferation of fibroblasts, diminish the new outgrowths of endothelial buds from blood vessels and stop the maturation of the fibroblasts already present in connective tissue[227]. Also when administered early after injury, corticosteroid delay the appearance of inflammatory cells, fibroblasts, the deposition of ground substance, collagen, regenerating capillaries, contraction, and epithelial migration[228]. These data

raised the interest on the use of steroids in the treatment and prevention of CC. The data available in the literature regarding the effect of steroids in the prevention and treatment of CC is spare and contradictory. The steroids have an important role in the earlier phases of wound healing[228], and the role of those effects on the early phase of breast capsule formation are also not understood nor explored. In study 5, breast implants with triamcinolone were introduced in the rabbit model.

1.7.1 Tissucol/Tisseel®

Fibrin glue consists of two components, a fibrinogen solution and a thrombin solution rich in calcium. Fibrin serves as a binding reservoir for several growth factors such as vascular endothelial growth factor (VEGF) [229], transforming growth factor-β1 [230] and basic fibroblastic growth factor (bFGF)[231]. Fibrin glue has been studied for decades for its use surgically as a hemostatic and sealant agent. It is routinely used in: gastrointestinal anastomosis, breast surgery, face-lifts, abdominoplasty, nerve repairs, graft securing, neurosurgery and ophthalmology [232],[233],[234],[235],[236],[237],[238],[239],[240],[241],[242]. More recently it has also gained attention as a possible means to deliver drug therapies[243]. For example, in a study by Zhibo and Miabo[244], release of lidocaine from fibrin glue for pain reduction was tested in humans after breast augmentation. Patients who received fibrin glue with lidocaine in the subpectoral pocket experienced less pain than those who received the same amount of lidocaine or fibrin glue alone.

To study the implications of wound healing in development of capsular contracture, the instillation of fibrin (Tissucol/Tisseel®) in the implant pocket, to induce hemostasis and as a tissue glue to bind the tissues together (adhesive properties), was performed (Studies 2 and 3); numerous reports have demonstrated that fibrin glue

application is an effective adhesive that is associated with improved parameters of wound healing[245],[246],[247],[248]. In Adams and Marisa *et al.*[84] (Publication I), we have demonstrated exactly the opposite; in this study, one experimental group has been instilled with 5 cc of fibrin glue [fibrin glue is prepared with 4 ml of rabbit cryo (Pel-Freez; Pel-Freez Biologicals, Rogers, Ark.), 500_l of 10% CaCl (Sigma- Tau Pharmaceuticals, Gaithersburg, Md.), 1000 units of thrombin (Monarch Pharmaceuticals, Bristol, Tenn.) in 1 ml of 50 mM TrisCl (Sigma), pH 7.4] into the implant pocket as a contracture inducing agent. Even if there was a non–statistically significant increase in capsular thickness in the Fibrin group, the degree of fibrosis was greater in the 8-week rabbit fibrin capsules and human contracture than in their counterparts (the 8-week rabbit control and human capsules, respectively). The purpose of this study is to clarify the impact of fibrin in contracture development. Incidentally, studies 1 and 2 were performed with fibrin (Tissucol/Tisseel®), to induce hemostasis and as a tissue glue to bind the tissues together (adhesive properties), which is different from the one used in publication I. As indicated by Sead *et al.*[249], fibrin sealant prepared from Tisseel kit without aprotinin has the ability to reduce extracellular matrix and TGF-β1 mRNA levels, especially from adhesion fibroblasts, which may indicate a role in reduction of postoperative adhesion development. As it has been demonstrated, TGF-β is a mediator in scar formation and in multiple fibrotic disorders. It has also been demonstrated that connective tissue growth factor (CTGF) is a downstream mediator of TGF-β and acts to stimulate wound contraction and fibrosis. It has been observed that local treatment with antagonists/anti-sense-oligonuceotides of TGF-β and CTGF at the time of surgery reduced CTGF levels in tissue and correlated with reduced capsular formation in a rat model. The study by Cole *et al.*[250] supports the use of fibrin to deliver MALP-2 and possibly other peptides, in an active form that might enhance

wound healing. In the increase understanding of the wound healing process, it becomes clear to Brissett *et al.*[251], that cellular recruitment and release of growth factors are paramount for normal healing to occur; a delay in this process can result in a chronic wound or excessive scar. Although the use of these preparatins (Tisseel and Vi-Guard) allows the closure of dead-space and approximation of the skin flaps, it is argued that these tissue adhesives produce such a dense architecture that angiogenesis and vascular ingrowth are inhibited; in addition, because these tissue adhesive do not possess growth factors or cytokines to actively recruit cells that are essential for wound healing, they are considered bioactively inert. The study by Petter-Puchner *et al.*[252] was designed to assess the impact of fibrin sealing with Tissucol/Tisseel® on adhesion formation to condensed polytetrafluoroethylene meshes as well as on tissue integration of these implants in experimental intra-abdominal peritoneal on lay mesh repair in rats. The authors concluded that Tissucol/Tisseel® improves the tissue integration and reduces early adhesion.

1.7.2 FloSeal®

FloSeal® does not contain any fibrinogen (different from the above fibrin sealant); it requires blood as a source for fibrinogen, for clot activation and is ineffective in the absence of any bleeding. FloSeal® is a combination of a gelatin-based matrix from bovine collagen containing microgranules, cross-linked with glutaraldehyde and human thrombin solution[253]. Upon contact with blood the gelatin particles swell and induce a tamponade-like effect. This characteristic allows it to be effective in controlling moderate arterial bleeding. Numerous reports have demonstrated that FloSeal® successfully reduces bleeding in cardiac surgery[254], urologic procedures[255],[256],[257], gynecology[258],[259] and neurosurgery[260]. Dogulu *et al.* [261], in a

pre-clinical model, concluded that the application of FloSeal® at a laminectomy site may be useful to decrease adhesion at the interface between the dura mater and epidural fibrosis.

1.7.3 Triamcinolone acetonide

The data available in the literature regarding the effect of steroids in the prevention and treatment of CC is spare and contradictory. Perrin[262] reported less than 5 percent of significant capsule formation on patients submitted to augmentation mammaplasty with inflatable breast prostheses filled with saline and a cortisone derivative, with no evidence of wound complications attributable to the steroid. This results were reinforced by Ksander[263] in a pre-clinical model with rats, where it was reported that saline implants filed with saline solution were harder and surrounded by a thicker capsular membrane than those filed with metilprednisolone sodium succinate, at 60 and 120 days. Caffee *et al.*[264] reported in a preclinical study, that triamcinolone in the pocket during surgery was ineffective for prevention of capsular contracture, but if injected 4 and 8 weeks postoperatively, the drug was able to completely eliminate contracture. Caffee *et al.*[264] assume that triamcinolone in the pocket was not effective because its effect does not last long enough, and the objection to this method has been the fact that the drug was given at the time of operation and was therefore most effective in the early phases of wound healing and less active in the latter stages when contracture is more likely to begin. However, betadine[62] and antibiotic breast irrigation[64],[87] were clinically associated with a low incidence of CC and more effective in the early phases of wound healing and less active in the latter stages. The majority of patients undergoing breast augmentation will never experience contracture, and therefore, it did not seem reasonable to apply an experimental invasive method to such a group only a

minority of patients who would potentially benefit. Caffee *et al.*[264] conclusions were based on indantation and applanation tanometry. There have been no further reports confirming that triamcinolone in the pocket during surgery was ineffective. Morover, Caffee *et al.* in 2002[265], and Sconfienza *et al.* in 2011[266], reported clinical success treating patients with CC with the injection of triamcinolone-acetonide between the capsule and the implant. Derendorf *et al.*[267] reported a plasma half-life after venous injection of 2h. Recently, Yilmaz *et al.*[268] performed an extensive review of human and experimental studies published on the pharmacokinetics of TA for the treatment of macular edema. The authors concluded that the pharmacokinetic profile of TA is unpredictable and the agent has a time-limited therapeutic action due to its relatively short half-life. This has led to the need for repeated injections to treat contracture or macular edema. The answer to the clinical efficiency of triamcinolone-acetonide with various doses is not known.

1.8. Chitosan and Chitooligosaccharides

Chitin, the polymer D-glucosamine in β (1,4) linkage, is the major component of exoskeleton of crustaceous and cell wall fungi[269]. Chitosan (CS) is the deacetylated product of chitin. Chitooligosaccharides (COS) are degraded products of chitosan, or the deacetylated and degraded products of chitin, by chemical and enzymatic hydrolysis. In the literature, the term chitosan is used to describe chitosan polymers with different molecular weight (50-2000 kDa), viscosity and degree of deacetilation (40-98%)[270]. Material with lower levels of deacetylation degrades more rapidly[271],[272],[273]. Chitosan has been the better researched version of the biopolymer because of its ready solubility in dilute acids rendering it more accessible for utilization and chemical reactions[274].

Chitosan and related chitooligosaccharides have intrinsic antibacterial and antifungal activities[217],[218],[219],[220], which permit the study of the infectious hypothesis. On other hand, its ability to bind to growth factors[221],[275], the hemostatic action[276], the ability to activate macrophages and cause cytokine stimulation[276] and to increase the production of TGF-β[277] allows the study of the hypertrophic scar hypothesis.

Chitosan can be processed in a variety of different shapes. These attributes make chitosan a promising biopolymer for tissue engineering due to its excellent biocompatibility. Chitosan applications include use in wound healing (full thickness skin defect, dermal burns)[218],[221],[278],[279], in target delivery of low molecular drugs[280], in orthopaedics (cartilage, anterior cruciate ligament, intervertebral disc, bone, osteomyelitis)[220],[281], in otologic diseases (tympanoplasty)[279] and in breast capsular contracture[282]. The combination of chitosan with materials is common in various reports[274]. The results published by Khor *et al.*[274], from cell line culture and animal model studies, indicated that chitin and chitosan materials were non-cytotoxic and suggest that these materials would provide tissue engineered implants that are biocompatible and viable. Baldrick *et al.*[276] observed that chitosan has local biological activity in the form of hemostatic action and, together with its ability to activate macrophages and cause cytokine stimulation (which has resulted in interest in medical device and wound healing applications), may result in a more careful assessment of its safety as a parenteral excipient.

Literature data reporting general toxicity testing for chitosan is limited[276]. An investigation of intestinal absorption of chitosan in rats showed that the material underwent digestion into low molecular weight substances within the gastrointestinal tract, and that they are distributed extensively in tissues[283]. Apparent toxicity was seen with 653-720 mg/Kg/day of COS in rats with side effects in skin and fur and decrease

bodyweight[284]; it is further suggested that increased platelet count, lymphocyte count and differential neutrophils count may be related to dermal inflammation. High dose effects were also seen in rabbits following intravenous dosing of chitosan, with deaths at 50 mg/Kg/day (but no effect at 4.5 mg/Kg/day)[285]; it was suggested that the finding was probably due to cell aggregation. Studies in dogs[286] showed evidence of toxicity following subcutaneous dosing with clinical signs (anorexia) from 30 mg/Kg/day, chemistry changes (especially neutrophilia) from 50 mg/Kg/day, and severe dyspneia and deaths from 150 mg/Kg/day; pathological examination showed severe pneumonia in the latter animal and it was suggested that this finding was possibly induced by immunological reaction and cytokine activation. Cytotoxicity was demonstrated with an inhibitory concentration (IC50) of 0.2 mg/ml for chitosan hydrochlorid with release of haemoglobin, damage of the erythrocyte membrane, cell aggregation and complete lysis[285]. Intratumoral injection of chitosan on tumor bearing mice, increases the rate of tumor growth, metastasis and the number of capillaries formed[287]. There were no reports in rabbits related with impregnated chitosan breast implants or with toxicity after chitosan implantation.

1.9 The New Zealand white rabbit

Adams and Marques *et al.*[84] (Publication I) reported a model to study capsule: the New Zealand white rabbit. The New Zealand white rabbit has the capability to support tissue expanders and breast implants, which is impossible in mice; porcine had limited reports.

This thesis is an extension of Adams and Marques *et al.*[84] study (Publication I). In this study New Zealand white rabbits (n = 32) were subdivided into experimental (n = 16) and control groups (n = 16). Each subgroup underwent placement of smooth

saline mini implants (30 cc). The experimental group underwent instillation of fibrin glue into the implant pocket as a capsular contracture-inducing agent. Rabbits were euthanized from 2 to 8 weeks after the procedure. Before the animals were euthanized, each implant was serially inflated with saline and a pressure-volume curve was developed using a Stryker® device to assess the degree of contracture. Representative capsule samples were collected and histologically examined. Normal and contracted human capsular tissue samples were also collected from patients undergoing breast implant revision and replacement procedures. Tissue samples were assessed histologically. Pressure-volume curves demonstrated a statistically significant increase in intracapsular pressure in the Fibrin group compared with the Control group. The Fibrin group had thicker, less transparent capsules than the Control group. Histological evaluation of the rabbit capsule was similar to that of the human capsule/contracture for the Control and the Fibrin groups. The authors concluded that pathological capsular contracture can be reliably induced in the rabbit. This animal model provides the framework for future investigations testing the effects of various systemic or local agents on reduction of capsular contracture.

In the discussion of this paper (Publication I) performed by Burkhardt[84], the author believe that if a rabbit model must be used for research, a more appropriate model is that reported by Shah *et al.*[288],[77], who used bacterial contamination to produce contracture. In opposite to our belief that the cause of contracture is multifactorial, to include hematoma, granuloma, foreign body reaction, hereditary factors and subclinical infection as any one of these factors may theoretically stimulate an internal hypertrophic scar response that then becomes a contracted capsule, Burkhardt believes that presumed cause is limited to infection or bacterial contamination.

The end result is that the histological analysis of the rabbit fibrin model was similar to human contracture but the limitation of this study was the inability to provide a clinical translation of this contracture model, as no rabbit developed a Baker II, III or IV. Moreover, this was a pilot study, and the fibrin modeling response in capsule formation deserves further studies.

This thesis is an extension of the Adams and Marques *et al.*[84] study (Publication I) to clarify the etiology of capsular contracture, based on this animal model, and with the hope of developing a clinical capsular contracture model.

1.10 The pig and the mice models

Two recent studies introduced a pre-clinical CC model; 1) Tamboto *et al.*[206] developed a pig model of CC with submammary pockets inoculated with *S. epidermidis* before miniature gel-filled implants introduction; 2) Katzel *et al.*[289] developed a mice model implanted with silicone gel implants then received a 10-Gy directed radiation dose from a slit-beam cesium source. These models brought to the science the possibility of further promissory studies.

2. Aims of the thesis

- Retrospective study in aesthetic and reconstructive groups of Portuguese women who received silicone textured breast implants within 1998 to 2004. Report the occurrence and severity of postoperative complications focused on capsular contracture. Analyse the impact of the follow up period, the Baker grade II subjects and factors that might contribute to the development of capsular contracture rates, namely estrogens and menopausal *status* (STUDY 1)
- Identify bacteria and fungi from operation air, rabbit's skin, tissue expanders, breast implants and removed capsules (STUDIES 2, 3, 4 and 5)
- Histological analysis of the capsule (STUDIES 2, 3, 4 and 5)
- Monitor the levels of interleukin-8 (IL-8) and tumor necrosis factor-α (TNF-α) in capsule extracellular fluid by microdialysis (STUDY 4 and 5)
- Identify the impact of hematoma in capsular contracture (STUDY 2)
- Identify the impact of coagulase-negative *Staphylococci* in capsular contracture (STUDY 3)
- Identify the impact of thrombin (FloSeal®) in capsular contracture (STUDY 2)
- Identify the impact of fibrin (Tissucol/Tisseel®) in capsular contracture (STUDIES 2 and 3)
- Identify the impact of chitosan in capsular contracture (STUDY 4)
- Identify the impact of triamcinolone acetonide in capsular contracture (STUDY 5)
- Clarify the etiology of capsular contracture (STUDIES 2, 3 and 4)

3. Material and methods

STUDY 1

Subjects and data collection

Existing medical records of women who had undergone breast implantation with customized textured silicone breast implants (Allergan, Santa Barbara, California, USA) between 1998 and 2004 in the Hospital of S. João (Oporto, Portugal) were examined. A total of 224 women were identified with 104 women who underwent cosmetic breast augmentation (Cosmetic group) and 120 women who underwent postmastectomy reconstruction of the breast (Reconstructive group).

The following data were collected from medical records: patient demographics, alcohol and medication habits, medical history, surgical procedures, incision location, implant device placement[290] and postoperative acute complications (hematoma, infection, and seroma). Postoperative chronic complication data (capsular contracture, folds, wrinkles, breast pain, and change of tactile sense) were not gathered from medical records. Self-reported complications related to satisfaction with implantation surgery were collected using a self-administered questionnaire. Women who answered the questionnaire were asked to attend a consultation to be further evaluated by two trained plastic surgeons in order to decrease subjectivity of this evaluation. The degree of late capsular contracture was assigned by the plastic surgeons according to Baker's classification[25].

Women from the initial group (157 of 224) completed the self-questionnaire and attended the consultation. The remaining 67 were then excluded (n = 35 women, Cosmetic group; n = 32, Reconstructive group) to remove any potential bias that might result from patients with incomplete data. Women were excluded due to loss of contact

as they moved out of Oporto or if no current mailing address or phone numbers were available at the time of the study. The Reconstructive group was comprised of 88 patients with 115 breast implants with 27 patients having received bilateral breast implants. The Cosmetic group had 69 patients with 136 breast implants from which 2 had a tuberous breast deformity, 1 had a unilateral aplasia and 1 had a Poland's syndrome. All cosmetic patients younger than 18 years old (n = 4) received implants following medical indication, namely severe asymmetry, aplasia of breast tissue or congenital malformation.

Statistical analysis

Postoperative local complications were analyzed independently for the entire study group and individual clinical treatment groups and reported *per* woman and *per* implantation operation (SPSS, Statistical Package for Social Sciences). Possible associations among recorded data sets of patients characteristics, surgical procedures and complications were evaluated using Pearson χ^2 testing and logistic regression modeling[291]. Trend analysis was performed using Chi−squared Automatic Interaction Detection (CHAID) method (SPSS, Statistical Package for Social Sciences, Chicago, IL)[292], using the likelihood ratio chi-square statistic as growing criteria, along with the Bonferroni 0.05 adjustment of probabilities, and setting the minimum size for parent and child nodes at 10 and 5, respectively. Relative risks (RR) and 95 percent confidence intervals (CI) were calculated for identified characteristics of interest to examine strength and precision of statistical associations.

CHAID has not been widely applied to trend analyses in plastic surgery investigations, but CHAID is one of the oldest tree-classification methods originally proposed by Kass[292]. In brief, CHAID is an exploratory method to examine

relationships between a dependent variable (*e.g.* capsular contracture) and a series of predictor variables (*e.g.*: type of cohort, age at surgery, follow up period, *etc.*) and their interactions. The CHAID algorithm creates adjustment cells by splitting a data set progressively via a classification tree structure where the most important predictor variables are chosen that to maximize a chi-square criterion. The most significant predictors defined the first split or the first branching of the tree. Progressive splits from the initial variables resulted in smaller and smaller branches. The result at the end of the tree building process is a series of groups that are different from one another on the dependent variable. Classification trees lend themselves to be displayed graphically and are far easier to interpret than numerical interpretation from tables.

STUDY 2

Eighteen (n = 18) New Zealand white female rabbits (3-4 kg) were implanted in an approved institutional animal care protocol, with textured saline tissue expanders (20 ml, Allergan, Santa Barbara, California, USA) with intact connecting tube and port. Prior to surgery the rabbit´s skin was washed with Betadine® Surgical Scrub containing 7.5% povidone-iodine, followed by Betadine® Solution containing 10% povidone-iodine (Purdue Products, Stamford, USA). The surgical procedure was performed in an animal operating theatre following aseptic rules. Penicillin G 40.000 u/Kg IM was administered just intraoperatively. Talc-free gloves were used at all times during the procedure. Pockets were developed in the *sub-panniculus carnosis* along the back region, with atraumatic dissection. Particular attention was given to hemostasis, under direct vision avoiding blunt instrumentation and there was no obvious bleeding. A new pair of talc-free gloves was used before tissue expanders insertion with minimal skin

contact. Each tissue expander was placed and filled up to 20 mls volume. Four expanders were placed per rabbit.

In each rabbit, 1 control and 3 experimental tissue expanders were placed. The experimental groups were: 1) sprayed with 1 ml of fibrin glue (Tisseel/Tissucol®; Baxter Healthcare Corporation, Vienna, Austria, Europe); 2) instillation of 2 ml of rabbit´s blood into the expander pocket to simulate a hematoma; 3) instillation of 5 ml of thrombin sealant (FloSeal®; Baxter Healthcare Corporation, Vienna, Austria, Europe) into the expander pocket.

A pressure measure device (Stryker® instruments, Michigan, USA) was connected to the tissue expander port and intra-expander pre-surgical pressure was recorded directly. Pressures were recorded after each 5 ml increments until tissue expanders were overfilled.

Rabbits were sacrificed at 2 or 4 weeks. Prior to sacrifice, each animal was anesthetized and the dorsal back area shaved. The pressure measure device was connected to the tissue expander port and intracapsular pressures were recorded 5 ml increments previously to any incision in the capsule. Capsule samples were submitted for histological and microbiological evaluation.

Microbiological Assessments

- Air

Operating room air samples (n = 36) were collected during all surgical procedures using the MAS 100-Eco® air sampler 00109227.0001 / 26299 at a flow rate of 100 L/min. Identification of bacterial and fungal isolates followed standard microbiological procedures. Gram positive cocci were characterized by biochemical methods. Catalase-positive and coagulase-positive isolates were reported as *Staphylococcus aureus*;

catalase-positive and coagulase-negative isolates were reported as coagulase-negative *Staphylococci*. Gram negative bacilli were characterized using the Vitek Two® with version VT2-R04.02 software. Fungi were characterized according to the macroscopic appearance and microscopic morphology.

- Rabbit skin

A total of 54 contact plates (18 brain-heart agar, 18 mannitol salt agar and 18 Sabouraud agar contact plates) were pressed to the shaved dorsal skin surfaces. Brain-heart and mannitol salt agar plates were incubated for 3 days at 28ºC; Sabouraud contact plates were incubated for 7 days at 28ºC. The identification of the bacteria and fungi followed the procedures reported above.

- Capsules and tissue expanders

Excised implants and representative capsule samples were incubated at 37ºC for 3 days in brain-heart broth and examined daily; changes in turbidity of the broth media were considered positive and were subcultured in solid agar media. Characterization of microbial isolates followed the above described procedures.

Histological Assessment

Capsule specimens were fixed with 10% buffered formalin and embedded in paraffin. Sections were stained with hematoxylin and eosin and evaluated histologically for tissue inflammation and capsular thickness. The type of inflammatory cells was grouped into 3 categories: 1) mononuclear (lymphocytes, plasmocytes and histiocytes); 2) mixed (mononuclear cells and eosinophils); and 3) polymorph (eosinophils and heterophils/neutrophils). Inflammatory infiltrate intensity was categorized according to the following criteria: absent (-); mild (+); moderate (++); and severe [(+++)[125]].

Samples were stained with Masson`s trichrome[293],[294] to characterize the connective tissue (loose or dense), organization of the collagen fibers (arranged in a parallel array or haphazard), angiogenesis (absent, mild, moderate or high) and fusiform cells density (mild, moderate or high) were observed. The dense connective tissue was semiquantitative analysed: a) dense ≤ 25%, thick collagen bundles less than 25%; b) dense 25-50%; c) dense 50-75%; e) dense >75%.

Statistical analysis

Data was grouped according to the type of product applied to the tissue expander, as none (Control), blood (Blood), Tissucol/Tisseel® (Fibrin) and FloSeal® (Thrombin), and analyzed separately for rabbits sacrificed at 2 or 4 weeks after surgery as well as for all 18 sacrificed rabbits. One-way analysis of variance was used to compare the intra-expander pressure prior to insertion. A two-tailed paired *t*-test and the nonparametric alternative Wilcoxon signed rank test were used to determine if continuous variables (intracapsular pressure and histological measured thickness) were significantly different between Control and experimental groups. Categorical variables were evaluated by Chi−square statistics and by Phi, Cramer's V and Contingency coefficients. Statistical significance was presumed at $p \leq 0.05$. Major trends within each group were further examined by the Chi−squared Automatic Interaction Detection (CHAID) method[292], using the likelihood ratio Chi-square statistic as growing criteria along with a Bonferroni 0.05 adjustment of probabilities. All analyses were carried out with the SPSS, Statistical Package for Social Sciences (SPSS, Version 16, Chicago, IL).

STUDY 3

Thirty-one (n = 31) New Zealand white female rabbits (3-4 kg) were implanted in an approved institutional animal care protocol with 1 textured tissue expander (non-filled with 20 ml, Allergan, Santa Barbara, CA) and 2 textured breast implants (90 ml, Allergan, Santa Barbara, CA). Prior to surgery the rabbit skin was washed with Betadine® Surgical Scrub contains 7.5% povidone-iodine, followed by Betadine® Solution containing 10% povidone-iodine (Purdue Products, Stamford, USA). The surgical procedure was performed in an animal operating theatre following aseptic rules. Penicillin G 40.000 u/Kg IM was administered just intraoperatively. Talc-free gloves were used at all times during the procedure. Pockets were developed in the *sub-panniculus carnosis* along the back region, with atraumatic dissection. Particular attention was given to hemostasis, under direct vision avoiding blunt instrumentation and there was no obvious bleeding. A sterile Op-site dressing was placed over the skin around the incision before the tissue expander and the implants insertion to eliminate contact with the skin[295]. A new pair of talc-free gloves was used to perform the tissue expander and the implants insertion.

The rabbits groups were: 1) untreated implants and expander (Control; n = 10); 2) implants sprayed each one with 2 ml of fibrin (Tisseel/Tissucol®; Baxter Healthcare Corporation, Vienna, Austria, Europe) and expander sprayed with 0.5 ml of fibrin and (Fibrin; n = 11); 3) implants each one inoculated with 100 microlitres of a suspension of coagulase-negative *Staphylococci* (10^8 CFU/ml - 0.5 density in McFarland scale) and expander with 2.5 x 10^7 CFU/ml (CoNS; n = 10).

Rabbits were sacrificed at 4 weeks. Prior to sacrifice, each animal was anesthetized and the dorsal back area shaved. A pressure measure device (Stryker® instruments, Michigan, USA) was connected to the tissue expander port and

intracapsular pressures were recorded at each 5 ml increments previously to any incision in the capsule. All capsule samples were submitted for histological and microbiological evaluation. All implants and expander devices were also submitted for microbiological evaluation.

Microbiological Assessments

As performed in STUDY 2.

- Air : air samples (n = 36)
- Rabbit skin: 93 contact plates (31 brain-heart agar, 31 mannitol salt agar and 31 Sabouraud agar contact plates)

Histological Assessment

As performed in STUDY 2.

Statistical analysis

Data were grouped according to the type of product applied to the breast implants, namely none (Control; n = 20), Tisseel/Tissucol® (Fibrin; n = 22) and coagulase-negative *Staphylococci* (CoNS; n = 20). One-way analyze of variance either parametric or nonparametric (Kruskal-Wallis H test) were performed to determine if continuous variables (intracapsular pressure and histological measured thickness) were equal, followed by post-hoc range tests to identify homogeneous subsets across groups. Two-tailed independent pair *t*-tests and the nonparametric alternative Mann-Whitney U tests were used. Categorical variables were evaluated by Chi−square statistics and by Phi, Cramer's V and Contingency coefficients. Statistical significance was presumed at $p \leq 0.05$. Major trends within each group were further examined by the Chi−squared

Automatic Interaction Detection (CHAID) method [292], using the likelihood ratio Chi-square statistic as growing criteria along with a Bonferroni 0.05 adjustment of probabilities. All analyses were carried out with the Statistical Package for Social Sciences (SPSS, Version 16, Chicago, IL).

STUDY 4

Eleven (n = 11) New Zealand white female rabbits (3-4 kg) were implanted according an approved institutional animal care protocol; each rabbit received 3 different textured breast implants (90 ml, Allergan, Santa Barbara, CA). Prior to surgery the rabbit skin was washed with Betadine® Surgical Scrub containing 7.5% povidone-iodine, followed by Betadine® Solution containing 10% povidone-iodine (Purdue Products, Stamford, USA). The surgical procedure was performed in an animal operating theatre following aseptic rules. Penicillin G 40.000 u/Kg IM was administered just intraoperatively. Talc-free gloves were used at all times during the procedure. Pockets were developed in the *sub-panniculus carnosis* along the back region, with atraumatic dissection. Particular attention was given to hemostasis, under direct vision avoiding blunt instrumentation and there was no obvious bleeding. A sterile Op-site dressing was placed over the skin around the incision before the implants insertion to eliminate contact with the skin[295]. A new pair of talc-free gloves was used to perform the implants insertion.

Each implant was placed beneath *panniculus carnosis* along the back (Figure 1A). The implant groups were: an untreated implant (Control); an implant impregnated with COS (MW 1.4 kDa, Nicechem, Shanghai, China); and an implant impregnated with LMWC (MW 107 kDa, Sigma-Aldrich, Sintra, Portugal). Both chitosan mixtures possessed deacetylation degree in the range of 80−85%. Implants were prepared by

immersion in either COS (20.0 mg/mL) or LMWC (10.0 mg/mL) solutions with pH adjusted to 5.8-5.9 for 2 hours. Implants were incubated at 37ºC in a flow chamber for 2 days, packed and sterilized by ethylene oxide.

Rabbits were sacrificed at 4 weeks. Prior to sacrifice, each animal was anesthetized and a 5 mm incision was made directly over the implant through skin, *panniculus carnosis* and capsule. A 100,000 molecular weight cutoff microdialysis probe (CMA Microdialysis, Stockholm, Sweden) was placed by the capsule implant interface and microdialysates were collected using sterile, normal saline solution (6 µl/min) for 1 hour. Whole blood was obtained by venipuncture and serum was collected after centrifugation (2000 $gmin^{-1}$, 4^0C). Capsule samples were submitted to histological and microbiological evaluations.

Microbiological Assessments

As performed in STUDIES 2 and 3.

- Air : air samples (n = 20)
- Rabbit skin: 33 contact plates (11 brain-heart agar, 11 mannitol salt agar and 11 Sabouraud agar contact plates)

Histological Assessment

As performed in STUDIES 2 and 3.

Microdialysis Assessment

The Invitrogen® Hu TNF-α US kit (Invitrogen®, Hu TNF-α Cat# KHC3014:1) is a solid phase sandwich Enzyme-Linked-Immuno-Sorbent-Assay (ELISA). An antibody specific for Hu TNF-α has been coated into wells of the microtiter strips

provided. The microdialysis fluid was pipetted into wells. During the first incubation, the Hu TNF-α antigen binds to the immobilized (capture) antibody on one site, and to the solution phase biotinylated antibody on a second site. After removal of excess second antibody, Strepavidin-Peroxidase (enzyme) was added which binds to the biotinylated antibody to complete the four-member sandwich. After a second incubation and washing to remove the unbound enzyme, a substrate solution was added, which was acted upon by the bound enzyme to produce color. The intensity of this colored product was directly proportional to the concentration of Hu TNF-α presented in the original specimen.

The protocol was repeated with the BioSource® Hu IL-8 US kit (BioSource®, Hu IL-8 Cat# KHC0083/KHC0084).

Statistical analysis

Data were grouped according to the type of product applied to the implant, namely none (Control), chitooligosaccharide mixture (COS) and low-molecular-weight-chitosan (Chitosan) and analyzed separately for the 11 sacrificed rabbits at 4 weeks after surgery. Two-tailed paired *t*-test and the nonparametric alternative Wilcoxon signed rank test were used to determine whether continuous variables (histologic measured thickness and dialysate levels of IL-8 and TNF-α) were likely to show differences between Control and experimental groups. Categorical variables were evaluated by Chi−square statistics and by Phi, Cramer's V and Contingency coefficients. Statistical significance was presumed at $p \leq 0.05$. Major trends within each group were further examined by the Chi−squared Automatic Interaction Detection (CHAID) method [292], using the likelihood ratio Chi-square statistic as growing criteria along with a

Bonferroni 0.05 adjustment of probabilities. All analyses were carried out with the SPSS, Statistical Package for Social Sciences (SPSS, Version 17, Chicago, IL).

STUDY 5

Nineteen (n = 19) New Zealand white female rabbits were implanted in an approved institutional animal care protocol with 1 textured tissue expander (non-filled with 20 ml, Allergan, Inc., Santa Barbara, CA) and 2 textured breast implants (90 ml, Allergan, Inc., Santa Barbara, CA). Prior to surgery, rabbit skin was washed with Betadine® Surgical Scrub contains 7.5% povidone-iodine, followed by Betadine® Solution containing 10% povidone-iodine (Purdue Pharma LP, Stamford, Connecticut). The surgical procedure was performed in an animal operating theatre following aseptic rules. Penicillin G 40.000 U/Kg was administered intramuscularly was administered just intraoperatively. Talk-free gloves were used at all times during the procedure. Two 5 cm incisions and one 2,5 cm incision were made directly over the skin and *sub-panniculus carnosus* to introduce the implants and the expander, respectively. Pockets were developed in the sub-panniculus carnosis along the back region, with atraumatic dissection. Particular attention was paid to hemostasis, under direct vision avoiding blunt instrumentation and there was no obvious bleeding. A sterile Op-site dressing was placed over the skin around the incision before the tissue expander and the implants insertion to eliminate contact with the skin. A new pair of talc-free gloves was used to perform the tissue expander and the implants insertion. Then, the implants and the tissue expander with intact connecting tube and port were introduced. In the experimental group, the introduction of triamcinolone-acetonide (Trigon® depot; Bristol-Myers Squibb) into the implant and expander pocket was performed. All wounds were closed with two planes of interrupted suture.

The rabbits groups were: 1) untreated implants and expander (Control; n = 10); 2) introduction of 1 ml (40 mg) of triamcinolone-acetonide into each implant pocket and 0.25 ml (10 mg) of triamcinolone-acetonide into each expander pocket (Triamcinolone; n = 9). No fluid suction was performed in order to retain the prevent dilution of the triamcinolone-acetonide (Trigon® depot; Bristol-Myers Squibb) in the surgical pocket.

Rabbits were sacrificed at 4 weeks. Prior to sacrifice, each animal was anesthetized and the dorsal back area shaved. A pressure measure device (Stryker Instruments, Kalamazoo, Michigan) was connected to the tissue expander port and intracapsular pressures were recorded at each 5 ml increments previously to any incision in the capsule. Then, a 5 mm incision was made directly over the implant through skin, panniculus carnosus and capsule. A 100,000 molecular weight cutoff microdialysis probe (CMA Microdialysis, Stockholm, Sweden) was placed by the capsule implant interface and microdialysates were collected using sterile, normal saline solution (6 µl/min) for 1 hour. Whole blood was obtained by venipuncture and serum was collected after centrifugation (2000 gmin-1, 40C). All capsule samples were submitted for histological and microbiological evaluation. All implants and expander devices were also submitted for microbiological evaluation.

Microbiological Assessments

As performed in STUDIES 2, 3 and 4.

- Air : air samples (n = 24)
- Rabbit skin: 57 contact plates (19 brain-heart agar, 19 mannitol salt agar and 19 Sabouraud agar contact plates)

Histological Assessment

As performed in STUDIES 2, 3 and 4.

Microdialysis Assessment

As performed in STUDY 4.

Statistical analysis

Data were analyzed by groups: Control (n = 20) and Triamcinolone (n = 18). One-way analysis of variance (parametric or nonparametric) was performed to check if the several means of continuous variables (histologic measured thickness and dialysate levels of IL-8 and TNF-α) were equal, followed by post-hoc range tests to identify homogeneous subsets across groups. A two-tailed independent pair *t*-test and the nonparametric alternative Mann-Whitney *U* test were used to determine whether such continuous variables were likely to show differences between control and experimental group. Categorical variables were evaluated by Chi–square statistics and by Phi, Cramer's V and Contingency coefficients. Statistical significance was presumed at $p \leq 0.05$ and all analyses were carried out with the SPSS program.

4. Results

STUDY 1

Baseline descriptive information for the Cosmetic and Reconstructive patient groups were presented in Tables IV and V, respectively.

Table IV. Baseline characteristics for the Cosmetic group.

Variable	No	%
Women with implants (breast implants)	69 (136)	
Age at surgery in years, mean (range)	31.0 (15–51)	
Follow-up period in months, mean (range)	35.4 (12–80)	
Implant placement		
• Subpectoral	9	13.0
• Subglandular	58	84.1
• Dual plane Tebbets	2	2.9
Incision placement		
• Inferior periareolar	7	10.1
• Axillary	21	30.4
• Inframammary	41	59.5
Contraceptive drugs		
• No	30	43.5
• Yes	39	56.5

Table V. Baseline characteristics for the Reconstructive group.

Variable	No	%
Women with implants (breast implants)	88 (115)	
Age at surgery in years, mean (range)	48.6 (25–73)	
Follow-up period in months, mean (range)	48.5 (12–96)	
Symmetrizing breast		
• No	28	31.8
• Breast implant (with or not mastopexy)	22	25
• Breast reduction	33	37.5
• Bilateral breast reconstruction	5	5.7
Hormone therapy[a]		
• No	85	96.6
• Yes	3	3.4

[a] Including contraceptive drugs or hormone replacement therapy

Cosmetic patients were younger at the time of surgery when compared with reconstructive patients (31.0 *vs*. 48.6 years). The average follow-up period was 35.6 months in the Cosmetic group when compared with 48.5 months in the Reconstructive group. Cosmetic patients reported contraceptive use (56.5%) while only 3.4% of reconstructive patients reported contraceptive use or hormone replacement therapy. Cosmetic patients also reported decrease use of psychotropic drugs (antidepressants, antianxiety and hypnotics drugs) compared with reconstructive patients (23.2% .*vs*. 52.3%, respectively). One woman from each group (n = 2) had a connective tissue disease (rheumatoid arthritis).

Among women in the Cosmetic group, the majority of silicone gel implants were placed subglandularly (84.1%) and the surgical approach was through the inframammary fold (58.0%). The majority of reconstructive patients had not received radiotherapy (85.2%) or tamoxifen (67.1%); chemotherapy was administered in 51.1%;

the reconstructed breast was on the left side in 52.3% of the patients and 68.2% were submitted to breast size symmetrization.

Clinical adverse events: acute

Acute complications were recorded in 20 reconstructive patients (8%) during the follow-up period, with complications recorded as seroma (8.0%), hematoma (4.5%) and perforation of the skin (3.2%).

Clinical adverse events: chronic

Chronic complication events were recorded and tabulated in Table VI.

Table VI. Chronic complications for both groups.

Chronic complications	Cosmetic group (N = 69)		Reconstructive group (N = 88)	
	No	**%**	**No**	**%**
Capsular contracture				
• No	57	82.6	46	52.3
• Unilateral	9	13.0	41	46.5
• Bilateral	3	4.4	1	1.2
Palpable implant folds				
• No	40	58.0	27	30.7
• Unilateral	12	17.4	48	54.5
• Bilateral	17	24.6	13	14.8
Visible skin wrinkles				
• No	59	85.5	72	81.8
• Unilateral	7	10.1	14	15.9
• Bilateral	3	4.4	2	2.3
Prolonged pain in the breast				
• No	59	85.5	78	88.7
• Unilateral	4	5.8	9	10.2
• Bilateral	6	8.7	1	1.1
Change of tactile sense				
• No	61	88.4	9	10.2
• Unilateral	4	5.8	67	76.1
• Bilateral	4	5.8	12	13.7

Overall, 81% (n = 127) of all women had 1 or more postoperative chronic events, ranging from less severe effects (*e.g.*: change in tactile sense) to complications requiring additional surgical interventions, such as severe capsular contracture. The

distribution of chronic complication frequency among women was: a) 23% of the patients had one complication; b) 31% of the patients had two complications; c) 27% of the patients had three 3 or more complications. From a temporal view of the clinical onset of chronic complications, 3% of the patients were diagnosed from 0-12 months postoperatively; 31% of the patients were diagnosed from 13-24 months; and 72% of the patients were diagnosed from 0-60 months.

The most frequent chronic adverse effect was palpable implant folds (47.8% of all cases), occurring in 42.0% of women from the Cosmetic group and in 69.3% from the Reconstructive group. Change of tactile sense also had a high incidence (41.0% of all cases) with 89.8% reporting changes in the Reconstructive group, not due to reconstruction but mastectomy. For this reason, capsular contracture was the second most common chronic complication, occurring in 34.4% of all women and in 23.1% of all implantations. Capsular contracture incidence rates were significantly different between the Cosmetic group (17.4% of women or 11.0% of implantations) and the Reconstructive group (47.7% of women or 37.4% of implantations; $p < 0.05$). Other chronic complications occurred less frequently (< 10% of all patients).

Furthermore, the occurrence of postoperative complications had a marked influence upon satisfaction index, *e.g.*, women without contracture were 1.6 times more likely to consider the outcome either good or very good compared to women with capsular contracture (RR = 1.6; 95% CI, 1.2, 2.2).

Capsular contracture characteristics

Baker capsular contracture grades for the cosmetic and reconstructive groups were presented in Table VII.

Table VII. Capsular Contracture *per* implant for both groups.

Grade	Cosmetic group	Reconstructive group
I	121 (89.0%)	72 (62.6%)
II	5 (3.7%)	9 (7.8%)
III	2 (1.4%)	12 (10.4%)
IV	8 (5.9%)	22 (19.1%)
Total	136 (100%)	115 (100%)

As a percent of patients, Reconstructive group had 7.4 and 3.2 fold great incidences of Baker III and IV grade capsular contractures compared to the Cosmetic group. When examined as a function of clinical time when Baker grades were assigned, 44 women (76%) of the 58 total patients were diagnosed 2 years after surgery. In detail, 5 (7%) women from the Cosmetic group and 28 (32%) from the Reconstructive group developed capsular contracture grade III/IV after the initial 2 years subsequent to implantation. Overall, the rate of grade III/IV capsular contracture *per* woman during the 8-year period of follow-up was 10.1% for patients undergoing cosmetic surgery, and 37.5% for breast reconstruction patients.

The occurrence of capsular contracture was associated with the duration of follow-up period and age at time of surgery (Table VIII).

Table VIII. Identified variables related to capsular contracture for the entire group (n = 157).

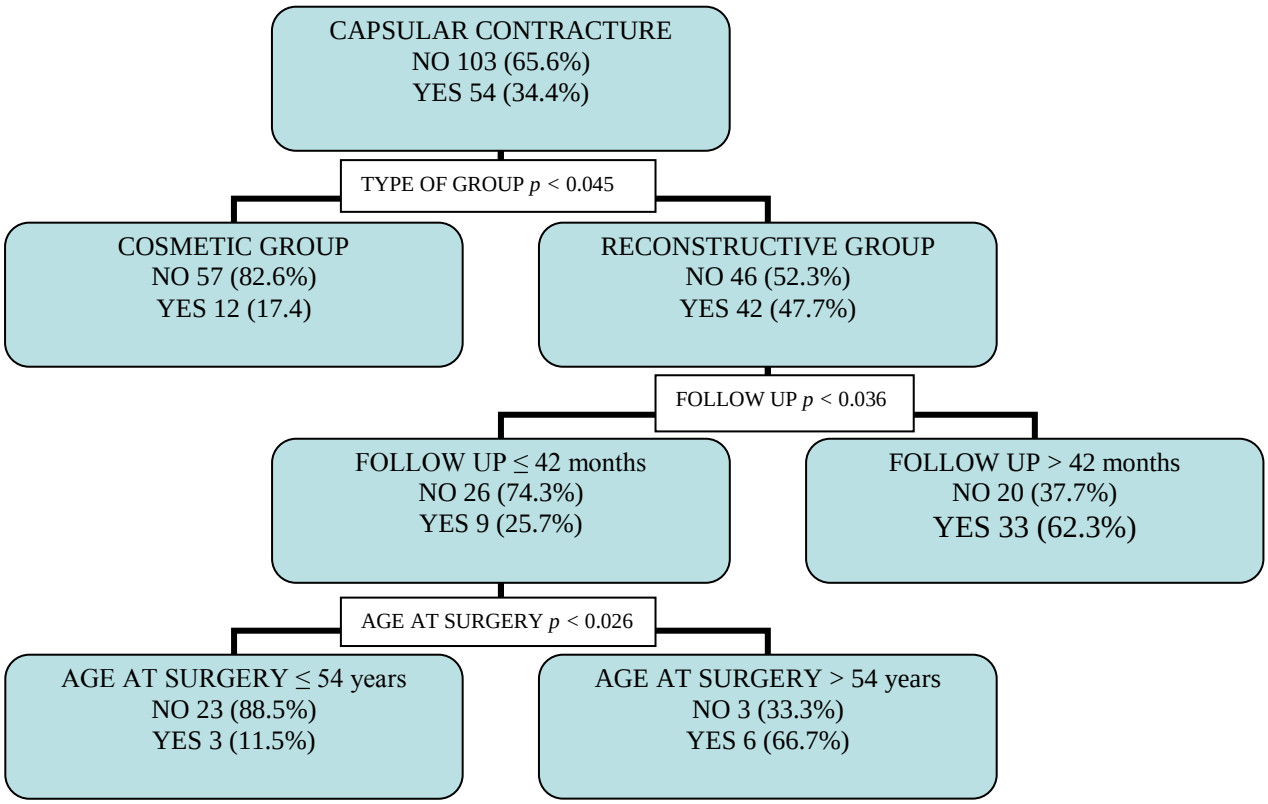
Variable	Capsular contracture (% of women)		*p* value
	No	Yes	
Follow-up period			< 0.001
• ≤ 42 months	40.1	10.8	
• > 42 months	25.5	23.6	
Age at Surgery			< 0.001
• ≤ 54 years	60.5	24.8	
• > 54 years	5.1	9.6	
Hormone therapy[a]			0.014
• No	21.7	29.3	
• Yes	43.9	5.1	
Type of group			< 0.001
• Reconstructive	29.3	26.8	
• Cosmetic	36.3	7.6	

[a] Including contraceptive drugs or hormone replacement therapy

Women with a follow-up period longer than 42-months (RR = 1.8; 95% CI, 1.3 to 2.4) or older women (RR = 3.6; 95% CI, 1.6 to 7.9 for an age of 54+ *versus* < 54 years) had increased incidences of capsular contracture ($p < 0.001$ for both comparisons). Moreover, an increased capsular contracture was detected in the Reconstruction group when compared to the Cosmetic group (RR = 1.7; 95% CI, 1.4 to 2.3; $p < 0.001$). No associations between capsular contracture cases and surgical procedures or other personal characteristics were observed.

Using the CHAID decision tree (Figure 3), the type of group was identified as the determining factor to develop capsular contracture. The first-level split produced two initial branches: Cosmetic (no capsular contracture; percentage = 82.6%) and Reconstructive (positive capsular contracture; percentage = 47.7%). The next splits indicated the best predictor variables for the Reconstructive group, as the follow-up period followed up by the age at surgery. Within that group, a follow-up period of 42 months or less was the best predictor for no capsular contracture (unadjusted percentage = 74.3%) while a follow-up of more than 42 months was predictive for positive capsular contracture (unadjusted percentage = 62.3%). For women with a follow-up of 42 months or less, capsular contracture was reported among 67.7% of women older than 54 years old compared to younger women (11.5%). The overall risk estimate according to the classification tree was 0.240 (standard error of risk estimate 0.034), indicating that 75.8% of the cases will be classified correctly using the decision algorithm based upon the current tree. The CHAID algorithm resulted in larger predictive values for occurrence of capsular contracture (72.2%) than LR (57.4%).

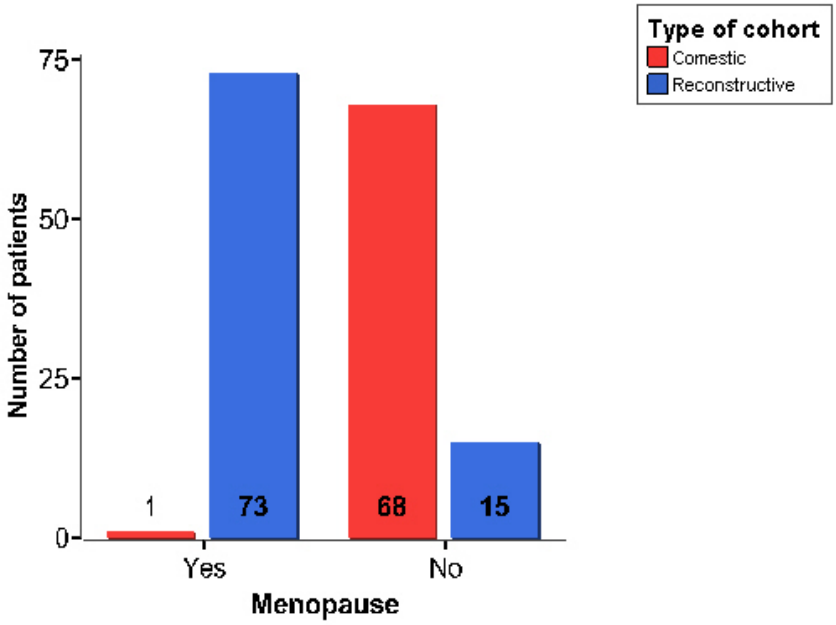
Figure 3. Prediction tree of capsular contracture by Chi-squared Automatic Interaction Detection algorithm.



A second CHAID decision tree analysis was performed with grade II subjects placed in the no capsular contracture group – similar to other reports – versus grade III and IV subjects. The first-level split produced two initial branches: Cosmetic (no capsular contracture or grade II; percentage = 89.9%) and Reconstructive (capsular contracture grade III or IV; percentage = 37.5%). The next split indicated the best predictor variable for the Reconstructive group, as the follow-up period. Within that group, a follow-up period of 64 months or less was the best predictor for no capsular contracture or grade II (unadjusted percentage = 73.4%) while a follow-up of more than 64 months was predictive for capsular contracture grade III or IV (unadjusted percentage = 66.7%). The overall risk estimate according to the classification tree was 0.255 (standard error of risk estimate 0.035), indicating that 79.6% of the cases will be classified correctly using the decision algorithm based upon the current tree.

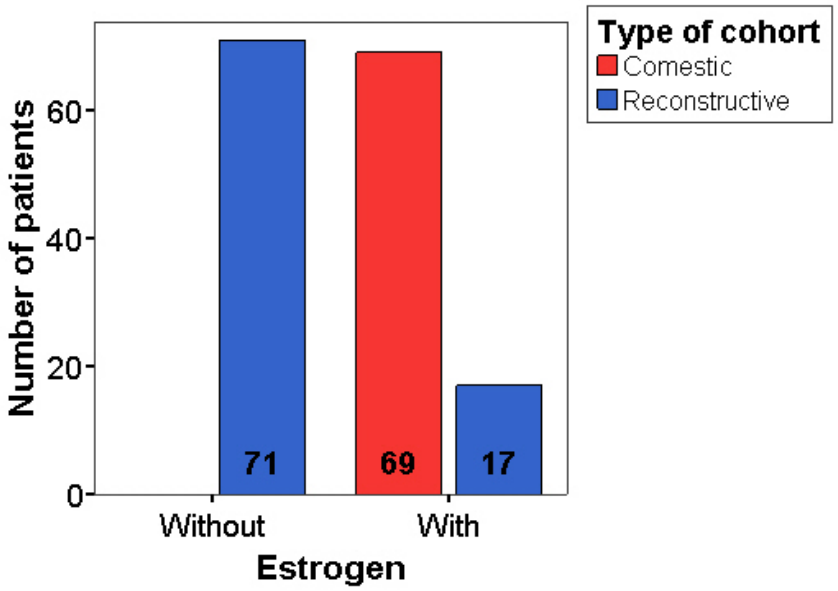
Exogenous hormone use was reported in 56.5% of cosmetic patients (n = 39) with one subject in menopause that used hormone replacement therapy; of the remaining 68 women, 38 used contraceptives (Figure 4). Only 3.4% of reconstructive patients (n = 3) used hormone therapy. Seventy-three patients were in menopause with 2 subjects using hormone replacement therapy. Fifteen women were premenopausal with one using contraceptives.

Figure 4. Graphic of patients with or without menopause *per* type of group.



Subjects who were premenopausal or postmenopausal using hormone therapy replacement, were grouped and analyzed as “estrogen protected” (Figure 5).

Figure 5. Graphic of patients protected or not by estrogen *per* type of group.



To clarify the relationships between menopause or women protected by estrogen with capsular contracture rates *per* type of group, 2 cross-tabulations were performed (Tables IX and X). No associations between capsular contracture and menopause or estrogen *status* were observed.

Table IX. Cross-tabulation between capsular contracture and menopause *per* type of group.

Type of group	Menopause		Capsular contracture		Total
			Yes	No	
Cosmetic	Menopause	Yes	0	1	1
		No	12	56	68
		Total	12	57	69
Reconstructive	Menopause	Yes	36	37	73
		No	6	9	15
		Total	42	46	88

Table X. Cross-tabulation between capsular contracture and being protected or not by estrogen *per* type of group.

Type of group	Protected by estrogen[a]		Capsular contracture		Total
			Yes	No	
Cosmetic	Protected by estrogen	Yes	12	57	69
		Total	12	57	69
Reconstructive	Protected by estrogen	Yes	35	36	71
		No	7	10	17
		Total	42	46	88

[a] Including all women before menopause or in menopause with hormone replacement therapy.

STUDY 2

Intracapsular pressure

No significant differences were observed regarding the pressure-volume curves between the Control and the experimental groups at baseline (tissue expander introduction) or at 2 weeks. At 4 weeks, rupture of 6 capsules in the Control group, 5 capsules in the Blood group and 1 capsule in Thrombin group, during pressure measurement were observed; no capsule ruptures in the Fibrin group were noted. To avoid too less sampling, the ruptured capsules were not excluded from statistical analyses but was stated that the pressure levels measured before capsule rupture were maintained after further additional saline was added. At 4 weeks, significant decreased intracapsular pressures were registered in the Fibrin ($p \leq 0.0006$) and Thrombin ($p \leq 0.003$) groups (Figure 6).

Figure 6. The pressure-volume curves at 4 weeks; there was a significant difference in intracapsular pressure in the Thrombin (FloSeal®) and Fibrin (Tissucol/Tisseel®) experimental groups.

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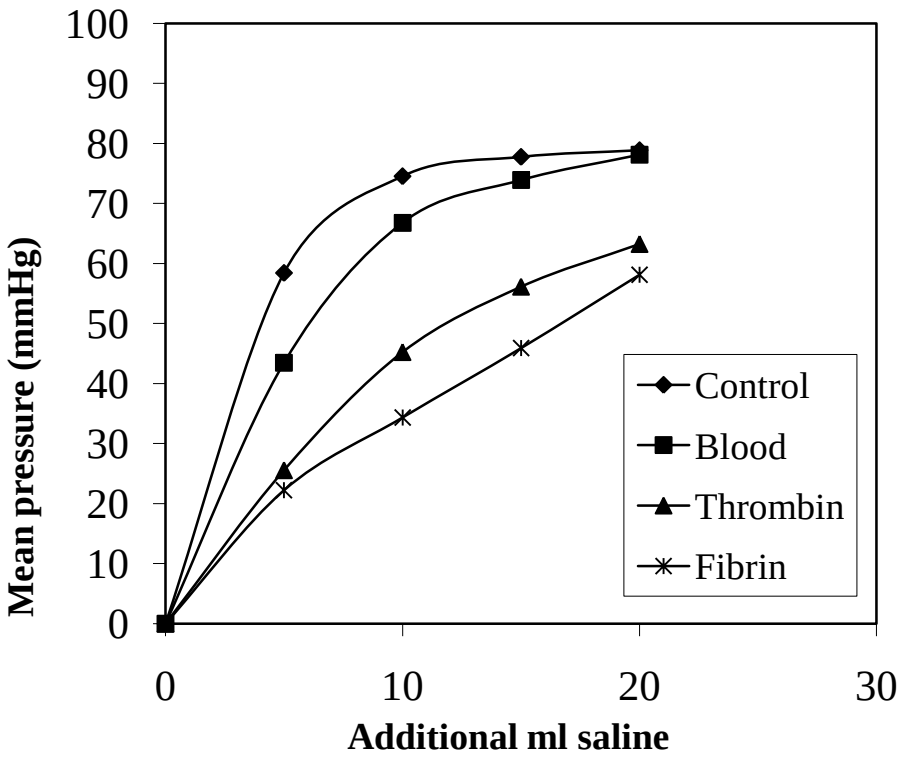


Histology

The average capsular thicknesses were similar among all groups at 2 and 4 weeks (Table XI). At 2 weeks, mixed type of inflammatory cells was predominantly observed in rabbit capsules and no statistically differences were found (Table XII). At 4 weeks, mononuclear type of inflammatory cells was predominant in the Control, Blood and Thrombin groups; in the Fibrin group mixed type of inflammatory cells was predominant but no statistically significant differences were observed (Table XII). Both at 2 and 4 weeks trends of intensity of inflammation showed no significant difference (Table XIII).

Table XI. Average capsular thickness of Control *versus* experimental groups.

Group	2 weeks (mm)	4 weeks (mm)
Control	0.83 ± 0.085	0.64 ± 0.078
Blood	1.02 ± 0.207	0.78 ± 0.572
Fibrin (Tissucol/Tisseel®)	0.89 ± 0.082	0.72 ± 0.083
Thrombin (FloSeal®)	0.90 ± 0.064	0.71 ± 0.105

Table XII. Outcomes for type of inflammatory cells of Control *versus* experimental groups.

Group	Type of inflammatory cells	2 weeks (%)	4 weeks (%)
Control	Mononuclear	22.2	55.6
	Polymorph	0	0
	Mixed	77.8	44.4
Blood	Mononuclear	33.3	55.6
	Polymorph	0	0
	Mixed	66.7	44.4
Fibrin (Tissucol/Tisseel®)	Mononuclear	11.1	22.2
	Polymorph	0	0
	Mixed	88.9	77.8
Thrombin (FloSeal®)	Mononuclear	22.2	77.8
	Polymorph	0	0
	Mixed	77.8	22.2

Table XIII. Outcomes for intensity of inflammation of Control *versus* experimental groups.

Group	Intensity	2 weeks (%)	4 weeks (%)
Control	Mild	11.1	55.6
	Moderate	77.8	44.4
	High	11.1	0
Blood	Mild	33.3	33.3
	Moderate	66.7	66.7
	High	0	0
Fibrin (Tissucol/Tisseel®)	Mild	0	22.2
	Moderate	66.7	33.3
	High	33.3	44.4
Thrombin (FloSeal®)	Mild	11.1	66.7
	Moderate	77.8	33.3
	High	11.1	0

Fibrosis was developed in all capsules at 2 and 4 weeks and no significant differences were observed regarding the organization of the collagen fibers between the Control and the experimental groups. At 2 weeks, dense >25% connective tissue in the Control group and loose or dense ≤25% connective tissue in the Blood group were observed (p = 0.023). Both at 2 and 4 weeks, increased angiogenesis was observed in the Control group (moderate or high) *versus* the Blood group (negative or mild) (p = 0.018). At 4 weeks, significant differences in the fusiform cells density were observed between the Control and the Blood groups (p = 0.047), with mild in the Control group and moderate in the Blood group.

Microbiology

Bacteria were isolated in 53% (38 of 72) of the capsules at 2 and 4 weeks, and in 47% (34 of 72) of tissue expanders. The isolates included: coagulase-negative *Staphylococci* (41%), *Escherichia coli* (10%), *Staphylococcus aureus* (8%), *Pseudomonas* spp. (0.7%), and other gram-negative bacilli (0.7%). In capsules, the predominant isolates were coagulase-negative *Staphylococci* detected in 53% (19 of 36) at 2 weeks and in 33% (12 of 36) at 4 weeks. In tissue expanders, coagulase-negative *Staphylococci* were found in 44% (16 of 36) at 2 weeks and in 22% (8 of 36) at 4 weeks. Capsules yielded a single isolate in 43% (31) of cases and more than one in 10% (7) of cases; tissue expanders yielded a single isolate in 32% (23) of cases and more than one in 15% (15) of cases. No fungi were recovered from the removed capsules or tissue expanders of all rabbits.

Similar bacterial isolates were cultured from the rabbit's skin. The predominant isolates were coagulase-negative *Staphylococci*, found in 16 of all 18 sacrificed rabbits (89%). Bacterial isolates from rabbit's skin were similar to those found in capsules and tissue expanders. Coagulase-negative *Staphylococci* were also isolated from all air samples. Other common airborne isolates included gram-positive bacilli and *Staphylococcus aureus;* less frequently *Penicillium* spp., *Aspergillus niger* and zygomicetes were recovered from the operation room air.

Statistical analyses revealed no significant differences in the frequency of culture positivity and the type of bacterial isolates among all the groups; also, no significant correlation between the microbiological and the histological data were found.

CHAID modeling associations

At 4 weeks statistical analysis with CHAID modeling showed association of intracapsular pressure measured at 20 ml, for the Control and the Fibrin groups. The determining factor for intracapsular pressure at 4 weeks was the type of inflammatory cells (Figure 7 a/b). The CHAID analysis showed in both trees that mixed type of inflammatory cells was correlated with decreased intracapsular pressure and mononuclear type of inflammatory cells was correlated with increased intracapsular pressure. In the Control tree, in the capsules with mononuclear type of inflammatory cells, the moderate inflammation was correlated with decreased pressure while capsules with mild inflammation had increased pressure.

CHAID classification analyses using intracapsular pressure measured at 20 ml, for the Fibrin and the Thrombin groups showed that the determining factor for intracapsular pressure at 4 weeks was the kind of bacteria isolated from tissue expanders (Figure 8 a/b). *Escherichia coli, Pseudomonas* spp. and negative cultures (other than *Staphylococcus and* no contaminated) were correlated with decreased intracapsular pressures. Coagulase-negative *Staphylococci* and *Staphylococcus aureus* (*Staphylococcus*) were correlated with increased intracapsular pressures.

Figure 7. Decision tree by CHAID algorithm for histological data at 4 weeks. (**a**) Control group; (**b**) experimental Fibrin (Tissucol/Tisseel®) group.

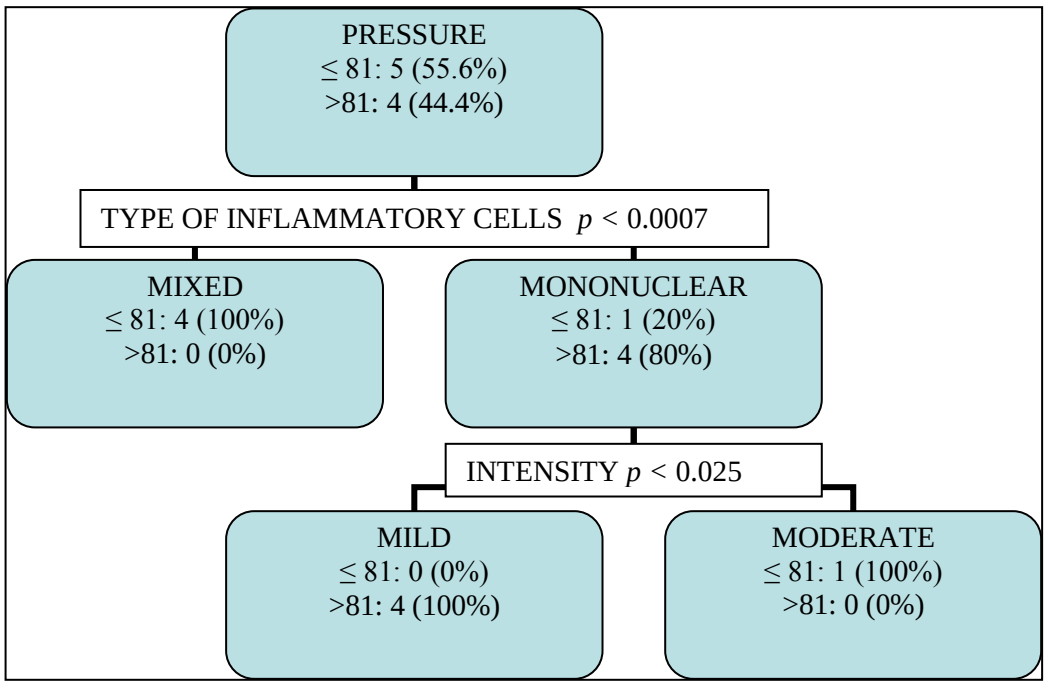


Figure 7 – (a)

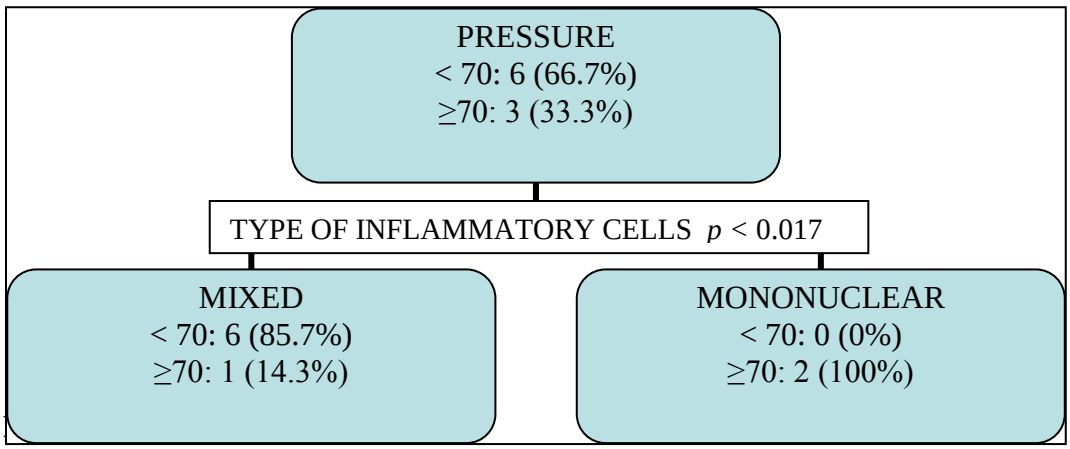


Figure 7 – (b)

Figure 8. Classification tree by CHAID algorithm for microbiological data at 4 weeks. (**a**) experimental Fibrin (Tissucol/Tisseel®) group; (**b**) experimental Thrombin (FloSeal®) group. **NO:** other than *Staphylococci* and no contaminated includes *Escherichia coli*, *Pseudomonas* spp. and negative cultures; **S:** *Staphylococci* includes coagulase-negative *Staphylococci* and *Staphylococcus aureus*.

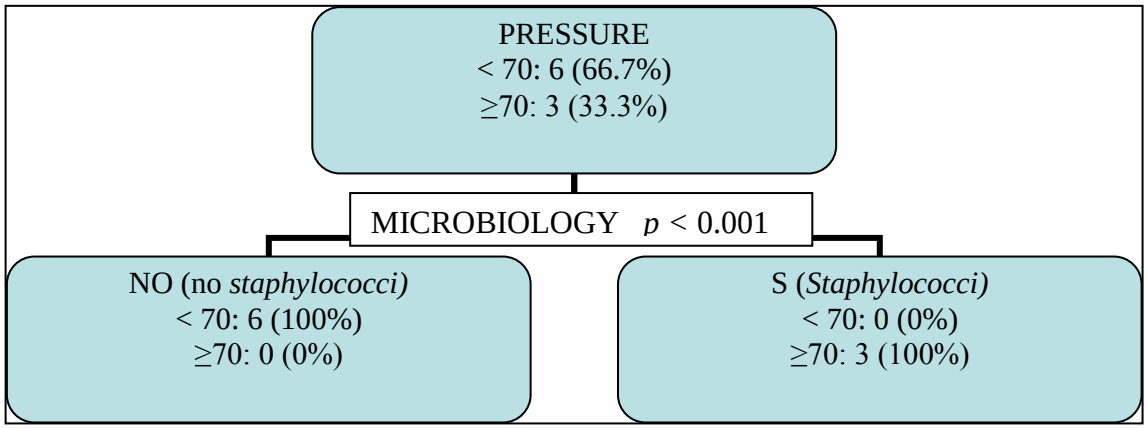


Figure 8 – (a)

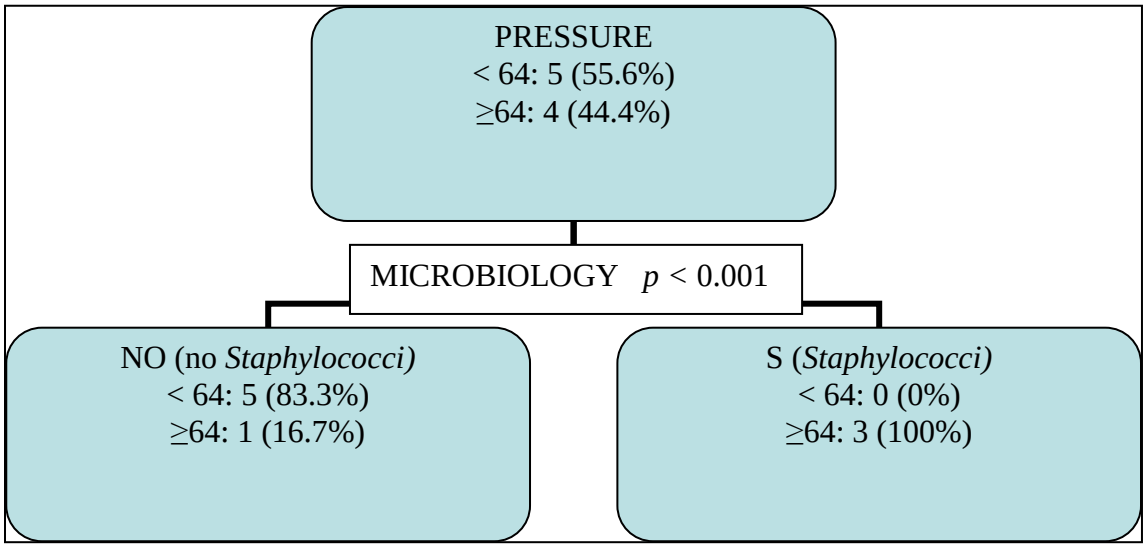


Figure 8 – (b)

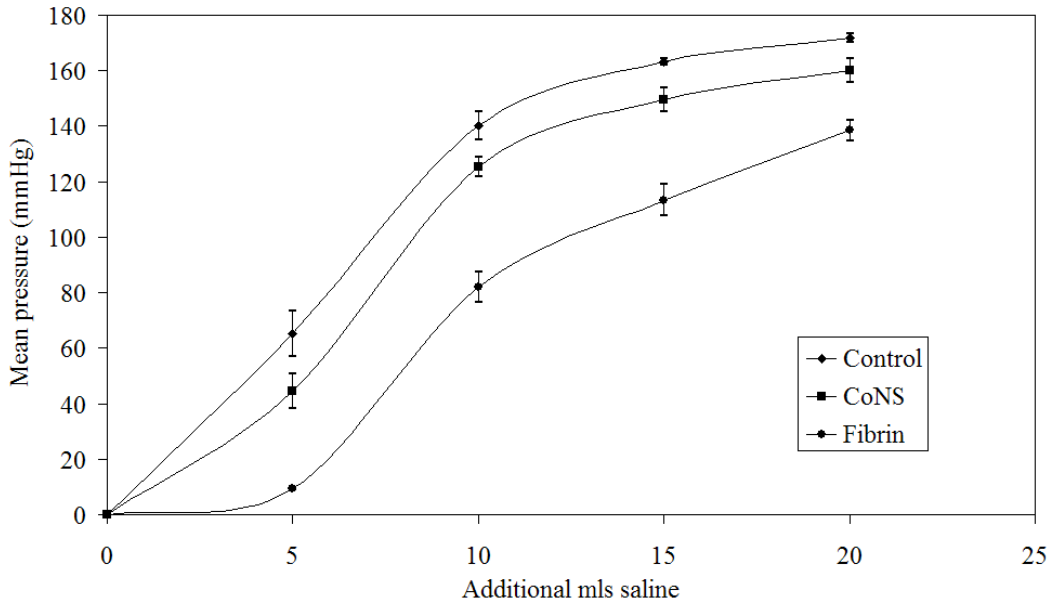
STUDY 3

Statistical analyses revealed no significant differences in histological and microbiological results between breast implants and tissue expanders (data not shown).

Intracapsular pressure

During pressure measurements, 5 (50%) capsules ruptured in the Control group and 5 (50%) capsules ruptured in the CoNS group. To avoid too less sampling, the ruptured capsules were not excluded from statistical analyses but, in such cases, the pressure value measured before rupturing was maintained after further additional ml saline added. Significant decreased intracapsular pressures were registered for the Fibrin group compared with the Control and the CoNS groups ($p \leq 0.001$; $p \leq 0.05$) (Figure 9). Statistical analyses revealed no significant differences between the CoNS and the Control groups (Figure 9).

Figure 9. The pressure-volume curves; there was a significant difference in intracapsular pressure in the Fibrin (Tissucol/Tisseel®) experimental group.



Histology

Average capsular thicknesses were 0.81 ± 0.21 mm, 0.47 ± 0.13 mm and 1.06 ± 0.29 mm in the Control, Fibrin and CoNS groups. Capsular thickness was not statistically homogeneous across the 3 groups ($p \leq 0.001$). Then, three subsets of similar means were found out by applying pos-hoc range tests, namely a first one comprising the Fibrin group (with the thinnest capsule), a second comprising the Control group, and a third one with the CoNS group (with the thickest capsule).

CHAID statistical modeling showed correlation between intracapsular pressure measured at 20 ml and thickness for the Control and the Fibrin groups (Figure 10 a/b); decreased intracapsular pressure was associated with thinner capsule for both groups, and the opposite was also true.

Figure 10. Decision tree by CHAID algorithm for thickness. (**a**) Control group; (**b**) experimental Fibrin (Tissucol/Tisseel®) group.

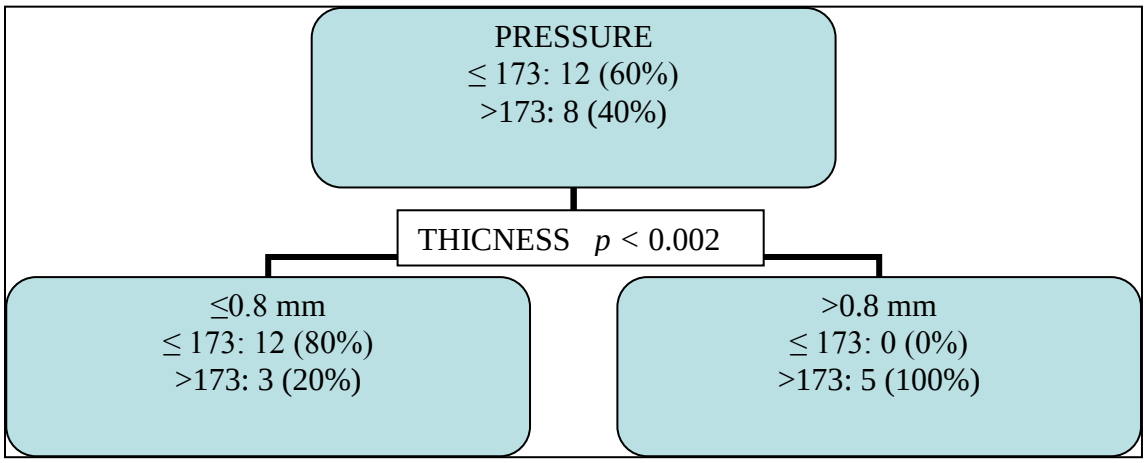


Figure 10- (**a**)

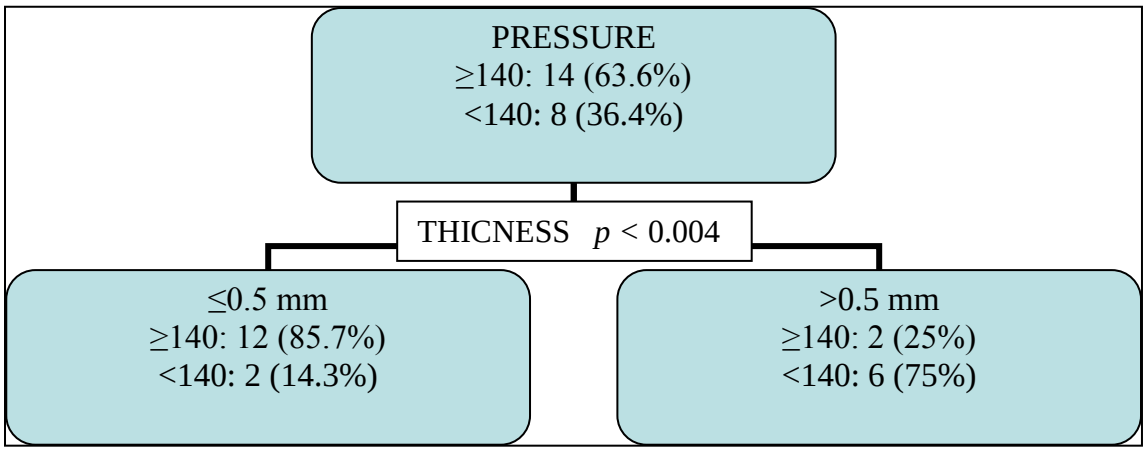


Figure 10- (b)

A mixed type of inflammatory cells was the most common finding in the Control and the Fibrin groups, but in the CoNS group, the polymorph type became predominant (Table XIV). Significant differences were observed between the Control and the CoNS groups (CoNS: $p = 0.0001$), between the CoNS and the Fibrin groups ($p = 0.0009$), but not between the Control and the Fibrin groups.

Intensity of inflammation was moderate in the Control and the Fibrin groups and mild in the CoNS group (Table XIV). Significant differences were found between the Control and the CoNS groups ($p = 0.011$), between the CoNS and the Fibrin groups ($p = 0.0058$) but not between the Control and the Fibrin groups. Significant correlations between the intensity of inflammation and the type of inflammatory cells for the Control ($p = 0.005$) and the Fibrin ($p = 0.006$) groups were observed.

Table XIV. Outcomes for capsule inflammation of Control *versus* experimental groups.

Group	Type of inflammatory cells	(%)	Intensity	(%)
Control	Mononuclear	25.0	Mild	30.0
	Polymorph	0	Moderate	70.0
	Mixed	75.0	High	0
Fibrin (Tissucol/Tisseel®)	Mononuclear	13.6	Mild	31.8
	Polymorph	13.6	Moderate	59.1
	Mixed	72.8	High	9.1
CoNS	Mononuclear	35.0	Mild	70.0
	Polymorph	50.0	Moderate	30.0
	Mixed	15.0	High	0

Fibrosis was detected in all capsules; no significant differences regarding the fusiform cells density among all groups were observed. Significant differences in the connective tissue were found between the Control and the Fibrin groups (p = 0.005), and between the CoNS and the Fibrin groups (p = 0.0007), with dense >25% connective tissue in the Control and the CoNS groups and loose or dense ≤25% connective tissue in the Fibrin group.

Significant differences in the organization of the collagen fibers were observed between the Control and the Fibrin groups (p = 0.019), and between the CoNS and the Fibrin groups (p = 0.0039), with haphazard collagen fibers in the Control and the CoNS groups and fibers arrayed parallel in the Fibrin group.

Significant differences in the angiogenesis were found between the Control and the Fibrin groups (p = 0.003), and between the CoNS and the Fibrin groups (p = 0.016),

with moderate or high in the Control and the CoNS groups and negative or mild in the Fibrin group.

Microbiology

Bacteria were isolated in 31% (19 of 62) of removed capsules, and in 84% (56 of 62) of the removed implants (Table XV). The predominant isolates were coagulase-negative *Staphylococci*, which were found in 16% (10 of 62) of all culture positive capsules, and in 60% (37 of 62) of culture positive implants. Overall, 97% and 90% of, respectively, culture positive capsules and implants yielded a single isolate, while 3% and 10% yielded two. No bacteria were detected on 69% of the removed capsules and on 16% of the removed implants. No fungi were recovered from the removed capsules or implants among all groups.

Table XV. Bacterial isolates from capsules and implants removed from the sacrificed rabbits[a].

Bacteria	Group[b]	Number of positive cultures	
		Capsules	Implants
Coagulase-negative *Staphylococci*	Control	2 (10%)	13 (65%)
	Fibrin	2 (9%)	9 (41%)
	CoNS	6 (30%)	15 (75%)
Staphylococcus aureus	Control	2 (10%)	2 (10%)
	Fibrin	1 (5%)	7 (32%)
	CoNS	0 (0%)	2 (10%)
Gram-positive bacilli	Control	1 (15%)	1 (5%)
	Fibrin	3 (14%)	4 (18%)
	CoNS	0 (0%)	2 (10%)
Micrococcus spp.	Control	0 (0%)	0 (0%)
	Fibrin	0 (0%)	0 (0%)
	CoNS	2 (10%)	1 (5%)

[a] 62 capsules and 62 implants were obtained from 31 rabbits
[b] Data collected from groups Control (10 rabbits; 20 capsules and 20 implants), Fibrin (11 rabbits; 22 capsules and 22 implants) and CoNS (10 rabbits; 20 capsules and 20 implants)

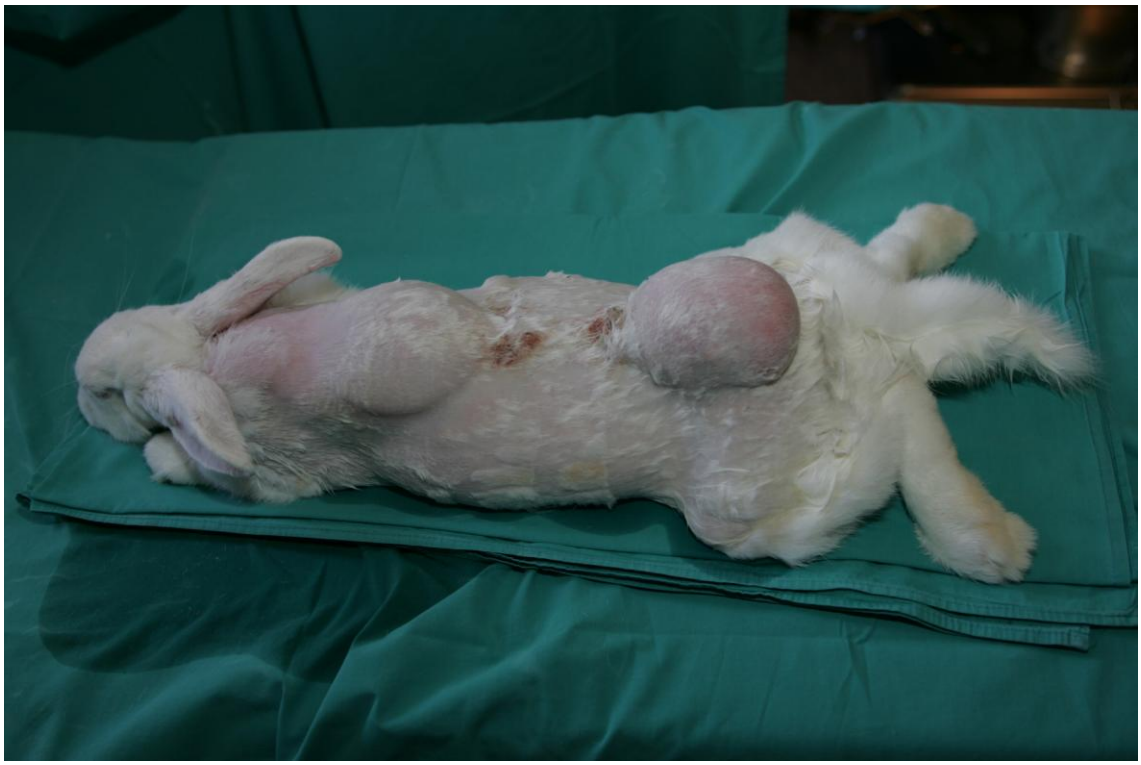
Statistical analysis revealed no significant differences in the type of bacteria and in the frequency of culture positivity among the study groups. Also, there was no significant association between microbiological and histological data.

Similar bacteria were isolated from the rabbit's skin. The predominant isolates were coagulase-negative *Staphylococci*, which was found in 37 of all 45 sacrificed rabbits (82%), followed by gram-positive bacilli (60%), *Staphylococcus aureus* (33%)

and *Micrococcus* spp. (9%). Other isolates found were *Enterococcus hermanii*, *S. harmolyticum* and *Proteus mirabilis*, though much less frequently. No skin sample was culture-negative while thirty-five samples yielded more than one isolate. The bacterial isolates from rabbit's skin were similar to those from the removed capsules and implants. Finally, coagulase-negative *Staphylococci* were also cultured from all the air samples; other airborne isolates were gram-positive and negative bacilli, such as *Micrococcus* spp., *Cryptococcus laurentii*, *Acinetobacter lwofii* and *Enterococcus agglomurans*. Fungal species, such as *Penicillium* spp., *Aspergillus niger*, *A. flavus* and *A. fumigatus* were recovered from the operation room air, *Penicillium* being the most common fungal isolate one.

In the CoNS group one animal developed a clinical contracture Baker grade IV, in one breast implant (Figure 11). The capsular thickness measured 1.70 mm and was the largest one among all capsules. The type of inflammatory cells was polymorph with moderate intensity. Histological evaluation of fibrosis revealed dense 25-50% connective tissue, haphazard collagen fibers, moderate fusiform cells density and moderate angiogenesis. Capsule and breast implant, were both infected with *Micrococcus* spp.; no other bacteria or fungi were detected.

Figure 11. Rabbit 31 from the CoNS group with a clinical contracture grade IV in the B implant.



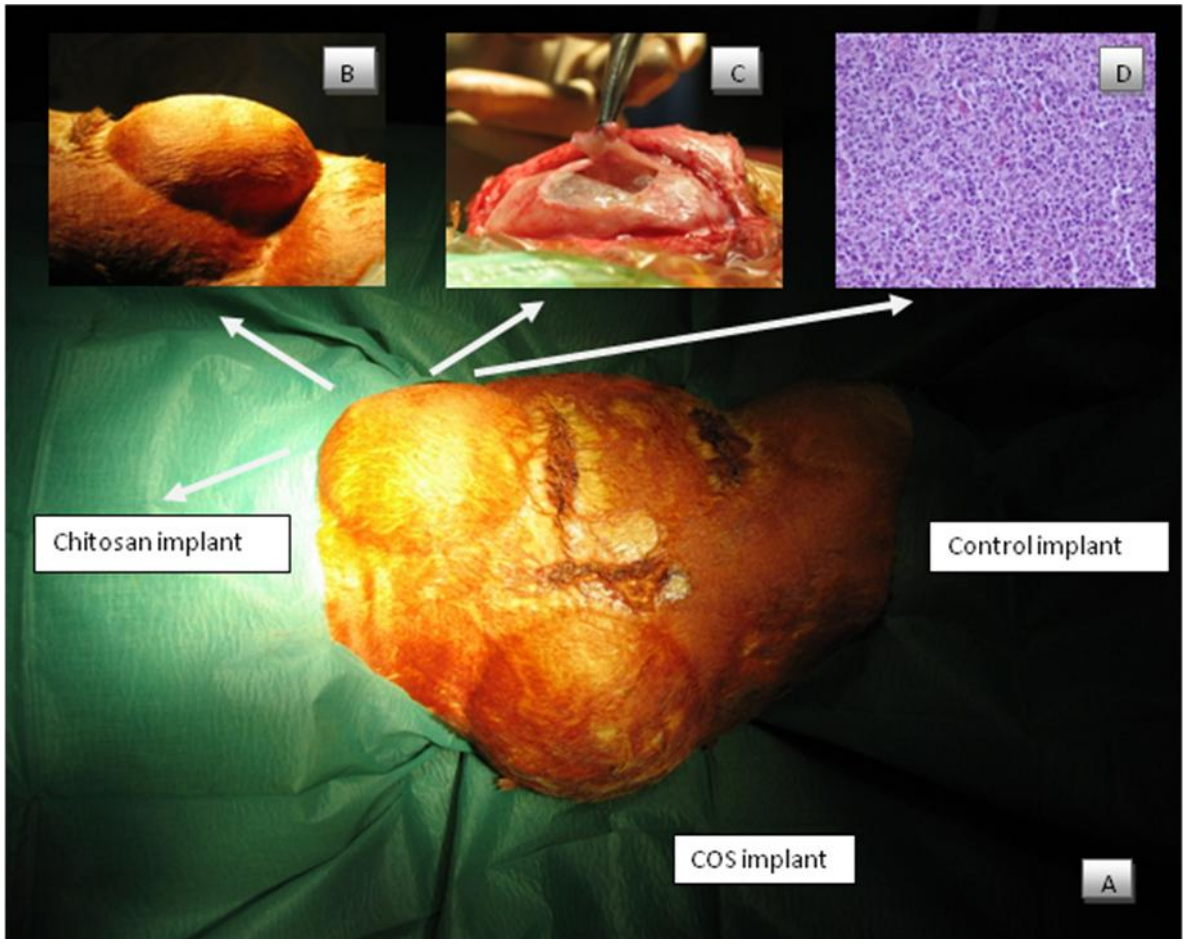
STUDY 4

Clinical

In the Control group, 1 of the 11 implants was ulcerated and none had developed clinical capsular contracture. In the COS group, 3 of the 11 implants were ulcerated and no implant had an observation of clinical capsular contracture. The Chitosan group had 1 ulcerated implant and all 11 implants had grade III/IV capsular contracture (Figure 12B). All Chitosan group capsules were extremely thick, opaque, stiff and resistant to cutting (Figure 12C); the implants were constricted and surface folding was observed.

Figure 12.

A) Rabbit with control implant, COS implant and Chitosan implant (contracture grade IV)
B) Chitosan implant- contracture grade IV
C) Chitosan implant - extremely thick, dense and opacity capsule
D) Chitosan implant - hematoxylin-eosin stain magnification 100x with apoptotic cells (cells have hiperchromatic and fragmented nuclei)



Histology

The average capsular thickness was 0.418 ± 0.160 mm in the Control group, 0.6364 ± 0.216 mm in the COS group, and 2.746 ± 0.817 mm in the Chitosan group. Capsular thicknesses were found to be statistically different among the 3 groups; capsular thicknesses from the Control group, were different from the COS group (p =

0.035) and from the Chitosan group (p= 0.003); capsular thicknesses were different between COS and Chitosan groups (p = 0.003).

No significant differences were observed regarding the type of inflammatory cells and intensity of capsule inflammation among the groups (Table XVI).

Table XVI. Outcomes for type and intensity of inflammatory cells of Control *versus* experimental groups.

Groups	Type of inflammatory cells	(%)	Intensity	(%)
Control	Mononuclear	9.1	Mild	72.7
	Polymorph	36.4	Moderate	27.3
	Mixed	54.5	High	0.0
COS	Mononuclear	9.1	Mild	54.5
	Polymorph	27.3	Moderate	45.5
	Mixed	63.6	High	0.0
Chitosan	Mononuclear	0.0	Mild	36.4
	Polymorph	45.5	Moderate	63.6
	Mixed	54.5	High	0.0

Apoptotic cells and necrosis (Figure 12D) were observed strongly in Chitosan group. Fibrosis was a component of all capsules and no significant difference was found regarding the organization of the collagen fibers (mainly arrayed in parallel in all groups), fusiform cells density and angiogenesis among all the groups. Regarding the characteristics of connective tissue (either loose or dense), significant differences were found between the Control and the Chitosan groups (p = 0.001); Control group had loose or dense ≤25% connective tissue and Chitosan group dense >25% connective tissue (mainly dense 25-50%).

Microbiology

Bacteria were isolated from 36.4% (12 of 33) capsules, and from 78.8% (26 of 33) implants. The organisms cultured (Table XVII) included coagulase-negative *Staphylococci*, *Staphylococcus aureus*, gram-negative bacilli and *Enterococcus* spp.. Among all the capsules that yielded bacteria, 11 of 12 capsules harboured coagulase-negative *Staphylococci* (91.7%) and *Enterococci* were associated with 1 capsule (8.3%). The same trend was observed in excised implants. In 20 of 26 implants that yielded bacteria, coagulase-negative *Staphylococci* were cultured from 76.9% and *Enterococcus* spp. was associated with 1 capsule (3.8%). In contrast to capsules, 4 of 26 bacterial contaminated implants harboured gram-negative bacilli (15.4%) and 1 of 26 *Staphylococcus aureus* (3.8%).

Table XVII. Bacterial isolates from capsules and implants samples removed from sacrificed rabbits.

Bacteria		Number of Positive Cultures	
		Capsules	**Implants**
Coagulase-negative *Staphylococci*	Control	4 (36.4%)	9 (81.8%)
	COS	5 (45.5%)	8 (72.7%)
	Chitosan	2 (18.2%)	3 (27.3%)
Staphylococcus aureus	Control	0 (0%)	0 (0%)
	COS	0 (0%)	1 (9.1%)
	Chitosan	0 (0%)	0 (0%)
Gram-negative bacilli	Control	0 (%)	2 (18.2%)
	COS	0 (%)	1 (9.1%)
	Chitosan	0 (0%)	1 (9.1%)
Enterococcus spp.	Control	0 (0%)	1 (9.1%)
	COS	1 (9.1%)	0 (%)
	Chitosan	0 (10%)	0 (%)

Overall, 39.4% (13 of 33) and 63.6% (21 of 33) of respectively culture positive capsules and implants yielded a single isolate, while 0% (0 of 33) and 9.1% (3 of 33) yielded more than one. No fungi were recovered from either capsules or implants.

No significant differences in the frequency of culture positivity and type of bacterial isolates were observed among all the study groups. No significant association between microbiological and histological data were also observed.

Considering rabbit's skin isolates, the predominant isolate was again coagulase-negative *Staphylococci*, which was formed in all rabbits. Bacterial isolates from skin were similar to those from capsules and implants. Coagulase-negative *Staphylococci* and gram-positive bacilli were isolated from all the air samples of the operation room, along with *Penicillium* spp. and *Aspergillus* spp..

Immunology

Interstitial fluid of IL-8 levels decreased from 89.4 ± 26.7 mg/ml in the Control group to 78.3 ± 32.7 mg/ml in the COS group, and to 66.8 ± 17.9 mg/ml in the Chitosan group. Significant differences were observed in IL-8 levels between the Control and the Chitosan groups ($p = 0.028$).

Levels of TNF-α decreased from 143.9 ± 123.8 mg/ml in the Control group to 96.8 ± 38.5 mg/ml in the COS group, and to 81.5 ± 31.8 mg/ml in the Chitosan group. Statistical analysis revealed no significant differences in the dialysate levels of TNF-α among all the groups. There was correlation between IL-8 and TNF-α in the Control group ($p < 0.001$), but it was not found in the COS ($p = 0.073$) and the Chitosan groups ($p = 0.099$).

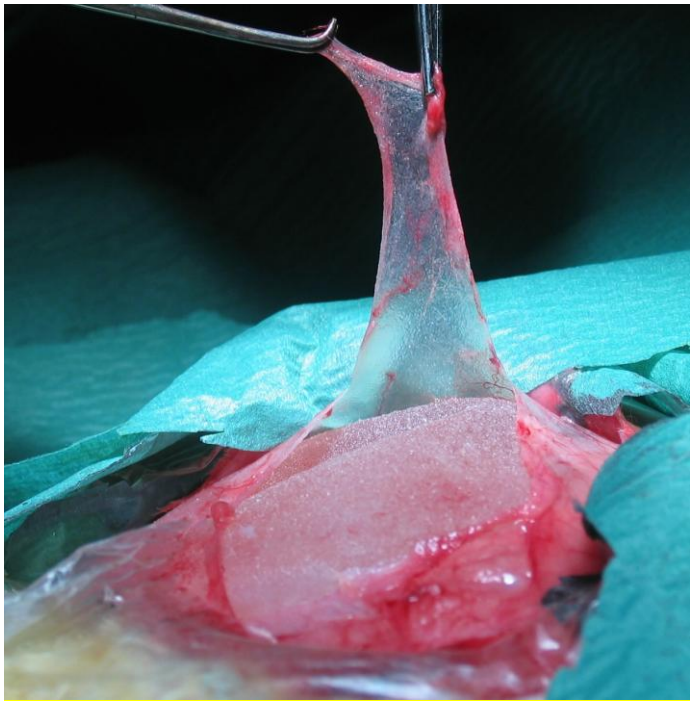
STUDY 5

Statistical analyses revealed no significant differences in histological and microbiological results between breast implants and tissue expanders (data not shown). The expanders were included in the protocol to determine the pressure-volume curves.

Clinical

In the Triamcinolone group (Figure 13) the capsule was thinner and more transparent than those of the Control group.

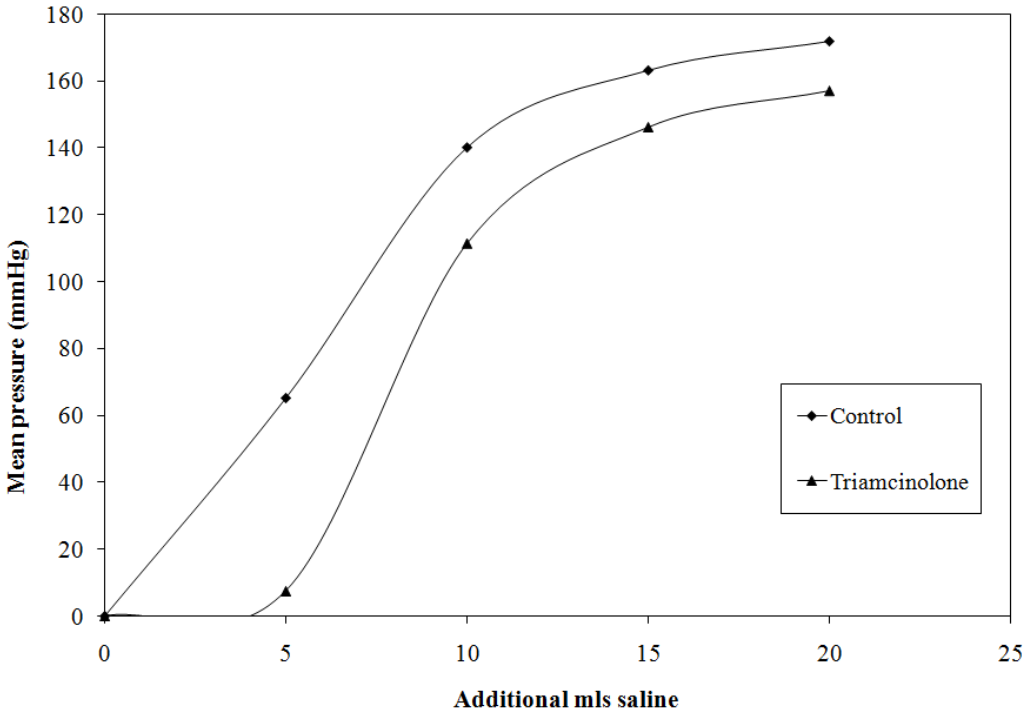
Figure 13. Capsule in the Triamcinolone experimental group.



Intracapsular pressure

During pressure measurements, 5 (50%) capsules ruptured in the Control group. To avoid too less sampling, the ruptured capsules were not excluded from statistical analyses but, in such cases, the pressure value measured before rupturing was maintained after further additional mls saline added. Pressure-volume curves were generated for all rabbits sacrificed. Statistical analyses revealed no significant differences between the Triamcinolone and the Control groups (Figure 14).

Figure 14. The pressure-volume curves.



Histology

Significant decreased capsular thickness was registered for the Triamcinolone group compared with the Control group ($p \leq 0.001$) (Table XVIII).

A mixed type of cells was the most common finding in the Control and the type mononuclear of cells was the most common finding in the Triamcinolone group (Table XVIII). Significant differences were found between the Control group and the Triamcinolone group ($p = 0.0003$).

Regarding the intensity of inflammation, a significant difference was observed between the Triamcinolone and Control groups (p =0.009), with mild in the Triamcinolone group and moderate in the Control group (Table XVIII).

No significant differences regarding the fusiform cells density, connective tissue, organization of the collagen fibers, between the Control and Triamcinolone groups were observed. Significant differences were found in angiogenesis between the Control group where it is basically moderate or high and the Triamcinolone ($p = 0.007$) group, where it was negative or mild.

Table XVIII. Outcomes for capsular thickness and inflammation of Control versus Triamcinolone Groups.

Group	Capsular thickness (mm)	Type of inflammatory cells	(%)	Intensity	(%)
Control	0.81 ± 0.209	Mononuclear Polymorph Mixed	25.0 0 75.0	Mild Moderate High	30.0 70.0 0
Triamcinolone	0.53 ± 0.136	Mononuclear Polymorph Mixed	83.3 0 16.7	Mild Moderate High	72.2 27.8 0

Microbiology

Statistical analysis revealed no significant difference in the type of bacteria and in the frequency of culture positivity bacteria between the Control and Triamcinolone groups regarding either implants or capsules. Also, there was no significant association between microbial presence and histological data. The predominant isolate was undoubtedly coagulase-negative staphylococci, which was identified predominantly in the removed implants (Table XIX).

Isolated bacteria from rabbits' skin and from the room air were statistically similar to those from the removed capsules and implants, being coagulase-negative staphylococci the prevailing one.

No fungi were recovered from the removed capsules or implants or skin samples of all rabbits. Fungal species, such as *Penicillium* spp. and *Aspergillus* were recovered from the operation room air.

Table XIX. Bacteria isolated from Capsule and Implant samples removed from all sacrificed Rabbits[a].

Bacteria	Group	Number of Positive Cultures	
		Capsules	Implants
Coagulase-negative staphylococci	Control Triamcinolone	2 (10%) 6 (33%)	13 (65%) 14 (78%)
Staphylococcus Aureus	Control Triamcinolone	2 (10%) 2 (11%)	2 (10%) 2 (11%)
Bacillus gram-positive	Control Triamcinolone	1 (15%) 2 (11%)	1 (5%) 2 (11%)

[a] Data collected from groups Control (10 rabbits; 20 capsules and 20 implants),and Triamcinolone (9 rabbits; 18 capsules and 18 implants) .

Immunology

The dialisate levels of IL-8 decreased from 115.56 ± 128.03 mg/ml in the Control group, to 54,41 ± 31.21 mg/ml in the Triamcinolone group. Statistical analysis revealed no significant difference in the dialisate levels of IL-8 between the Control and the Triamcinolone groups.

The dialisate levels of TNF-α decreased from 328.62 ± 307.55 mg/ml in the Control group to 148.9177 ± 211.92273 mg/ml in the Triamcinolone group. Statistical analysis revealed no significant difference in the dialisate levels of TNF-α between the Control and the Triamcinolone group.

There is correlation between IL-8 and TNF- α in the Control group ($p < 0.001$) and in the Triamcinolone group ($p = 0.036$)

5. Discussion

STUDY 1

Capsular contracture in a Portuguese population

In our report, the occurrence of local complications, the frequency, severity and long-term sequela were in the reported range as described in other studies[12],[13],[14],[15],[16],[17],[18],[19].

TableIII[12],[13],[14],[18],[19],[57] ,[85],[86],[87],[88],[89],[90],[91],[92],[93],[94] demonstrates that reported capsular contracture rates vary widely due to authors' reporting various Baker classification rates and follow-up time periods. These data showed incidence complications were elevated in reconstruction patients compared to cosmetic patients[95],[13]. No acute complications occurred in the Cosmetic group and all chronic complications were less prevalent in this group. In our study, women with breast implants due to cosmetic reasons had a lower body mass index than women with breast reconstruction, similarly to previous study which compared breast augmentation with breast reduction and general population[9].

In this study, 16% of capsular contracture (Baker III to IV) was diagnosed after a 1.6-year period following initial breast implantation. There were no significant associations between surgical route or implant placement, and any postoperative complication. Like Henriksen *et al.*[58], no significant associations were observed between body index mass, smoking habits, alcohol consumption, hormone therapy and capsular contracture in our study groups.

Capsular contracture, type of cohort, Baker II subjects and follow-up period

Capsular contracture may be apparent within the first year after implantation[13],[14],[20],[58]. However, in our study, about 76% of cases of capsular contracture (Baker II to IV) appeared just following 2 years; 10.1% and 37.5% of severe capsular contracture (Baker III to IV) occurred in the Cosmetic and Reconstructive groups, respectively, during the 8 years period of follow-up. Breiting *et al.* [9] reported 18% of severe breast pain, indicative of severe capsular contracture and, in a previous study, involving a subgroup of this population they had diagnosed 45% of capsular contracture (Baker II to IV) after a 5 years period following breast implantation[96]. Capsular contracture may also be symptomatic several years after surgery[9],[20],[58],[59].

Using the CHAID decision tree, the determining factor for capsular contracture was the type of group; the next splits indicated the best predictor variables for the Reconstructive group, as being the follow-up period*;* once considered no capsular contracture *versus* capsular contracture the follow-up period should be longer than 3 years and 6 months. However, if considering no capsular contracture including grade II subjects *versus* grade III or IV subjects, a longer follow up period of 5 years and 4 months was determined. It is interesting that both CHAID tree decision analyses had the same qualitative splits but with longer follow up time periods in grade III or IV subjects. This is expected as breast capsule formation is thought to develop from grade II to grade III, and grade III to grade IV. These results underscore the importance of considering grade II as an important clinical observation that should be included in the capsular contracture analyses. Thus, we believe that a follow-up period longer than 42 months from grade II and reconstructive patients should be considered when studying local complications among women receiving breast implants.

Estrogens, menopause and capsular contracture

It is well known the protective role of estrogens in the progression of liver fibrosis[97],[98] and the fact that estrogen deprivation was being associated with declining dermal collagen content and impaired wound healing[99], nevertheless there are no reports concerned with menopause nor estrogens *versus* capsular contracture. The authors for the first time report no association between capsular contracture and menopause or estrogen *status*. Therefore, the pathophysiology of capsule formation and subsequent contracture developing metabolic pathways are not estrogen derived.

Limitation and strength of the study

The main limitation of this study was the relatively small sample size and thus limited statistical power to observe relationships with rare outcomes, especially in the Cosmetic group.

One strength of this study was the statistical analyses of these data among the 2 groups using the CHAID method, a sophisticated algorithm used in many other disciplines, adjusting the probability of a single variable among multiple variables.

STUDY 2

The major findings of our study were observed on capsules and tissue expanders in the rabbits sacrificed at 4 weeks. Compared to the Control group, in the Fibrin and the Thrombin groups significantly decreased intracapsular pressures were measured. The Fibrin group was the only group without capsule ruptures during the pressure measurement. For both the Control and the Fibrin groups, mixed type of inflammatory cells was correlated with decreased intracapsular pressures while mononuclear type of inflammatory cells was correlated with increased intracapsular pressures. For both the

Fibrin and the Thrombin groups, other bacteria than *Staphylococci* or negative cultures were correlated with decreased intracapsular pressures and *Staphylococci* were correlated with increased intracapsular pressures. In the Blood group increased fusiform cells density were observed compared to the Control group. Increased angiogenesis was observed in the Control group compared to the Blood group. Average capsular thicknesses, type and intensity of the inflammatory cells, connective tissue and organization of the collagen fibers were similar among all groups. Also the bacterial isolates from capsules, tissue expanders and rabbit skin were similar among the four groups. In capsules, tissue expanders and rabbit skin the predominant isolates were coagulase-negative *Staphylococci*, which were also isolated from all air samples. No fungi were recovered from capsules, tissue expanders or rabbit's skin, although being isolated from all air samples.

It should be addressed that this study was performed with tissue expanders to measure the capsule pressure directly, o achieve more accurate results[144]. Similar capsules and increased pressure levels were observed in both the Control and Blood groups. Based on wound healing principles we may conclude that increased pressure levels and capsule rupture rates were correlated with contracture[145]. The fact that increased angiogenesis is related with fibrosis was demonstrated, supporting the major trends observed in the capsule contracture development in the Control group of this study[129],[144],[296].

FloSeal® requires blood for activity; with the Thrombin group results we may conclude that an active hemostasis is indispensable to prevent capsular contracture, although unnecessary with a hemostatic commercial product.

On the other hand, the Fibrin group had mixed type of inflammatory cells correlated with decreased intracapsular pressures compared with the Control group,

which is consistent with other reports observing that the activation of fibrosis in the early implant period may be the major mechanism for capsular contracture development[125]. In our study, type of inflammatory cells was not significantly correlated with capsular thickness which is consistent with Siggelkow *et al.*[125] results. Sead *et al.*[249] studied fibrin sealant prepared from Tisseel kit without aprotinin and observed the ability to reduce extracellular matrix and TGF-β1, especially from adhesion fibroblasts, which may indicate a role in reduction of postoperative adhesion development. It is well known that fibrosis is associated with excessive collagen extracellular matrix (ECM) formation and cells proliferation and activation of myofibroblasts. In this context, macrophages and mast cells have been implicated as important participants in the inflammatory process involving fibrosis[124]. Macrophages contribute to this process by the production of TGF-β1 and IL-6[162]. In the study by Ruiz-de-Erenchun *et al.*[297], TGF-β1 inhibitor peptide applied in a matrix with tetraglycerol dipalmitate was significantly effective in achieving a reduction in periprosthetic fibrosis after placement of silicone implants. Interestingly, in the Fibrin group, mixed type of inflammatory cells was correlated with decreased intracapsular pressures, however if infected by *Staphylococcus* the intracapsular pressures increased. Our results suggest the role of fibrin in preventing capsular contracture; and that the bacterial colonization of mammary implants may be partially responsible for capsule contracture, and coagulase-negative *Staphylococci* may play a relevant role [71],[72],[130],[131],[132],[133],[134],[135],[136],[137],[138],[139],[140],[141],[142]. It is reported in literature that infection of implanted medical devices was commonly mediated by formation of bacterial biofilms[298],[299],[300],[301]. However, Pajkos[74] reported that biofilm was demonstrated with scanning electron microscopy in a single culture-negative sample. Interestingly was the fact that extensive amorphous biological deposits were observed

with scanning electron microscopy, even in the absence of bacterial structures. Moreover, because of the low pathogenicity of coagulase-negative *Staphylococci* and the existence of microorganisms in a dormant phase within the biofilm around the implant, capsular contracture does not usually clinically manifest until some remote time after placement of mammary implants[74],[298],[299],[300],[301]. For all these reasons, in a pre-clinical study, the authors did not consider the biofilm investigation. All the methods to biofilm investigation are very expensive, are not routinely used and the follow-up period should be really longer.

We sacrificed the rabbits at 2 and 4 weeks to study the capsule formation and try to understand how it is possible to model wound healing formation[145]. Our study demonstrated very similar wound healing results at 2 weeks among all the groups, consistent with Adams and Marques *et al.*[84] report (Publication I). Our results differed from Adams and Marques *et al.*[84] study where intracapsular pressures were increased with fibrin glue application while in our results intracapsular pressure decreased. This may be explained in part as our data were collected at 4 weeks while Adams and Marques *et al.* [84] data examined more mature capsules at 8 weeks. On other hand, in this previous study Adams and Marques *et al.*[84] applied an autologous fibrin glue of unknown fibrin concentration into the implant pocket while in this experimental design we sprayed a commercial fibrin product widely studied and used in clinical practice (Tisseel/Tissucol®) in Europe and USA to reduce polypropylene meshes adhesions[252],[302]. To the best of our knowledge, this is the first report examining capsular formation with a commercial available fibrin product (Tissucol/Tisseel®). In addition, this is the first study with investigation of bacterial contamination from rabbit's skin and operation room air.

The authors performed the study on a New Zealand white rabbit animal model, an extension of Adams *et al.*[84] study, with the capacity to support four tissue expanders, which is impossible in mice. There are limited reports with the use of porcine.

One limitation of this study was the use of tissue expanders to measure the pressure directly using ports, instead of commercial silicone breast implants with ports, not available in this size. One strength of this study was the statistical analyses of these data among the 4 groups using the CHAID method, modeling a single variable among multiple variables.

Other parameters considered to be addressed in future studies: longer follow-up time period; breast implants sprayed with fibrin (Tissucol/Tisseel®); focus on fibrosis that may influence or modulate capsule contracture.

STUDY 3

Significant results were demonstrated in each of the experimental groups. In the Fibrin group the data showed significantly decreased intracapsular pressures and capsular thicknesses without any capsule rupture, compared to the Control and the CoNS groups. For the Fibrin and the Control groups, decreased intracapsular pressures were correlated with thinner capsules. A mixed type of inflammatory cells was the most common finding for both Fibrin and Control groups. In the Fibrin group, loose or dense ≤ 25% connective tissue was observed compared to the Control and the CoNS groups that had dense >25% connective tissue. In the Fibrin group, negative or mild angiogenesis was observed compared with the Control and the CoNS groups with moderate or high angiogenesis. No significant differences regarding fusiform cells density were observed between the Fibrin and Control groups.

In the CoNS group, increased capsular thickness was measured compared to the Control group. A polymorph type of inflammatory cells was the most common observation in the CoNS group, significantly different from the Control group. Regarding fusiform cells density, connective tissue, organization of the collagen fibers and angiogenesis, similar results were observed for both CoNS and Control groups.

Similar bacterial isolates were observed among all the study groups, regarding either implants or capsules. Implants were 2.7 times more frequently infected than capsules. The predominant isolates were coagulase-negative *Staphylococci*, which were present 3.8 times more in implants compared to capsules. There was no significant association between microbiological and histological data. Bacteria isolates from rabbit´s skin were similar to those isolated from capsules and implants. As expected, the predominant isolate in rabbit´s skin, as in implants and capsules, were coagulase-negative *Staphylococci.* Unexpectedly, *Micrococcus* spp. were isolated from rabbit´s skin specimens, operating room air samples and from one rabbit; in this specific rabbit, *Micrococcus* spp. were detected on the capsule, but not on the implant surface, and this capsule did not develop capsular contracture. Interestingly, on the contrallateral implant in the same rabbit, a *Micrococcus* spp. isolate was detected on both implant surface and capsule which was associated with clinical Baker grade IV capsule contracture development (Figure 11). To the best of our knowledge, this is the first report that shows a direct association between the presence of *Micrococcus* spp. and clinical capsule contracture, in a rabbit model. Fungi were isolated from the operation room air samples but not from the rabbit´s skin, capsules or implants. Even with similar bacteria types observed among all the groups, regarding implants or capsules, fibrin still modulates the capsule formation.

Our results support the probable role of fibrin as an agent that may modify capsule formation and subsequent capsule contracture, with decreased capsule thicknesses and pressures, loose or dense ≤ 25% connective tissue and negative or mild angiogenesis. The decrease of intracapsular pressure correlating with thinner capsules was also consistent with other clinical contracture reports[125],[126],[144],[303]. In addition to these results, the dense connective tissue and increased angiogenesis related with capsular contracture has already been demonstrated in other reports as achieved in our Control and CoNS groups[126],[129],[296]. The organization of the collagen fibers (parallel or haphazard) in capsular contracture is controversy; our results are similar to the study of Karaçal *et al*.[144].

The cytokine transforming growth factor beta 1 (TGF-β1) is a central mediator of fibrosis[304],[305],[306]. Some reports focused on fibrin properties for enhanced wound healing by the reduction of collagen extracellular matrix and decreased TGF-β1[157],[162],[249],[250]. TGF-β1 inhibitor peptide was significantly effective in achieving a reduction in fibrosis in silicone breast implants[297]. The use of fibrin-containing preparations (Tisseel® and Vi-Guard®) allow the closure of dead-space and approximation of the skin flaps, and it is argued that fibrin-containing tissue adhesives produced such a dense architecture that angiogenesis and vascular ingrowth were inhibited[251].To the best of our knowledge, this is the first pre-clinical study with a commercial fibrin compound (Tissucol/Tisseel®), sprayed to a textured silicone breast implant.

According to our results, bacterial infection of breast implants was more common than capsules infection and the predominant isolates were coagulase-negative *Staphylococci*. This is consistent with the fact that coagulase-negative *Staphylococci*, a commensal bacteria of the skin, are the predominant cause of biomaterial-associated

infection, commonly mediated by the formation of biofilms[298],[299],[300],[301],[307],[308]. The major pathogenicity is related to extensive biofilm formation on solid surfaces, which is extremely difficult to treat with antibiotics, thereby necessitating invasive procedures to remove the infected tissue or devices[309],[310],[311]. A strong correlation between the presence of biofilm (particularly by *S. epidermidis*) and the presence of significant capsular contracture were also reported[74]. They assumed that biofilm on the outer surface of the implant, once established, acts as a focus of irritation and chronic inflammation, leading to accelerated capsular contracture[74]. However, our results are contradictory to this report[74]. In the study by Pajkos *et al.*[74], the rate of recovery bacteria from the implant surface was lower than the rate of recovery from the capsule surface, but the authors explain that there was a greater sensitivity in detecting bacterial growth on capsules.

The clinical contracture Baker grade IV developed in one implant had the thickest capsule (Figure 11) among all capsules studied and was unusual as the contracture developed quickly with an acute inflammation. Histological evaluation of fibrosis in this capsule contracture revealed dense 25-50% connective tissue, haphazard collagen fibers and moderate angiogenesis. Unexpectedly, both the capsule and implant were infected only with *Micrococcus* spp., a low pathogenic agent. As far as we know, there are few reports concluding that *Micrococcus* spp. may have a true etiologic role in infection[312] and mediated by formation of bacterial biofilms[313],[314].

Our fibrin results are contradictory to our previous report[84] (Publication I), but consistent with another pre-clinical study[315] (Study 2; Publication III). This may be explained as in this previous published study[84] (Publication I), where we applied an autologous fibrin glue of unknown fibrin concentration into the implant pocket while in this experimental design we sprayed a commercial fibrin product widely study and used

in clinical practice (Tisseel/Tissucol®) in Europe and USA to reduce polypropylene meshes adhesions[252],[302], to reduce the incidence of posterior spinal epidural adhesion formation[236], and to reduce the recurrence rate of pterygium after surgery[242]. Another explanation was the application mechanism (manual with a syringe *versus* sprayed). A previous study found that a thin layer of glue is preferable to a thick one[316]; a thin layer of fibrin glue may support the healing process, whereas a thick layer of adhesive inhibits skin graft healing[317]. Moreover was the fact that in this study capsule pressure was measured directly in tissue expanders, to achieve more accurate results [144]. The fibrin glue is used by its properties as a hemostatic agent[318]; for enhanced wound healing by the reduction of collagen extracellular matrix and decreased TGF-β1 (mediator of fibrosis)[157],[162],[249],[250]; to prevent adhesions[252],[302]; widely use in ophthalmology[237],[238],[239],[240],[241],[319]; used as a drug delivery system such as antibiotic[320]; and our preclinical animal model results, make fibrin glue a promissing agent to prevent capsular contracture. Furthermore, fibrin glue was already used in a clinical model after breast augmentation as a drug delivery system[244].

The limitation of this study was the use of one tissue expander *per* rabbit just to measure the pressure directly using port. To correlate intracapsular pressure from tissue expanders with histological and microbiological results from breast implants, we performed statistical analyses that revealed no significant differences in histological and microbiological results between breast implants and tissue expanders. However, silicone breast implants with ports 90 ml size would be better to achieve more accurate results but are not commercially available. One strength of this study was the statistical analyses of these data among the 3 groups using the CHAID method, a sophisticated algorithm used in many other disciplines, adjusting the probability of a single variable among multiple variables.

Possible future studies would include: 1) a prospective clinical study comparing a women control group with a experimental group with Tissucol/Tisseel® sprayed to a silicone breast implants/pocket, with a follow-up period longer than 42 months[321](Study1; Publication II); and 2) analyze *S. epidermidis* and *Micrococcus* spp. biofilm development in a pre-clinical study with silicone breast implants with ports sprayed with Tissucol/Tisseel® and infected with bacteria

STUDY 4

In this study, we report the development of capsular contracture in a rabbit model associated with chitosan. All Chitosan group implants had clinical Baker grade III/IV breast contractures with significantly thicker capsules than non-treated implants. Chitosan exposed capsules were opaque, stiff and resistant to cutting and considerable shrinkage, and folding of the implant surfaces were observed that may indicate the constricting nature of fibrous implant capsules. Control group had thin capsule and loose or dense ≤ 25% connective tissue compared to the dense > 25% connective tissue observed in the Chitosan group. This is consistent with the fact that the major component of chitosan, glucosamine, forms cartilage tissue and is also present in tendons and ligaments[146]. Collagenous layer of granulation tissue is increased by chitosan applications; according to this finding, chitosan may stimulate fibroblast proliferation and extracellular matrix production[287]. Chitosan induced an accelerated wound healing process which increased TGF-β1 responsible for several proinflammatory regulatory influences, including cell migration, granulation tissue formation and increased collagen production[277] and, recognized as a central mediator of fibrosis[155].

A mixed or polymorph type of inflammatory cells was the most common finding in all rabbit capsules and inflammation intensity was moderate or mild in all capsules which was expected as chitosan is chemoattractant for neutrophils[220],[322]. Chitosan enhances the function of inflammatory cells such us polymorphonuclear leukocytes (PMN), macrophages, fibroblasts (production of IL-8), angioendothelial cells[287] and LMWC has a systemic effect[283]. Apoptotic cells and necrosis were observed strongly in Chitosan capsules which were consistent with other reports[323],[324].

Statistical analyses revealed no significant differences in the frequency of culture positivity and types of bacteria among all the groups. Interestingly, no significant associations between microbiological and histological data were observed in any group. Similar bacterial isolates were cultured from rabbits' skin and air samples and the predominant isolates were coagulase-negative *Staphylococci*. The antimicrobial activity of chitosan and its derivatives against several bacterial species has been recognized and considered as one of the most important properties linked directly to their possible biological applications[217],[218],[219],[220]; however, the new interest on chitosan as a drug deliverer such us antibiotics questioned the high efficacy of chitosan alone as an antibacterial agent[325],[326],[327],[328]. This study supports that capsular contracture formation was not the result of bacterial infection alone, in contrast to the infectious hypothesis which has been championed and consistently supported by Burkhardt[61],[63],[84].

To gain insight into the inflammatory process, the major biomarkers, TNF-α and of IL-8, were measured. This is the first report examining extracellular levels of IL-8 and TNF-α in a breast capsule implant environment. Microdialysate levels of IL-8 were decreased ($p< 0.05$) in the Chitosan group compared to the Control group. No significant differences in the microdialysate levels of TNF-α were observed among the

groups. In the Control group, a correlation between IL-8 and TNF-α was observed; no significant correlation between IL-8 and TNFα levels were observed in the experimental groups.

We originally hypothesized that serum concentrations of the inflammatory mediators would be significantly increased in the Chitosan group due to the expected greater inflammatory response with Chitosan as this molecule promotes the production of IL-8[287]. These data did not support the hypothesis but were consistent with Tilg *et al.*[329] study which reported increased IL-8 and TNF-α levels in bacterial infection and decreased IL-8 and TNF-α levels in acute rejection. Interestingly, we now report clinical Baker grade III/IV breast capsule contractures in all rabbits exposed to chitosan associated with polymorph and mixed inflammation and not due to a bacterial infection. Not all Chitosan implants were infected and IL-8 and TNF-α were decreased in the Chitosan group. Molecular regulation of IL-8 production has been studied *in vitro* and TNF-α has proven to be a major regulatory molecule. It is not surprising that *in vivo* IL-8 and TNF-α serum levels were also significantly correlated in the Control group. Correlation between IL-8 and TNF-α was well established in the case of bacterial infection, less pronounced in cytomegalovirus hepatitis and not apparent in acute cellular liver rejection episodes. Lack of correlation in acute rejection was also associated with low levels of IL-8[329]. This suggests that in contrast to bacterial infection, countering cytokines may be active in capsular contracture (at least promoted by chitosan), and down-regulating IL-8 transcription and/or translation. So far, no reports exist on production and regulation of IL-8 in capsular contracture. Recent studies have additionally demonstrated that COS displayed anti-inflammatory properties in immunocytes including the inhibition of nitricoxide, the down-regulation of IL-6 and TNF-α and the increase of cell viability of neutrophils[330],[331]. Additionally, IL-8 was

induced by a wide range of stimuli, including lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria and TNF-α. The study by Lund *et al.*[190] concluded that LPS induced IL-8 release in monocytes, while TNF-α was a good inductor of IL-8 in PMN. In the chitosan contracture model, we had decreased levels of IL-8 and it was possible to conclude that there was no Gram-negative bacteria infection to induce IL-8. On the other hand, chitosan increased the production of TGF-β1[277], a central mediator of fibrosis; the degree of capsular contracture is directly related to an increased level of TGF-β[125]. Even with contradictory studies about the role of TNF-α, Moritomo *et al.*[332] concluded that TNF-α played a pivot role in the maintenance of hemostasis and tissue repair by inhibiting TGF-β1.

Our data support the theory that chitosan initiates capsular contracture response due to a toxic local effect that resulted in an impaired wound healing response. An earlier series of pilot studies were performed with much higher levels of chitosan (data not shown). Using similar experimental protocol in the rabbit model, implants exposed to 25.0 mg/mL levels were used. The majority of animals expired within a short time period; surviving animals had decreased weight (15-25.8%) compared to baseline body weights with leucocitosis and decreased hemoglobin. At autopsy, fat biopsies were atrophied and liver specimens had lymphoid infiltration in portal spaces. We report toxicity with 25.0 mg/mL of implanted LMWC *per* rabbit. The study design was modified to test decreased chitosan levels that were not systemically toxic to the animals. In the reported data, all animals were clinically healthy. Literature data reporting general toxicity testing for chitosan is limited[276] and our results are consistent with the few papers about chitosan toxicity[283],[284],[285],[286],[287].

In several important studies[73],[77] the same rabbit had different implants. Darouich *et al.*[73] with the objective to examine *in vivo* the antimicrobial efficacy of

minocycline/rifanpin-impreganated saline-filled silicone implants, used the same rabbit to place 4 implants (2 antimicrobe-impregnated and 2 control implants were placed in each rabbit). In the study by Shah *et al*.[77], with the objective to examine in vivo the infectious hypothesis, each rabbit underwent a *Staphylococcus epidermidis* contaminated implant and a control implant. However, due to the systemic influence of chitosan, the use of 3 different implants in the same rabbit, in our study, obviously may confounded the results. To clarify this issue, a Control limb study was performed (data not shown) and compared with the Control group of this study. Using similar experimental protocol, 10 rabbits were implanted with 2 textured breast implants. Interestingly, on the Control group from this study the capsular thickness was lower than in the Control limb group (0.81 ± 0.21 mm) (p = 0.001). No significant differences were observed regarding the intensity of inflammation, characteristics of connective tissue (either loose or dense), fusiform cells density and angiogenesis between the groups; significant differences were observed with respect to the type of inflammatory cells, with mixed type of inflammatory cells in 54.5% of the Control group of this study and mononuclear type of inflammatory cells in 55.6% of the Control limb group (p = 0.017); significant differences were observed in the organization of the collagen fibers, which were arrayed in sequence in the Control group of this study and haphazard in the Control limb group (p = 0.007). Statistical analysis revealed no significant differences in the type of bacteria and frequencies between the control group of this study and the control limb group. A decreased levels of IL-8 (p = 0.016) and TNF-α (p = 0.001), were observed in the Control group of this study when compared with the Control limb group, which prove the systemic influence of chitosan.

In the discussion of our previous paper[84] (Publication I), Burkhard considered that if a rabbit model must be used for research, a more appropriate model was that

reported by Shah *et al.*[77],[288] who used bacterial contamination to produce contracture. In that study[77], 16 New Zealand white rabbits underwent each one, a *Staphylococcus epidermidis* contaminated implant and a control implant. The capsules were dissected at 2, 4, 6 and 8 weeks. Capsules on the contaminated side were 2 to 3 times thicker than those on the control side, and did not change thickness with time. Capsules on the contaminated side consisted of densely packed longitudinally oriented thick bundles of collagen fibers; there was a large cellular infiltration with leukocytes and macrophages. In contrast, the capsules on the control side were thinner and consisted of loosely organized connective-tissue fibers predominantly parallel to the prosthesis surface. Bacteriologic cultures on the contaminated side consistently isolated *Staphylococcus epidermidis* with occasional diphtheroides, while the control side showed no bacterial growth. As Prantl *et al.*[333] we believe that subclinical infection with chronic inflammation represents one of the possible important reasons for the development of capsular contracture. We also hypothesize that all possible causes of fibrosis result in the common key factor of pathological response with the development of chronic inflammation. Prantl *et al.*[333] included only those implants with high gel cohesiveness (third-generation implants); in these implants, the silicone filler presumably does not leak from the shell into the tissue in case of implant rupture; surprising, in 67% of their specimens, they detected vacuolated macrophages with microcystic structures containing silicone, and in 54% of the specimens, the capsular tissue contained empty spaces of varying sizes of silicone particles. It remains unclear whether these silicone structures represented friction particules from the surface of the implant or particules in the implant filler. Heppleston and Styles[334] study performed *in vitro* experiments demonstrating that silica damages macrophages, which subsequently produces TGF-β1 which stimulates fibroblast to produce collagen. However, since the study by Shah *et*

al.[77], and as far as we know, even with many publications with infected implants, there was no translation of the Baker classification in a pre-clinical model.

An infection-induced contracture limb study was performed (data not shown) and compared with the Chitosan group of this study. Using similar experimental protocol, 10 rabbits were implanted with 2 textured breast implants, each one with a suspension of 100 microlitres of coagulase-negative *Staphylococci* (10^8 CFU/ml - 0.5 density in McFarland scale). Histologically, the average capsular thickness was 1.065 ± 0.287 mm in the infection-induced contracture limb group (CoNS group) and 2.746 ± 0.817 mm in the Chitosan group. Capsular thicknesses were found to be statistically different among the two groups (p = 0.00003). A significant difference was also observed regarding the type of inflammatory cells among the two groups (p = 0.021), with a polymorph type predominant in the CoNS group, and mixed type predominant in the Chitosan group. No significant differences were found between the two groups regarding the intensity of capsule inflammation. Significant differences in the angiogenesis were found between the CoNS and Chitosan groups (p = 0.004), with equally absent/mild and moderate/high in the CoNS group but only high in the Chitosan group, as well as in the synovial metaplasia (p = 0.043) which was always absent in the Chitosan group but present in some cases of the CoNS group. However, no significant differences were found between the two groups regarding the characteristics of the connective tissue, organization of the collagen fibers (parallel or haphazard) and fusiform cells density. Histologically, this type of capsular contracture induced by chitosan is different than those induced by infection, in some aspects: 1) the capsule was thicker; 2) the mixed type of inflammatory cells was predominant; 3) the angiogenesis was high; 4) the synovial metaplasia was absent. This study reported to science a pre-clinical non-infectious model of capsular contracture and further studies are necessary.

We sacrificed rabbits at 4 weeks to study early capsule formation and to understand how it is possible to model wound healing formation[145]. The point is well taken for longer time periods and is currently planned for future experiments. However, long term differences in capsule structures under these experimental challenges do result from different wound healing trajectories from day 0. Our strategy was to examine these early differences with methods that were sensitive to detect histological or biomarker changes. There is no answer how long enough is necessary in a pre-clinical model. In a clinical model the authors propose a follow-up period longer than 42 months[321] (Study 1; Publication II). However, it might be expected that the finding of a dense collagenous capsule would increase with time, reflecting a continued stimulus toward a fibroplasia and ultimately collagen remodelling[335],[336],[337].

The weakness of this study was the relatively small size and the lack of capsule immunohistochemestry detection of IL-8 and TNF-α in tissue specimens. Nevertheless, the release of IL-8 and TNF-α into the circulation represented a "spillage" of factors rather than a direct signal driving inflammation and leukocyte recruitment; the use of microdialysis was appropriate for determining tissue concentration of cytokines such us IL-8 and TNF-α. Because of the proximity of the sampling site to the source of the cytokine, microdialysis may provide a means of sensitively detecting relative changes of inflammatory mediators' concentration with experimental treatments.

Possible future studies would include: 1) silicone breast implants with ports (to measure the capsule pressure directly) impregnated with low molecular weight chitosan (LMWC) implanted *per* rabbit; detection of IL-8, TNF-α, TGF-β1 and determination of fibrosis index; 2) With the same protocol analyze silicone breast implants with ports impregnated with low molecular weight chitosan (LMWC) and sprayed with Tissucol/Tisseel®[315],[338] . In summary, a capsular contracture animal model was

observed when implants were impregnated with chitosan and not due to a bacterial infection. This report suggests a new approach of studying capsular contracture using a pre-clinical animal model.

STUDY 5

The capsule is composed by a layer of fibrous dense connective tissue[339], and is an integrant part of the wound healing process. To understand the formation of this late complication, and the potential therapeutic roles of both pharmacological and non pharmacological approaches, it is crucial to know the physiological mechanisms that are behind this process.

Wound healing has been divided into three distinct phases: inflammation, proliferation and maturation[340]. The first phase of wound healing, which courses immediately upon injury through day 4 to 6, is characterized firstly by hemostasis, an important event that serves as the initiating step for the healing process; and an inflammatory response. The second phase of wound healing (proliferative phase) is characterized by epithelization, angiogenisis and provisional matrix formation, and courses from day 4 through 14, overlapping the phase 1 and 3. Fibroblasts and endothelial cells are the predominant cells proliferating during this phase. Maturation and remodeling (phase 3), occurring from day 8 through 1 year, is characterized by the deposition of collagen in an organized and well-mannered network[145].

As seen before, corticosteroids are known to have an important role on modelling wound healing, as they can stop the growth of granulation completely, the proliferation of fibroblasts, diminish the new outgrowths of endothelial buds from blood vessels and stop the maturation of the fibroblasts already present in connective tissue[227]. Also when administered early after injury, corticosteroid delay the

appearance of inflammatory cells, fibroblasts, the deposition of ground substance, collagen, regenerating capillaries, contraction and epithelial migration[228]. As so, steroids can have an important role in CC formation, in both early and late phases of fibrous phase formation.

The efficacy of triamcinolone on treating and preventing CC in women has been reported[265], [266]. However, this still represents an off-label practice and further studies are required to validate the efficacy of this approach. Both works have limitations: are non-randomised, with no control group, with a limited follow-up period[265],[266] and none of them have, as an objective, to comprove which is the mechanism of action of this compound in capsular contracture formation.

A comprehensive understanding on the effects of this compound on the mechanisms of capsular formation; a knowledge on the systemic side effects and potential adverse events are, in the authors opinion, crucial for the improvement of TA in clinical activity.

This study arises as the first one analyzing the impact of TA in early capsule formation. The authors examined the effects of TA on pressure, histological, microbiological and immunological characteristics of capsules, in an animal model, in order to understand the role of this steroid in early capsule formation, and the possible role in the prevention of CC.

In our study, TA was found to decrease capsular thickness both on macroscopic and microscopic examination, when compared to the Control group. These findings were also associated with decreased inflammation and angiogenesis, as it was expected as steroids are anti-inflammatory drugs, capable of delaying the appearance of inflammatory cells and diminish the proliferation of endothelium from blood vessels[227] and regeneration of capillaries[228]. Although no significance was found in the

intracapsular pressure between groups, it was observed a tendency to lower pressures (and no capsule rupture during the pressure measurement) in the Triamcinolone group when compared to the Control group (Figure 14). Also both cytokine markers (IL-8 and TNF-α) were lower in the Triamcinolone group, even without statistic significance. No significant differences were observed in fusiform cells densities, connective tissue and organization of the collagen fibers. Taken together, these results suggest that washing the pocket intraoperatively with TA has a role on capsule formation, and might prevent CC.

As Caffee *et al.*[264], we were not able to observe a significant decreased capsular pressure in the group treated with triamcinolone in the time of implant placement. However in our study we go further and we analyzed not only the pressure, an unquestionable indicator of capsular contracture, but also other characteristics that are related with the formation of this pathology, as a continuous process. The breast capsule begins being formed after implant placement, however, in the clinical practice, the contracture is a late complication, and a follow-up as longer as 42 months (Study 1; Publication II)[321], is required to the diagnostic of this entity. In preclinical models, there is no consensus on the timing for sacrifice and timing for representative stages for capsule formation.We were not able to observe significant differences on pressure between the groups, probably because we sacrificed animals too early to the complete development of this complication. However, we were able to observe the early alterations that are not characteristic of CC as thinner and more transparent capsules on macroscopic and microscopic evaluation, and decreased inflammation and angiogenesis. It might be expected and is reasonable to assume that a more dense collagenous capsule with increased thickness would be present with longer incubation times, reflecting a

continued stimulus toward a fibroplasia and ultimately collagen remodelling[336],[335],[337].

Caffee *et al.* reported in both preclinical[264] and clinical[265] studies that triamcinolone injected postoperatively was able to eliminate CC and prevent the recurrence of this condition. Those findings are confirmed by Sconfienza *et al.*[266], that was able to demonstrate that US-guided injection of triamcinolone acetonide in the peri-implant pouch of women with augmented or reconstructed breast affected by Baker grade IV CC is effective in reducing capsular contraction. Both authors concluded that triamcinolone was effective in the late stages of capsule formation. With our study we were able to observe that triamcinolone is probably not only effective when injected postoperative, but it also as a role in the early phases of the development of capsular contracture.

In a previous report (Study 3; Publication 4)[338] using the same protocol, the authors were also able to find another compound, fibrin (Tissucol/Tisseel), that was associated with a lower incidence of CC when sprayed in the pocket/implant during surgery and more effective in the early phases of wound healing than in later phases. It was found that fibrin[338] , was able to decrease intracapsular pressures when compared to Control ($p \leq 0.001$ - data not shown), and the capsular thickness were decreased (0.47 ± 0.129 mm) ($p \leq 0.001$) as in Triamcinolone group.

TNF-α plays an important role in the wound healing process: it is produced by activated macrophages, platelets, keratinocytes, and other tissues and it stimulates mesenchymal, epithelial, and endothelial cell growth and endothelial cell chemotaxis[341],[342]. During the inflammatory phase, it is one of the main responsible for neutrophils drawn into the injured area[343], macrophages generation of NO[344] and damaged extracellular matrix digestion by matrix metalloproteinase[345]. During the

second phase TNF-α upregulates KGF gene expression in fibroblasts, upregulate integrins, a matrix component that serves to anchor cells to the provisional matrix, stimulates epithelial proliferation[341] and is also a potent promoter of angiogenesis. TNF-α is known to be a growth factor for normal human fibroblast, and promotes the synthesis of collagen and prostaglandin E2. IL-8, enhances neutrophil adherence, chemotaxis, and granule release; and enhances epithelization during wound healing[341],[346]. TNF-α levels were reported to be markedly elevated in fibrotic diseases as liver fibrosis, and is considered a mediator of fibrosis such as TGF-β1[346]. Moritomo *et al*.[332] concluded that TNF-α played a pivot role in the maintenance of hemostasis and tissue repair by inhibiting TGF-β1. We were not able to find significant differences in IL-8 and TNF-α levels, although decreased levels were observed in the group treated with triamcinolone, possibly reflecting a role of this drug in modulation of wound healing process and fibrotic response in the presence of the implant. More studies, with longer follow-up and increasing doses of the compound are needed to confirm this data.

On the other hand, a significant correlation was also found between IL-8 and TNF-α in both groups. This was not unexpected as correlations between IL-8 and TNF-α with bacterial infections have been reported[329]. We did not find any differences in the microbiology cultures between groups but further studies are necessary to clarify if triamcinolone increases the risk of infection.

With fibrin it was observed a significant decreased on TNF-α (140.9 ± 165.9 mg/ml) and IL-8 (23.9 ± 43.4 mg/ml) levels (p = 0.003; p = 0.048) supporting the possible role of this compound in the early capsule formation and in the reduction of collagen extracellular matrix. No correlation between IL-8 and TNF- α was observed in the Fibrin group which suggests a possible antibacterial role of fibrin[338].

The main limitations of this study are: 1) inappropriate dosage in this model system; rabbits have much faster basal metabolic rates than human, and as such, it is presumed that rabbits have shorter drug half-lives[268]; 2) unknown pharmacokinetics of triamcinolone in the capsule pocket and subsequent metabolism, although triamcinolone modelling may be based on systemic steroid modelling[347]; 3) short follow-up, as capsular contracture usually takes more than four weeks on developing; and 4) the use of one tissue expander per rabbit to directly measure internal expander pressures using the port. Silicone breast implants with ports with a 90 ml volume capacity would be optimal to achieve more accurate results but are not commercially available. In addition, the preclinical model would not support the use of multiple large expanders or implants over long time periods.

Our data support future studies examining triamcinolone as a potential agent on preventing CC.

Possible future studies may include: 1) pre-clinical study with silicone breast implants with ports (to measure the capsule pressure directly) with introduction of 1.5 ml (60 mg) of triamcinolone-acetonide into each implant pocket and sacrifice the animals at a much longer time point with detection of IL-8, TNF-α, TGF-β1 and determination of fibrosis index; and 2) with the same protocol, assess the effects of of saline or other vehicles of triamcinolone-acetonide on pressure/volume curves. We believe that a pre-clinical study higher dose of triamcinolone-acetonide introduced into the implant pocket and a longer follow-up time period will support the growing body of evidence that triamcinolone-acetonide mitigates capsular contracture.

In summary, our results suggest that triamcinolone has a role in early capsule formation, and it may have a role in the prophylactic management of this complication. Obviously, it role is centred on the management of the factors related to wound

healing[124], and it is important to exclude a deleterious role in the factors related to infection that are also known to increase CC[62],[64],[79],[21],[206]. The clinical use of triamcinolone-acetonide may prove to be a reliable and safe intraoperative method to prevent capsular contracture in women undergoing breast implants. The ultimate goal is to translate these preclinical results to the clinic as these finding may help not only patients with breast implants, but to all patients with any device in which capsule contracture around that device may lead to an adverse clinical event.

6. Conclusions

- The authors for the first time report no association between capsular contracture and menopause or estrogen *status*. (Study 1; Publication II)
- Our data suggest that grade II subjects should be included in a capsule contracture analyses and a follow-up period longer than 42 months (3 years and 6 months) should be considered. (Study 1; Publication II)
- The authors document a pre-clinical capsular contracture with thicker capsule, mixed or polymorph inflammatory cells, dense >25% connective tissue, haphazard collagen fibers or arrayed in sequence, moderate or high angiogenesis. (Studies 3 and 4; Publications IV and V)
- It is not appropriate to sacrifice the animals at 2 weeks. (Study 2; Publication III)
- Bacteria from rabbit´s skin and operation air were similar to those from removed capsules, tissue expanders and breast implants, but, the fungi were just isolated in the air samples. (Studies 2, 3, 4 and 5; Publications III, IV, V and VI)
- Interestingly, in the Fibrin (Tissucol/Tisseel®) group mixed type of inflammatory cells were correlated with decreased intracapsular pressures, however if infected by *Staphylococcus* the intracapsular pressures increased. Our results suggest the role of fibrin (Tissucol/Tisseel®) in preventing capsular contracture; and that the bacterial colonization of mammary implants may be partially responsible for capsule contracture, and coagulase-negative *Staphylococci* may play a large role. (Studies 2 and 3; Publications III and IV)
- The role of *Micrococcus spp.* in the pathogenesis of capsular contracture deserves further study. (Study 3; Publication IV)
- Based on the Blood and the Thrombin (FloSeal®) groups results we may conclude

that an active hemostasis is indispensable to prevent capsular contracture, although unnecessary with a hemostatic commercial product. (Study 2; Publication III)

- The fibrin (Tissucol/Tisseel®) benefits enhance wound healing reducing capsular contracture and the *minor* adverse events observed make this drug an attractive alternative for use in women undergoing breast implantation. (Studies 2 and 3; Publications III and IV)
- A capsular contracture animal model related with chitosan and not due to an infection. (Study 4; Publication V)
- The use of microdialysis is appropriate for determining the concentration of cytokines such us IL-8 and TNF-α and could provide a means of sensitively detecting relative changes of inflammatory mediators' concentration with experimental treatments. (Study 4 and 5; Publication V and VI)
- Our data support the theory that chitosan initiates capsular contracture response due to a toxic local effect that resulted in an impaired wound healing response. (Study 4; Publication V)
- Triamcinolone-acetonide during breast implantation influences the early capsule formation and may reduce capsular contracture. (Study 5; Publication VI)
- Capsular contracture is multifactorial including any bias which promotes a subclinical toxic effect. (Studies 2, 3 and 4; Publications III, IV and V)

Financial disclosure and products page

The present study was carried out at the Faculty of Medicine, University of Oporto, Portugal (Department of Experimental Surgery and Department of Microbiology), the Centro Hospitalar of São João, Oporto, Portugal (Department of Plastic and Reconstructive Surgery and Department of Pathology), the Faculty of Sciences, University of Oporto, Portugal (Department of Chemistry), the Biotechnology School, University of Oporto, Oporto, Portugal and the UT Southwestern Medical School at Dallas, Texas, USA (Department of Plastic Surgery and Research, Nancy L. & Perry Bass Advanced Wound Healing Laboratory).

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the best Aesthetic Facial Surgeons and is famous for resident's mentorship, scientific publications and has their own research lab - the Nancy L. & Perry Bass Advanced Wound Healing Laboratory. I forwarded my *Curriculum Vitae* and a letter to Research Professor Spencer Brown, and also a letter to Dr. Rod Rohrich. One week later, I was in the operating room, in the middle of 2 surgeries, and I received a phone call from Professor Spencer Brown, the Director of the Research Department of Plastic Surgery at UT Southwestern Medical School in Dallas! He was so friendly with such encouragement during this phone call (without knowing me at all!) which was, to me, unbelievable! Once again, I was so afraid to disappoint, but accepted and started my research projects with Professor Spencer Brown in 2003. His far-reaching vision, not only in science, but also in life, has been a true inspiration to me.

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Original publications

Publication I

A Rabbit Model for Capsular Contracture: Development and Clinical Implications

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Background: Capsular contracture remains one of the most common complications involving aesthetic and reconstructive breast surgery; however, its cause, prevention, and treatment remain to be fully elucidated. Presently, there is no accurate and reproducible pathologic in vitro or in vivo model examining capsular contracture. The purpose of this study was to establish an effective pathologic capsular contracture animal model that mimics the formation of capsular contracture response in humans.

Methods: New Zealand White rabbits (n = 32) were subdivided into experimental (n = 16) and control groups (n = 16). Each subgroup underwent placement of smooth saline mini implants (30 cc) beneath the panniculus carnosus in the dorsal region of the back. In addition, the experimental group underwent instillation of fibrin glue into the implant pocket as a capsular contracture–inducing agent. Rabbits were euthanized from 2 to 8 weeks after the procedure. Before the animals were euthanized, each implant was serially inflated with saline and a pressure-volume curve was developed using a Stryker device to assess the degree of contracture. Representative capsule samples were collected and histologically examined. Normal and contracted human capsular tissue samples were also collected from patients undergoing breast implant revision and replacement procedures. Tissue samples were assessed histologically.

Results: Pressure-volume curves demonstrated a statistically significantly increased intracapsular pressure in the experimental group compared with the control group. The experimental subgroup had thicker, less transparent capsules than the control group. Histologic evaluation of the rabbit capsule was similar to that of the human capsule for the control and experimental subgroups.

Conclusions: The authors conclude that pathologic capsular contracture can be reliably induced in the rabbit. This animal model provides the framework for future investigations testing the effects of various systemic or local agents on reduction of capsular contracture. (*Plast. Reconstr. Surg.* 117: 1214, 2006.)

Breast implant capsular contracture remains one of the most common complications for both aesthetic and reconstructive breast surgery. Despite the importance of this problem, the cause and treatment have remained unresolved for the past 40 years. Further complicating this problem is that there are currently no reliable in vitro or in vivo models producing capsular contracture. Various animal models have been reported in previous studies; however, most lack the ability to produce the pathologic state of contracture and, thus, correlation of proposed treatments for clinical capsular contracture are invalid in this setting.

Histologically, the human breast capsular tissue is composed of an inner layer of fibrocytes and histiocytes, which is surrounded by a thicker layer of collagen bundles arranged in a parallel array.[1,2] The outer layer is more vascular and is composed of loose connective tissue. Although intuitively and clinically most would consider the degree of capsule thickness to be commensurate with the severity of capsular contracture, this has never been definitively proven, and some reports have found no correlation among contamination, thickness, and clinical contracture.[3]

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The literature is replete with earlier studies that attempted to detect differences in capsule characteristics between those formed around smooth versus textured implants. Both gross and histologic sections revealed a thicker capsule, with increased cellularity surrounding the textured implants[4,5]; however, other reports have produced contradictory results.[6,7] Equally perplexing is the incongruity between studies with animal models compared with human clinical studies.[4,5,8] Current data have yet to determine the exact cause for contracture and thus no completely effective prophylaxis or therapy has been developed.[5–9] Compounding the problem is the use of various animal models for analysis of capsular contracture when the animals themselves do not produce a pathologic capsular state.[6,10,11]

Furthermore, a large body of conflicting data exist on the mechanisms and various cell types involved with the formation of the host capsular contracture tissue response. As with any condition where the cause is unknown, there exists a multitude of treatment modalities offered based on anecdotal or clinically based experience. The bulk of the literature on this subject is retrospective, unblinded, uncontrolled, and rarely uses elegant scientific methodology.

The purpose of this study was to develop a pathologic, reproducible, and reliable animal model for capsular contracture that is similar to human breast capsular contracture tissue. This information can be used to help systematically determine the cause of this problem and to allow options for prevention and potential treatment of capsular contracture.

MATERIALS AND METHODS

Thirty-two New Zealand White rabbits underwent implantation with customized smooth saline mini implants (30 cc; McGhan Medical, Santa Barbara, Calif.) under an approved institutional animal care protocol. Each implant was placed in the subpanniculus carnosus plane in the dorsal back region and filled to the manufacturer's recommended 30-cc fill volume. One implant was placed per rabbit, using sterile surgical technique.

The rabbits were divided into an experimental (n = 16) and control subgroups (n = 16). The experimental subgroup also underwent instillation of 5 cc of fibrin glue [fibrin glue is prepared with 4 ml of rabbit cryo (Pel-Freez; Pel-Freez Biologicals, Rogers, Ark.), 500 μl of 10% CaCl (Sigma-Tau Pharmaceuticals, Gaithersburg, Md.), 1000 units of thrombin (Monarch Pharmaceuticals, Bristol, Tenn.) in 1 ml of 50 mM TrisCl (Sigma), pH 7.4] into the implant pocket as a contracture-inducing agent. The incision was closed in two layers with subdermal 4-0 Vicryl (Ethicon, Inc., Somerville, N.J.) and 4-0 interrupted nylon suture.

Rabbits were killed at 2 or 8 weeks. Before the animals were killed, each animal was anesthetized and the dorsal back area was shaved. A small incision was made directly over the implant fill valve through skin, panniculus carnosus, and capsule. The incision traversing the capsule was sufficiently small (<3 mm) to not impede the accurate assessment of intracapsular pressure. The Stryker device was connected to the valve and opening intracapsular pressure was recorded (Fig. 1). Subsequent pressures at 2-cc increments were recorded after equilibration as the implants were overfilled. Representative capsule samples were

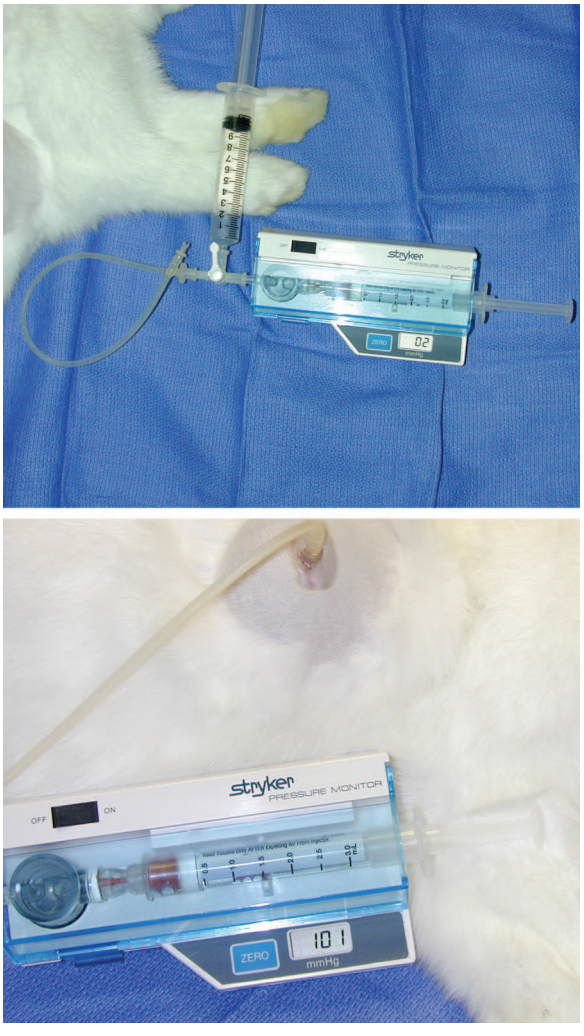

Fig. 1. (*Above*) Stryker pressure monitor setup next to the implanted mini implant. (*Below*) Stryker pressure monitor connected to the mini implant through a small capsular window.

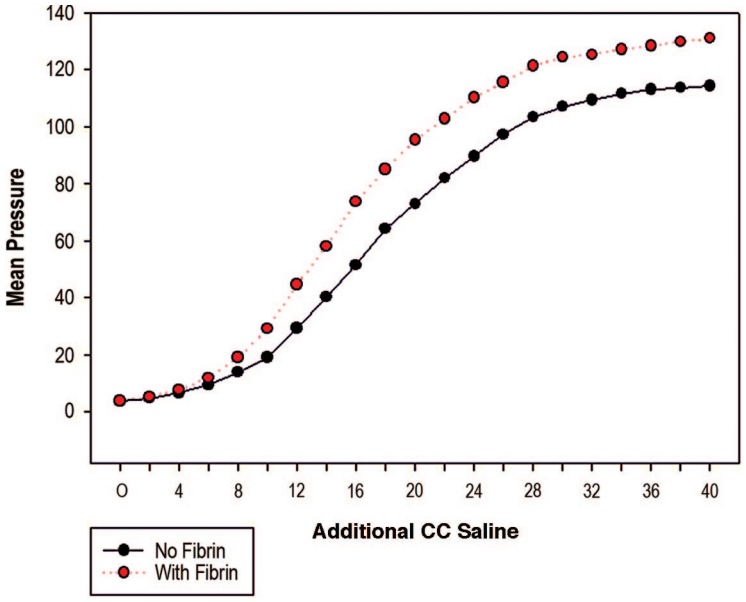

Fig. 2. The pressure-volume curve at 8 weeks; there was a significant increase in intracapsular pressure in the experimental group.

submitted in formalin for histologic evaluation for tissue architecture and capsular thickness.

Human breast capsular tissue samples from clinically normal breasts (implantation time, 6 months) and pathologically contracted capsule (Baker III/IV; implantation time, 5 to 6 months) were collected and processed using standard hematoxylin and eosin staining. The histologic sections were reviewed by a blinded pathologist and the morphologic characteristics of the human capsule samples were characterized.

Statistics comparing the intracapsular pressures were performed using the two-tailed t test demonstrating a significant difference between the experimental and control groups. Statistical significance is defined as $p < 0.05$.

RESULTS

The pressure-volume curve was generated at 2 and 8 weeks (Fig. 2). There was no significant difference between the experimental and control groups at 2 weeks; however, at 8 weeks there was a significant increase in intracapsular pressure in the experimental group. On gross examination of the capsules, the control group capsule appeared more transparent and had less vessel predominance on the capsular surface (Fig. 3, *above*). The experimental group (Fig. 3, *below*) had a more opacified capsule and in many cases appeared thicker. The average capsular thickness (histologically measured) was 0.6 mm in the rabbit control group, 1.0 mm in the rabbit experimental group and in human capsules, and 2.5 mm in human capsule contractures. There was a non–statistically significant increase in capsular thickness in the experimental group.

Histology

Hematoxylin and eosin sections of rabbit control capsules at 8 weeks, rabbit contractures at 8 weeks, human capsules, and human contractures were compared. Synovial-like reaction of fibrohistiocytic cells (synovial metaplasia) was most pronounced in the rabbit control capsule at 8 weeks, focal in the rabbit contracture at 8 weeks, and absent in the human contractures and control capsules (which is not unexpected, as synovial metaplasia is reported to be present in only 50 percent of cases).[12]

Inflammation (consisting of lymphocytes, histiocytes, and eosinophils) was moderate in the 8-week rabbit control capsule and mild in the 8-week rabbit contracture. The human capsule demonstrated minimal inflammation, whereas the human contracture showed mild inflammation. The degree of fibrosis was greater in the 8-week rabbit contracture and human contracture (Fig. 4) than in their counterparts (the 8-week rabbit control and human capsules, respectively).

DISCUSSION

Capsular contracture is the most common complication involving aesthetic and reconstructive breast surgery, with a reported incidence rang-

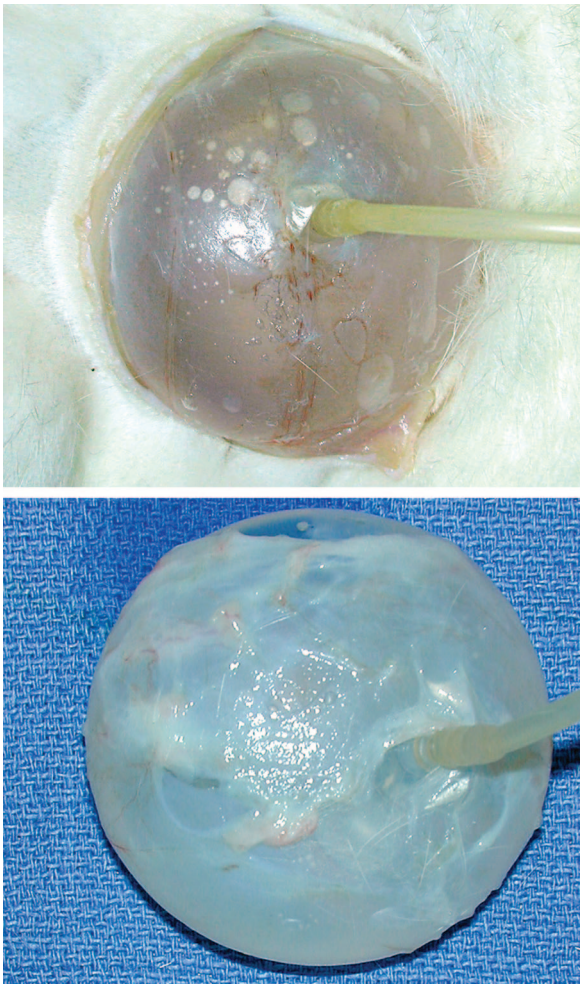

Fig. 3. (*Above*) Rabbit capsule at 8 weeks (control; the capsule is transparent and has less vessel predominance on the capsular surface). (*Below*) Rabbit capsule at 8 weeks (with fibrin glue); the capsule is more opacified and thicker.

ing from 0.6 to 50 percent.[13,14] An incidence of 8 to 15 percent[15–17] may be cited as a more scientific appraisal. Clinically, capsular contracture manifests on a continuum with varying degrees of severity, and is typically measured subjectively by means of the Baker classification. Furthermore, contracture may become clinically evident from weeks to years after implantation.

Capsular contracture is the formation of fibrous scar tissue investing a foreign body or surgically implanted device. Artificial joints or heart valves, central venous catheter ports, breast implants, and a multitude of additional surgical devices have been involved in the development of capsule formation and its adverse consequences. Capsule formation presumably plays a vital role in the host's response to a foreign body. Nevertheless, the results of this process may pose potential serious health risks or adverse aesthetic sequelae.

The true cause of capsular contracture remains elusive.[18] Two prevailing theories have emerged: the *infectious hypothesis* and the *hypertrophic scar hypothesis*. The infectious hypothesis, which has been championed by Burkhardt and supported by others,[19–23] implicates subclinical infection in the development of capsular contracture. *Staphylococcus epidermidis*, which is the most common organism isolated from nipple secretions, is the most common organism cultured from capsules excised during open capsulotomies. Furthermore, acceleration of capsule formation around silicone implants by addition of *Staphylococcus aureus* as an independent variable has been reported.[24]

The hypertrophic scar hypothesis attempts to implicate noninfectious stimuli, namely, hemato-

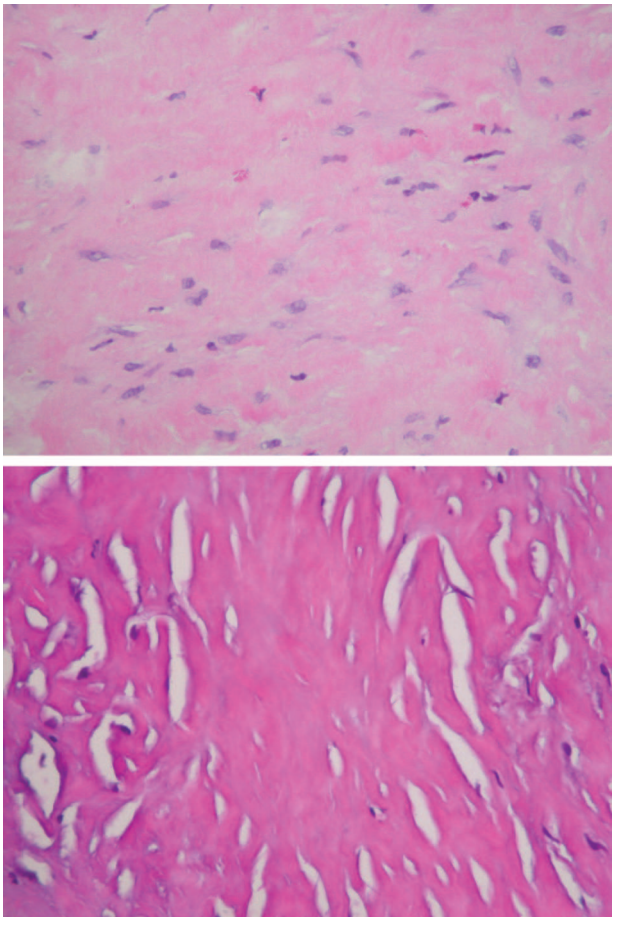

Fig. 4. (*Above*) Experimental group rabbit contracture at 8 weeks (original magnification, ×40) showing areas of more dense fibrous deposition in the mildly cellular mid zone; fibroblasts are widely separated by spindled fibroblasts. (*Below*) Human capsule contracture (original magnification, ×40) showing hypocellular fibrous mid zone; central area shows dense collagen without any fibroblasts; fibroblasts at periphery are widely separated by thick, dense bands of collagen fibers.

mas, granulomas, or hereditary factors, which confer a foreign body reaction and resultant formation of a hypertrophic scar around an implanted device. The underlying mechanism behind this process involves the activation of the myofibroblast cells within the capsule, which supposed contractile elements exert the force necessary to produce capsular contracture. Myofibroblasts contain the contractile elements actin and myosin and have been identified inconsistently within the capsules of implanted devices; however, they have proven difficult to culture and study in detail and, when found in the capsule, are found in exceedingly small quantities, are located sporadically throughout the capsule, and are not found to attach to each other. This scenario poses an inconsistent model for the development of contractile forces necessary to produce contracture.

The purpose of this study was to consider a novel pathologic animal model for capsular contracture. The fibrin glue inducing agent was discovered serendipitously in our laboratory; however, this places ample amounts of fibrinogen around the implant, and the critical role of fibrinogen in capsule formation has been scientifically established independent of our work.[25] This agent merely reliably produces conditions that are likely to result in a contracted capsule.

Many different animal models for contracture studies have been reported[6,8,10,19,24–28]; however, the majority consider the effect of a given therapy on *normal* capsule formation.[6,10,11,19,29,30] This minimizes and likely invalidates the significance/conclusions of many of these previous studies, as therapy needs to be directed at a pathologic capsule. Other reports have used bacteria to stimulate the formation of pathologic capsules; however, the reproducibility and control of this model have not been validated.[19] It is our opinion that the cause of contracture is multifactorial. In humans, there exist capsular contracture–inciting agents that, for known or unknown reasons, result in a contracture (i.e., hematoma, infection). The fibrin glue inducing agent is no different. This agent simply facilitates conditions that already are known to produce capsule formation in a predictable fashion.

Furthermore, the correlation between animal contracture and that of humans has not been substantiated. In fact, several studies using the rabbit model have found contradictory results from our clinical observation in humans.[7,8] Most of these studies have reported more pathologic capsules in rabbits using textured implants,[4,5] when it is generally accepted that textured implants produce less contracture in humans. The reason for this is largely unknown; however, the use of a nonpathologic animal model is likely a major issue.

The histologic findings demonstrate a similar increase in fibrosis in rabbit and human contracted capsule compared with respective controls. The differences in synovial metaplasia in the specimens constitute a histologic detail that carries no clinicopathologic significance; however, they were reported for the sake of completeness. The end result is that the histologic analysis of the rabbit contracture model is similar to human contracture.

We report for the first time, to the best of our knowledge, a breast capsular contracture animal model that mimics the histologic characteristics of human breast capsular tissue. The degree of inflammation and fibrosis over time in the rabbit contracture appears to correlate with those of the human contracture, suggesting that the rabbit capsule may be an optimal animal model for the changes seen in human contractures. Despite these findings, we acknowledge that the ultimate model for the study of capsular contracture is the human model, and all animal models, including this one, will need to ultimately reconcile this fact.

CONCLUSIONS

Our model does produce pathologic and nonpathologic capsules histologically similar to the human pathologic and nonpathologic capsule. Interestingly, our contracture-inducing agent (fibrin) has been implicated as a key player in the formation of capsule formation in prior studies.[28] Plastic surgeons have endured 40 years of darkness in their true understanding of capsular contracture. We hope this model may not only provide a platform for future investigation but allow us all to see the "light" and provide insight into the true cause of breast implant capsular contracture.

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A Rabbit Model for Capsular Contracture: Development and Clinical Implications

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Tucson, Ariz.

I have admired the work that Dr. Adams and his team have published previously, and compliment them once again on their carefully controlled methodology and their nicely presented results. Well-meaning colleagues often have constructive disagreements, however, and I do have some with Dr. Adams. Readers will have to decide.

An accurate, reliable, and reproducible animal model for capsular contracture is indeed needed. The authors do report an apparently reliable and reproducible method for producing measurable contracture around saline mini implants in rabbits. Whether the model is accurate is discussed below. The experiment included a control group, the measurement of contracture was objective and reproducible, and the histology of the capsules was consistent with capsules from around implants in humans. It is good work, and the authors should be congratulated.

I do not believe, however, that this is an accurate model of the contracture process that occurs around breast implants in humans. For this reason, I question the usefulness of this and other similar animal research models in advancing our understanding and (it is hoped) preventing human contracture. This is a fundamental dissent, and requires further explanation.

The authors note correctly that capsule formation is a normal physiologic response and that our focus should be on capsular *contracture* rather than on the capsule formation itself, a distinction that has sometimes been ignored in our literature and deserves emphasis. Although acknowledging infection as one probable cause, however, they believe the cause of contracture is "multifactorial," to include hematoma, granuloma, foreign body reaction, and hereditary factors, any one of which may theoretically stimulate an internal hypertrophic scar response that then becomes a contracted capsule. This assumption is central to the potential usefulness of their rabbit model: if contracture is just the final common pathway for expression of a whole market basket of presumed nonbacterial causes, using fibrin glue to produce contracture in rabbits and then developing treatments that modify this tissue response is a rational approach. If the presumed cause is limited to infection or bacterial contamination, however (and I confess some personal bias here), I do not believe that working with glue-induced contracture in rabbits will lead to remedies that are transferable to humans. If we examine published evidence, the burden of proof falls clearly on those who assume that the cause is multifactorial and therefore reasonably duplicated by this rabbit response to fibrin glue.

Evidence for a bacterial etiology for human contracture is abundant. Bacteria (mainly *Staphylococcus epidermidis*) have been cultured from 67[1] to 95 percent[2] of contractures, 97 percent of human breast milk samples,[3] 50 percent of intraoperative cultures of retromammary implant pockets,[4] and 50 percent of cultures of biopsy specimens from uninfected breasts.[5] Periprosthetic lactoceles, demonstrating a clear connection between the periprosthetic space and the ductal system of the breast, are well documented in our literature.[6,7] To this I would add a personal observation that contractures in my own practice often occur long after the initial surgery, following pregnancy and lactation. Bacterial contamination of mini inflatable implants with *S. epidermidis* causes contracture in rabbits that is reduced by intraluminal antibiotics.[8,9]

What is the evidence for other causes? An "inherent" or genetic tendency is clearly inconsistent with a preponderance of unilateral contracture (do the right and left breasts really have different genes or different tissue responses?). Although previous authors have implicated inadequate initial dissection,[10] foreign body reaction,[11] hematoma,[12] and the myofibroblast,[13] the relevance of these studies, using the advantage of today's knowledge, is quite thin.

If we are in fact dealing with a tissue response to a securely buried, contaminated foreign body, we must be especially cautious about attempts to modify that tissue response. Intraluminal steroids[14] were a notable disaster that few of us veterans would care to revisit. I do not pretend to

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have the answer, but I believe that if a rabbit model must be used for research, a more appropriate model is that reported by Shah et al.,[8,9] who used bacterial contamination to produce contracture. I do question whether such studies as the one at hand can lead to progress in the prevention of human contracture, which I believe is the result of contamination from a uniquely human environment of open, epithelium-lined, bacteria-filled breast ducts that simply cannot (as yet) be duplicated on the backs of rabbits.

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Publication II

Long-Term Follow-Up of Breast Capsule Contracture Rates in Cosmetic and Reconstructive Cases

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Background: Silicone gel breast implants are associated with long-term adverse events, including capsular contracture, with reported incidence rates as high as 50 percent. However, it is not clear how long the follow-up period should be and whether there is any association with estrogen or menopausal status. In addition, the placement of Baker grade II subjects in the majority of reports has been in data sets of controls instead of capsular contracture.

Methods: A retrospective medical study (1998 to 2004) was performed in women ($n = 157$) who received textured silicone breast implants for aesthetic or reconstructive procedures at the Hospital of S. João (Portugal). Medical data were collected that included the following: patient demographics, history, lifestyle factors, surgical procedures, and postoperative complications. Statistical analyses included Pearson chi-square testing, logistic regression modeling, and *chi*-squared *a*utomatic *i*nteraction *d*etection (*CHAID*) methods.

Results: The reconstructive cohort had a great incidence of capsular contracture compared with the cosmetic cohort. If one considered no capsular contracture versus capsular contracture, the follow-up period should be longer than 42 months. However, if considering no capsular contracture and grade II subjects versus grade III or IV subjects, a longer follow-up period of 64 months was determined. There was no association between capsular contracture and menopause/estrogen status.

Conclusions: Increased frequencies of capsular contracture were recorded in breast reconstruction that were not attributable to estrogen or menopausal status. On the basis of these results, the authors propose a follow-up period longer than 42 months and the inclusion of Baker grade II subjects. (*Plast. Reconstr. Surg.* 126: 769, 2010.)

Silicone gel breast implants for cosmetic augmentation and breast reconstruction have been implanted worldwide since 1962.[1] Multiple investigations have to date been alert for the potential adverse health effects of silicone breast implants.[2–12] Additional reports have focused on postoperative local complications and patient safety issues in women receiving silicone breast implants.[13–20]

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Capsular contracture is the most common and severe complication associated with silicone breast implants,[13–20] despite innovations in shell surface textures, implant shapes, inner gel composition, surgical implantation techniques, and pocket irrigation.[21–41] In cosmetic and reconstructive breast surgery reports, the incidence of capsular contracture ranged widely from 0 to 50 percent of implantations.[13–20,24,33,42–46]

The Baker classification system defines stages of breast capsule clinical presentation into distinct grades.[46] Grade II is the first stage of capsular contracture, and clinical interpretation of grade II may be highly dependent on individual surgeons'

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opinions. Although the clinical impact of grade II is relevant to the continuum of breast capsule formation, nevertheless, the majority of retrospective and prospective reports do not include grade II subjects as breast capsule cases.[47–50] The exclusion of grade II subjects in these reports may result in underreporting of capsular contracture rates.

In this study, we report the occurrence and severity of postoperative complications in a cohort of Portuguese women who received silicone textured breast implants between 1998 and 2004. Also, factors that might contribute to the development of capsular contracture rates (including grade II subjects) were considered along temporal trends with estrogens and menopausal status.

PATIENTS AND METHODS

Subjects and Data Collection

The study was approved by the Portuguese Institutional Review Board for Human Subjects. Existing medical records of women who had undergone breast implantation with customized textured silicone breast implants (McGhan Medical, Santa Barbara, Calif.) between 1998 and 2004 in the Hospital of S. João (Porto, Portugal) were examined. A total of 224 women were identified, with 104 women who had undergone cosmetic breast augmentation (cosmetic cohort) and 120 women who had undergone postmastectomy reconstruction of the breast (reconstructive cohort).

From medical records, the following data were collected: patient demographics, alcohol and medication use, medical history, surgical procedures, incision location, implant device placement,[51] and postoperative *acute* complications (hematoma, infection, or seroma). Postoperative *chronic* complication (capsular contracture, folds, wrinkles, breast pain, and change of tactile sense) data were not gathered from medical records. Self-reported complications related to satisfaction with implantation surgery were collected using a self-administered questionnaire. Women who answered the questionnaire were asked to attend a consultation to be further evaluated by the two trained plastic surgeons to decrease subjectivity of this evaluation. The degree of late capsular contracture was assigned by the plastic surgeons according to the Baker classification.[46]

Women from the initial cohort (157 of 224) completed the self-questionnaire and attended the consultation. The remaining 67 were then excluded ($n = 35$ women, cosmetic cohort; $n = 32$, reconstructive cohort) to remove any potential bias that might result from patients with incomplete data. Women were excluded because of the loss of contact as they moved out of Porto or because no current mailing address or phone numbers were available at the time of the study. The reconstructive cohort was composed of 88 patients with 115 breast implants and with 27 patients having received bilateral breast implants. The cosmetic cohort had 69 patients with 136 breast implants: 62 patients with 124 breast implants, two of whom had a tuberous breast deformity and unilateral aplasia; and seven patients with 12 breast implants, with one woman having Poland syndrome. All cosmetic patients younger than 18 years old ($n = 4$) had received implants following medical indication, namely, severe asymmetry, aplasia of breast tissue, or congenital malformation.

Statistical Analysis

Postoperative local complications were analyzed independently for the entire study cohort and individual clinical treatment cohorts and reported per woman and per implantation operation (SPSS, Inc., Chicago, Ill.). Possible associations among recorded data sets of patient characteristics, surgical procedures, and complications were evaluated using Pearson chi-square testing and logistic regression modeling.[52] Trend analysis was performed using the *ch*i-squared *a*utomatic *i*nteraction *d*etection (*CHAID*) method (SPSS),[53] using the likelihood ratio chi-square statistic as growing criteria, along with the Bonferroni 0.05 adjustment of probabilities, and setting the minimum size for parent and child nodes at 10 and 5, respectively. Relative risks and 95 percent confidence intervals were calculated for identified characteristics of interest to examine strength and precision of statistical associations.

CHAID has not been widely applied to trend analyses in plastic surgery investigations, but CHAID is one of the oldest tree-classification methods originally proposed by Biggs et al.[53] In brief, CHAID is an exploratory method to examine relationships between a dependent variable (e.g., capsular contracture) and a series of predictor variables (e.g., type of cohort, age at surgery, follow-up period) and their interactions. The CHAID algorithm created adjustment cells by splitting a data set progressively by means of a classification tree structure where the most important predictor variables were chosen to maximize a chi-square criterion. The most significant predictors defined the first split or the first branching of the tree. Progressive splits from the initial variables resulted in smaller and smaller branches. The result at the end of the tree-building process is a series of groups that were different from one

another on the dependent variable. Classification trees lend themselves to be displayed graphically and are far easier to interpret than numerical interpretation from tables.

RESULTS

Baseline descriptive data for the cosmetic and reconstructive patient cohorts are listed in Tables 1 and 2, respectively. Cosmetic patients were younger at the time of surgery compared with reconstructive patients (31.0 versus 48.6 years). The average follow-up period was 35.4 months in the cosmetic group compared with 48.5 months in the reconstructive group. Contraceptive use was reported by 56.5 percent of cosmetic patients, whereas only 3.4 percent of reconstructive patients reported contraceptive use or hormone replacement therapy. Cosmetic patients also reported decreased use of psychotropic drugs (e.g., antidepressants, antianxiety, and hypnotics drugs) compared with reconstructive patients (23.2 percent versus 52.3 percent, respectively). One woman from each cohort ($n = 2$) had a connective tissue disease (rheumatoid arthritis).

Among women in the cosmetic cohort, the majority of silicone gel implants were placed subglandularly (84.1 percent), and the surgical approach was through the inframammary fold (59.4 percent). The majority of reconstructive patients had not received radiotherapy (85.2 percent) or tamoxifen (67.1 percent); chemotherapy was administered in 51.1 percent; the reconstructed breast was the left side in 52.3 percent of the patients, and 68.2 percent submitted to breast size symmetrization.

Table 1. Baseline Characteristics for the Cosmetic Cohort

Variable	No.	%
No. of women with implants (no. of breast implants)	69 (136)	
Age at surgery, years		
Mean	31.0	
Range	15–51	
Follow-up period, months		
Mean	35.4	
Range	12–80	
Implant placement		
Subpectoral	9	13.0
Subglandular	58	84.1
Dual-plane Tebbetts	2	2.9
Incision placement		
Inferior periareolar	7	10.1
Axillary	21	30.4
Inframammary	41	59.4
Contraceptive drugs		
No	30	43.5
Yes	39	56.5

Table 2. Baseline Characteristics for the Reconstructive Cohort

Variable	No.	%
No. of women with implants (no. of breast implants)	88 (115)	
Age at surgery, years		
Mean	48.6	
Range	25–73	
Follow-up period, months		
Mean	48.5	
Range	12–96	
Symmetrizing breast		
No	28	31.8
Breast implant (with or without mastopexy)	22	25
Breast reduction	33	37.5
Bilateral breast reconstruction	5	5.7
Hormone therapy*		
No	85	96.6
Yes	3	3.4

*Including contraceptive drugs or hormone replacement therapy.

Acute Clinical Adverse Events

Acute complications were recorded in 20 reconstructive patients (8 percent) during the follow-up period, with complications recorded as seroma (8.0 percent), hematoma (4.5 percent), and perforation of the skin (3.2 percent) (data not shown).

Chronic Clinical Adverse Events

Chronic complication events were recorded and are listed in Table 3. Overall, 81 percent ($n = 127$) of all women had one or more postoperative chronic events, ranging from less severe effects (e.g., change in tactile sense) to complications requiring additional surgical interventions, such as severe capsular contracture. The distribution of chronic complication frequency among women was as follows: 23 percent of the patients had one complication; 31 percent of the patients had two complications; and 27 percent of the patients had three or more complications. From a temporal view of the clinical onset of chronic complications, 3 percent of the patients were diagnosed from 0 to 12 months postoperatively; 31 percent of the patients were diagnosed from 13 to 24 months; and 72 percent of the patients were diagnosed from 24 to 60 months.

The most frequent chronic adverse effect was palpable implant folds (47.8 percent of all cases), occurring in 42.0 percent of women from the cosmetic cohort and in 69.3 percent from the reconstructive cohort. Change of tactile sense also had a high incidence (41.0 percent of all cases), with 89.8 percent in the reconstructive cohort reporting changes. Capsular contracture was the second most common chronic complication, occurring in

Table 3. Chronic Complications for Both Cohorts

Chronic Complications	Cosmetic Cohort (n = 69) No.	%	Reconstructive Cohort (n = 88) No.	%
Capsular contracture				
No	57	82.6	46	52.3
Unilateral	9	13.0	41	46.5
Bilateral	3	4.4	1	1.2
Palpable implant folds				
No	40	58.0	27	30.7
Unilateral	12	17.4	48	54.5
Bilateral	17	24.6	13	14.8
Visible skin wrinkles				
No	59	85.5	72	81.8
Unilateral	7	10.1	14	15.9
Bilateral	3	4.4	2	2.3
Prolonged pain in the breast				
No	59	85.5	78	88.7
Unilateral	4	5.8	9	10.2
Bilateral	6	8.7	1	1.1
Change of tactile sense				
No	61	88.4	9	10.2
Unilateral	4	5.8	67	76.1
Bilateral	4	5.8	12	13.7

*All reported cases were unilateral.

34.4 percent of all women and in 23.1 percent of all implantations. Capsular contracture incidence rates were significantly different between the cosmetic cohort (17.4 percent of women or 11.0 percent of implantations) and the reconstructive cohort (47.7 percent of women or 37.4 percent of implantations; $p < 0.05$). Other chronic complications occurred less frequently (>10 percent of all patients).

Furthermore, the occurrence of postoperative complications had a marked influence on satisfaction index; for example, women without contracture were 1.6 times more likely to consider the outcome either good or very good compared with women with capsular contracture (relative risk, 1.6; 95 percent confidence interval, 1.2 to 2.2).

Capsular Contracture Characteristics

Baker capsular contracture grades for the cosmetic and reconstructive cohorts are listed in Table 4. As a percentage of patients, the reconstructive cohort had 7.4- and 3.2-fold greater incidences of Baker grade III and IV capsular contractures compared with the cosmetic cohort. When examined as a function of clinical time when Baker grades were assigned, 44 women (76 percent) of the 58 total patients were diagnosed after 2 years after surgery. In detail, five women (7 percent) from the cosmetic cohort and 28 women (32 percent) from the reconstructive cohort developed capsular contracture grade III/IV after the initial 2 years after implantation. Overall, the rate of grade III/IV capsular contracture per woman during the 8-year period of follow-up was 10.1 percent for patients undergoing aesthetic surgery and 37.5 percent for breast reconstruction patients.

Table 4. Capsular Contracture per Implant for Both Cohorts

Grade*	Cosmetic Cohort (%)	Reconstructive Cohort (%)
I	121 (89.0)	72 (62.6)
II	5 (3.7)	9 (7.8)
III	2 (1.4)	12 (10.4)
IV	8 (5.9)	22 (19.1)
Total	136 (100)	115 (100)

*According to the Baker classification.

The occurrence of capsular contracture was associated with the duration of follow-up and age at the time of surgery (Table 5). Women with a follow-up period longer than 42 months (relative risk, 1.8; 95 percent confidence interval, 1.3 to 2.4) or older women (relative risk, 3.6; 95 percent confidence interval, 1.6 to 7.9 for age 54 years or older versus younger than 54 years) had increased incidences of capsular contracture ($p < 0.001$ for both comparisons). Moreover, increased capsular contracture occurred in the reconstruction group of patients compared with the cosmetic cohort. (relative risk, 1.7; 95 percent confidence interval, 1.4 to 2.3; $p < 0.001$). No associations between capsular contracture cases and surgical procedures or other personal characteristics were observed.

Using the CHAID decision tree (Fig. 1), the type of cohort was identified as the determining

Table 5. Identified Variables Related to Capsular Contracture for the Entire Cohort (n = 157)

Variable	Capsular Contracture (% of Women) No	Yes	p
Follow-up period			
≤42 months	40.1	10.8	<0.001
>42 months	25.5	23.6	
Age at surgery			
≤54 years	60.5	24.8	<0.001
>54 years	5.1	9.6	
Hormone therapy*			
No	21.7	29.3	0.014
Yes	43.9	5.1	
Type of cohort			
Reconstructive	29.3	26.8	<0.001
Cosmetic	36.3	7.6	

*Including contraceptive drugs or hormone replacement therapy.

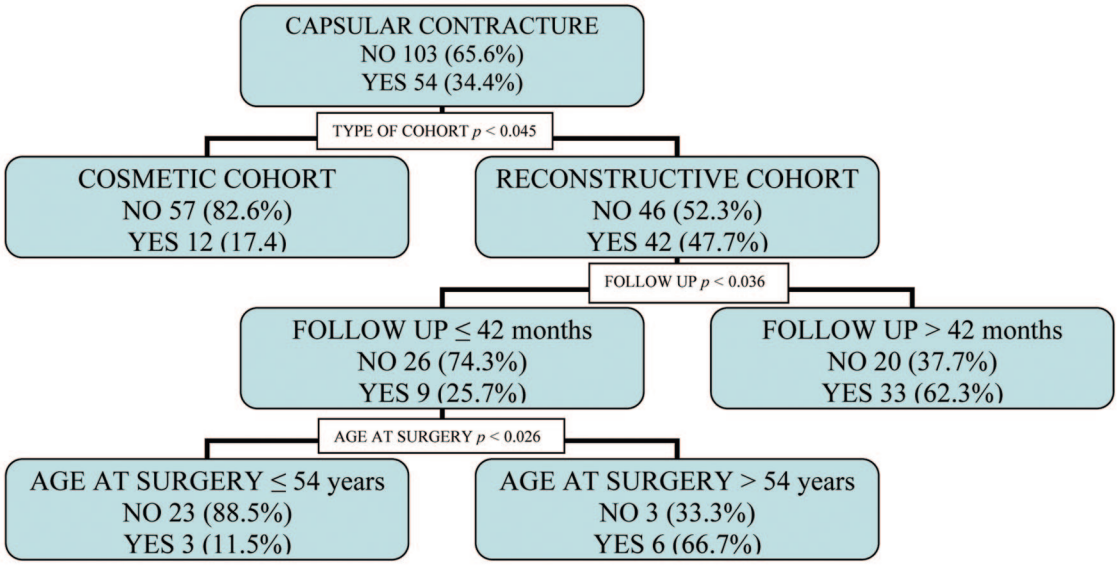

Fig. 1. Prediction tree of capsular contracture by chi-square automatic interaction detection algorithm. The first-level split produced two initial branches: cosmetic and reconstructive. The type of cohort was identified as the determining factor for developing capsular contracture, and the reconstructive group is predictive for positive capsular contracture. The next splits indicated the best predictor variables for the reconstructive group, as the follow-up period followed by the age at surgery. Within that group, a follow-up period of 42 months or less was the best predictor for no capsular contracture and a follow-up of more than 42 months was predictive for positive capsular contracture. For women with a follow-up of 42 months or less, capsular contracture was reported among 67.7 percent of women older than 54 years old compared with younger women (11.5 percent).

factor for developing capsular contracture. The first-level split produced two initial branches: cosmetic (no capsular contracture; 82.6 percent) and reconstructive (positive capsular contracture; 47.7 percent). The next splits indicated best predictor variables for the cohort reconstructive group, as the follow-up period followed by age at surgery. Within that group, a follow-up period of 42 months or less was the best predictor for no capsular contracture (unadjusted, 74.3 percent) and a follow-up of more than 42 months was predictive for positive capsular contracture (unadjusted, 62.3 percent). For women with a follow-up of 42 months or less, capsular contracture was reported among 67.7 percent of women older than 54 years old compared with younger women (11.5 percent). The overall risk estimate according to the classification tree was 0.240 (standard error of risk estimate, 0.034), indicating that 75.8 percent of the cases will be classified correctly by using the decision algorithm based on the current tree. The CHAID algorithm resulted in larger predictive values for occurrence of capsular contracture (72.2 percent) than logistic regression (57.4 percent).

A second CHAID decision tree analysis was performed with grade II subjects placed in the no capsular contracture group—similar to other reports—versus grade III and IV subjects. The first-level split produced two initial branches: cosmetic (no capsular contracture or grade II; 89.9 percent) and reconstructive (capsular contracture grade III or IV; 37.5 percent). The next split indicated the best predictor variable for the reconstructive group, as the follow-up period. Within that group, a follow-up period of 64 months or less was the best predictor for no capsular contracture or grade II (unadjusted, 73.4 percent), whereas a follow-up of more than 64 months was predictive for capsular contracture grade III or IV (unadjusted, 66.7 percent). The overall risk estimate according to the classification tree was 0.255 (standard error of risk estimate, 0.035), indicating that 79.6 percent of the cases will be classified correctly by using the decision algorithm based on the current tree.

Exogenous hormone use was reported in 56.5 percent of cosmetic patients ($n = 39$), with one subject in menopause that used hormone therapy replacement; of the remaining 68 women, 38 used contraceptives (Fig. 2). Only 3.4 percent of reconstructive patients ($n = 3$) used hormone therapy. Seventy-three patients were in menopause, with two subjects who used hormone replacement ther-

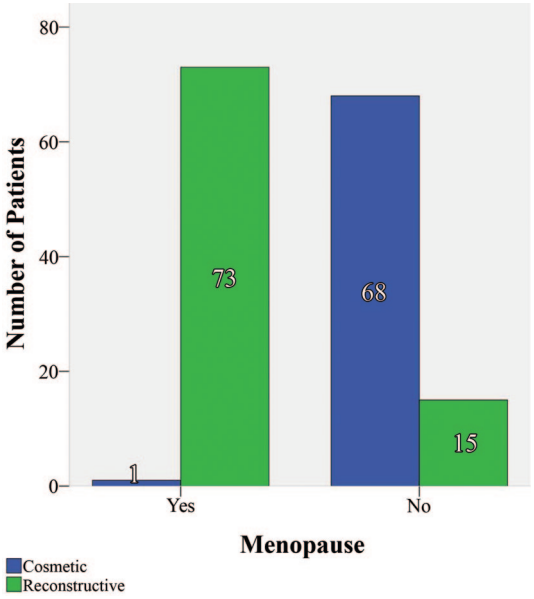

Fig. 2. Patients with or without menopause according to type of cohort.

apy. Fifteen women were premenopausal, with one who used contraceptives.

Subjects who were premenopausal or postmenopausal women using hormone therapy replacement were grouped and analyzed as "estrogen protected" (Fig. 3). To clarify the relationships between menopause or women protected by estrogen with capsular contracture rates according to type of cohort, two cross-tabulations were performed (Tables 6 and 7).

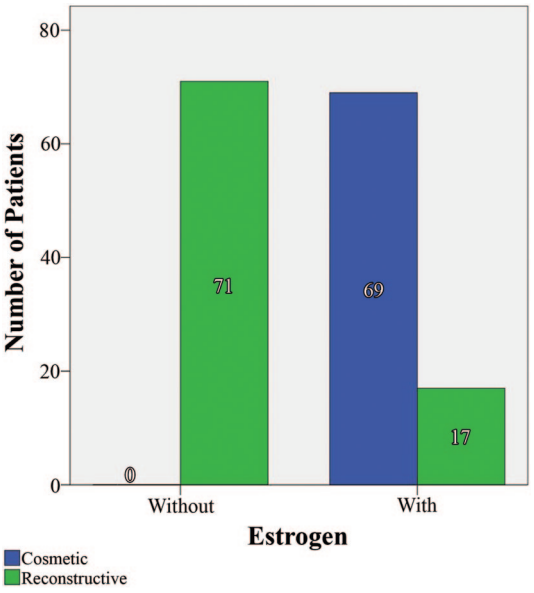

Fig. 3. Patients protected or not by estrogen according to type of cohort.

Table 6. Cross-Tabulation between Capsular Contracture and Menopause According to Type of Cohort

	Capsular Contracture		
Menopause	**Yes**	**No**	**Total**
Cosmetic cohort			
Yes	0	1	1
No	12	56	68
Total	12	57	69
Reconstructive cohort			
Yes	36	37	73
No	6	9	15
Total	42	46	88

Table 7. Cross-Tabulation between Capsular Contracture and Being Protected or Not by Estrogen According to Type of Cohort

	Capsular Contracture		
Protected by Estrogen*	**Yes**	**No**	**Total**
Cosmetic cohort			
Yes	12	57	69
Total	12	57	69
Reconstructive cohort			
Yes	35	36	71
No	7	10	17
Total	42	46	88

*Including all women before menopause or in menopause with hormone replacement therapy.

No associations between capsular contracture and menopause or estrogen status were observed.

DISCUSSION

Despite significant surgical efforts and precautions, capsular contracture continues to occur,[23,29,31,54–56] and the true cause of capsular contracture remains elusive.[21,23,24,39–42,55–72] In our report, the occurrence of local complications and the frequency, severity, and long-term sequelae were in the reported range as described in other studies.[13–20] Table 8 [13–15,19,20,47–50,73–79] demonstrates that reported capsular contracture rates vary widely because of authors reporting various Baker classification rates and follow-up time periods. These data showed that the incidence of complications was elevated in reconstruction patients compared with cosmetic augmentation patients.[14,80] No acute complications occurred in the aesthetic cohort, and all chronic complications were less prevalent in our study group. In our study, women with breast implants for cosmetic reasons had a lower body mass index than women who had undergone

Table 8. Average Follow-Up versus Capsular Contracture

Study	Type of Study	No. of Patients	Average Follow-Up	Capsular Contracture
Spear et al., 2003[74]	Prospective	85 cosmetic revisions	11.5 mo	2% Baker grade II; no Baker grade III–IV
Adams et al., 2006[49]	Prospective	235 (172 cosmetic primary augmentation; 63 reconstructive)	14 mo	Baker grade III–IV: 1.8% cosmetic primary augmentation, 9.5% reconstructive
Henriksen et al., 2005[80]	Retrospective	2277	19.5 mo	4.3% (Baker grade II–IV)
Brown et al., 2005[19]	Retrospective	150 (118 cosmetic; 32 reconstructive)	21 mo	Cosmetic, 2 cases; reconstructive, 3 cases; just Baker grade II; no cases of Baker grade III–IV
Fruhstorfer et al., 2004[13]	Prospective	35	23 mo	0%
Henriksen et al., 2003[14]	Prospective	1090	2 yr	4.1% (Baker grade II–IV)
Cunningham et al., 2007[47]	Prospective	955 (572 primary augmentation; 123 revision-augmentation; 191 reconstruction; 69 revision-reconstruction)	2 yr	Baker grade III–IV: 0.8% primary augmentation, 5.4 revision-augmentation, 2.2% primary reconstruction, 6% revision-reconstruction
Camirand et al., 1999[75]	Prospective	830	2.39 yr	0%
Seify et al., 2005[76]	Retrospective	44	34 mo	20% (Baker grade II–IV)
Cunningham et al., 2007[48]	Prospective	1007 (551 primary augmentation; 146 revision-augmentation; 251 reconstruction; 59 revision-reconstruction)	3 yr	Baker grade III–IV: 8.1% primary augmentation, 18.9 revision-augmentation, 8.3% primary reconstruction, 16.3% revision-reconstruction
Bengtson et al., 2007[77]	Prospective	941 (492 cosmetic primary augmentation; 225 reconstructive; 224 revisions)	3 yr	Baker grade III–IV: 5.9%
Spear et al., 2007[50]	Prospective	940 (455 cosmetic primary augmentation; 98 reconstructive; 162 revisions)	6 yr	Baker grade III–IV: 14.8% primary augmentation, 20.5% revision-augmentation, 15.9% primary reconstruction
Kjøller et al., 2001[73]	Retrospective	754	7 yr	11.4% of implantations
Kulmala et al., 2004[20]	Retrospective	685	10.9 yr	17.7% (15.4% of implantations) Baker grade II–IV
Hölmich et al., 2007[78]	Retrospective	190	19 yr	62%
Handel et al., 2006[79]	Retrospective	1529 (825 cosmetic; 264 reconstructive)	23.3 yr	Baker grade III–IV per 1000 patient-months: 1.99 cosmetic, 5.37 reconstructive, 4.36 revision

breast reconstruction, similar to previous studies[10] that compared breast augmentation with breast reduction and the general population.

In this study, 16 percent of capsular contractures (Baker grade III to IV) of the breast was diagnosed after a 1.6-year period after initial breast implantation. In the cosmetic study group, no significant associations were formed between surgical route or implant placement and any postoperative complication. Like Henriksen et al.,[81] no significant associations were observed between body index mass, smoking habits, alcohol consumption, hormone therapy, and capsular contracture in our study groups.

Capsular contracture may be apparent within the first year after implantation.[14,15,33,81] However, in our study, approximately 76 percent of cases of capsular contracture (Baker grade II to IV) ap-

peared just after 2 years; 10.1 percent and 37.5 percent of severe capsular contracture (Baker grade III to IV) occurred in the aesthetic and reconstructive cohorts, respectively, during the 8-year period of follow-up. Breiting et al.[10] reported an 18 percent rate of severe breast pain, indicative of severe capsular contracture, and in a previous study involving a subgroup of this population, they had diagnosed a 45 percent rate of capsular contracture (Baker grade II to IV) of the breast after a 5-year period after breast implantation.[82] Capsular contracture may also be symptomatic several years after surgery.[10,33,81,83]

Using the CHAID decision tree, the determining factor for capsular contracture was the type of cohort. The next splits indicated the best predictor variables for the cohort reconstructive level being the follow-up period; if one considered no capsular contracture versus capsular contracture, the follow-up period should be longer than 3 years 6 months. However, if considering no capsular contracture including grade II subjects versus grade III or IV subjects, a longer follow-up period of 5 years 4 months was determined. It is interesting that both CHAID tree decision analyses had the same qualitative splits but with longer follow-up periods in grade III or IV subjects. This is expected, as breast capsule formation is thought to develop from grade II to grade III, and from grade III to grade IV. These results underscore the importance of considering grade II as an important clinical observation that should be included in the capsular contracture analyses. Thus, we believe that a long follow-up period from grade II and reconstructive patients should be considered when studying local complications among women receiving breast implants and other female age-related factors such as menopause.

The protective role of estrogens in the progression of liver fibrosis[84,85] and the fact that estrogen deprivation was being associated with declining dermal collagen content and impaired wound healing is well known[86]; nevertheless, there are no reports concerned with menopause or estrogens versus capsular contracture. The main limitation of this study is the relatively small sample size and thus limited statistical power for observing relationships with rare outcomes, especially in the cosmetic cohort.

The authors for the first time report no association between capsular contracture and menopause or estrogen status. Therefore, the pathophysiology of capsule formation and subsequent contracture developing metabolic pathways are not estrogen derived. Our data suggest that grade II subjects should be included in a capsule contracture analyses and a follow-up period longer than 42 months should be considered. Our hope is that the breast contracture "riddle" will be solved in our lifetime so that our patients will not have to confront recurrent and intractable capsular contractures.[87]

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Publication III

Effects of Fibrin, Thrombin, and Blood on Breast Capsule Formation in a Preclinical Model

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Effects of Fibrin, Thrombin, and Blood on Breast Capsule Formation in a Preclinical Model

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Abstract

Background: The root cause of capsular contracture (CC) associated with breast implants is unknown. Recent evidence points to the possible role of fibrin and bacteria in CC formation.

Objectives: The authors sought to determine whether fibrin, thrombin, and blood modulated the histological and microbiological outcomes of breast implant capsule formation in a rabbit model.

Methods: The authors carried out a case-control study to assess the influence of fibrin, thrombin, and blood on capsule wound healing in a rabbit model. Eighteen New Zealand white rabbits received four tissue expanders. One expander acted as a control, whereas the other expander pockets received one of the following: fibrin glue, rabbit blood, or thrombin sealant. Intracapsular pressure/volume curves were compared among the groups, and histological and microbiological evaluations were performed (capsules, tissue expanders, rabbit skin, and air). The rabbits were euthanized at two or four weeks.

Results: At four weeks, the fibrin and thrombin expanders demonstrated significantly decreased intracapsular pressure compared to the control group. In the control and fibrin groups, mixed inflammation correlated with decreased intracapsular pressure, whereas mononuclear inflammation correlated with increased intracapsular pressure. The predominant isolate in the capsules, tissue expanders, and rabbit skin was coagulase-negative staphylococci. For fibrin and thrombin, both cultures that showed an organism other than staphylococci and cultures that were negative were associated with decreased intracapsular pressure, whereas cultures positive for staphylococci were associated with increased intracapsular pressure.

Conclusions: Fibrin application during breast implantation may reduce rates of CC, but the presence of staphylococci is associated with increased capsule pressure even in the presence of fibrin, so care should be taken to avoid bacterial contamination.

Keywords

capsule, tissue expander, fibrin, thrombin, blood, coagulase-negative staphylococci

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Fibrosis is a major global health problem, but its cause, pathogenesis, and diagnosis are not completely understood. Fibrosis may occur as a consequence of multiple pathologic conditions, including keloids, Dupuytren contracture, postoperative adhesions, burns, postinfection liver fibrosis, silica dust, asbestos, antibiotic bleomycin, scleroderma, cardiac pacemakers, polypropylene meshes, and—of special interest to aesthetic surgeons—breast implant capsular contracture (CC).[1-3]

The cause of CC remains largely undetermined, with clinically-reported incidences ranging from 8% to 45%.[4-9] Prior investigations of CC have focused on microorganisms found in the capsule or outer implant surface,[10-22] on inflammatory responses,[1,23,24] and on histological characteristics of the capsule.[25-32] Two reports found correlations between CC and hematoma.[32,33]

Histologically, human CC tissue comprises an inner layer of fibrocytes and histiocytes, surrounded by a thicker layer

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of collagen bundles arranged in a parallel array.[34,35] The outer layer is more vascular and contains loose connective tissue. From a clinical perspective, authors seems to agree that the degree of capsule thickness is proportionate to the severity of the CC, but this has never been definitively proven and some reports found no correlations among microbial contamination, thickness, and CC.[36] However, we do know that transforming growth factor beta 1 (TGF-β1), connective tissue growth factor, osteopontin, interleukin-4 (IL-4), IL-6, IL-10, IL-13, IL-21, basic fibroblast growth factor, epidermal growth factor, insulin-like growth factor 1, platelet-derived growth factor, oncostatin M, and endothelin 1 (see Sticherling[37]) all promote fibrosis.[1]

Numerous reports have associated fibrin glue with improved parameters of wound healing[38-41] and reduced quantity and consistency of adhesions, even in the case of polypropylene meshes.[2,3] To the best of our knowledge, only one study focused on the impact of autologous fibrin glue on capsule formation as a contracture-inducing agent, and no reports exist for commercially-available fibrin products.[26] In preclinical reports, several molecules were found to reduce CC[27,42,43]; nevertheless, these compounds are not currently available in clinical practice. However, the fibrin-containing commercial products are widely used clinically and are an attractive adjunct for patients receiving breast implants.

The purpose of this study was to perform a comprehensive evaluation (based on a rabbit model[26]) of the relationships among intracapsular pressure, histological characteristics, and infection surrounding the tissue expander in the capsule, in the rabbit skin, and in the operating room air. To clarify whether hematoma is associated with CC, the study was conducted with tissue expanders surrounded by rabbit blood to simulate a hematoma, as well as with tissue expanders in the presence of thrombin (FloSeal, Baxter US, Deerfield, Illinois), an absorbable hemostatic agent that contains no fibrinogen and requires contact with blood for the clot to be activated. To study the implications of wound healing in development of CC, the implant pocket was instilled with fibrin (Tissucol/Tisseel, Baxter US), a hemostatic agent with adhesive properties.

METHODS

Eighteen New Zealand white female rabbits (3-4 kg) were implanted with textured saline tissue expanders (20 mL, Allergan, Santa Barbara, California) with intact connecting tubes and ports, in accordance with an approved institutional animal care protocol. Before surgery, the animals were washed with Betadine surgical scrub (Purdue Pharma LP, Stamford, Connecticut), which contains 7.5% povidone-iodine, and their skin disinfected with Betadine solution, which contains 10% povidone-iodine. The surgical procedure was performed in a veterinary operating room with aseptic techniques. Penicillin G (40,000 U/kg) was immediately administered intramuscularly to the subjects was intraoperatively. Talc-free gloves were worn at all times during the procedure. Pockets were atraumatically dissected under direct vision in the *subpanniculus carnosus* along the back region of each rabbit. Attention was paid to hemostasis and blunt instrumentation was avoided; there was no obvious bleeding. A new pair of talc-free gloves was placed on the surgeon's hands before tissue expander insertion, with minimal skin contact.

One control and three experimental tissue expanders were placed in each rabbit. The experimental expanders received one of the following: 1 mL of fibrin glue spray (Tisseel/Tissucol), 2 mL of rabbit blood to simulate a hematoma, or 5 mL of thrombin sealant (FloSeal) in the expander pocket. A pressure-measuring device (Stryker Instruments, Kalamazoo, Michigan) was connected to each tissue expander port. Intraexpander pressure was recorded immediately before filling and in 5-mL increments until the tissue expanders were overfilled. Each tissue expander was filled to 20 mL.

The rabbits were euthanized at two or four weeks. Beforehand, each animal was anesthetized and the dorsal back area was shaved. The pressure monitor was connected again to the tissue expander port, and intracapsular pressures were recorded in 5-mL increments as the expander was drained, before any incision in the capsule. Capsule samples were submitted for histological and microbiological evaluation.

Microbiological Assessments

Air. Operating room air samples (n = 36) were collected during all procedures with the MAS 100-Eco air sampler (EMD Chemicals, Inc., Gibbstown, New Jersey) at a flow rate of 100 L per minute. Identification of bacterial and fungal isolates followed standard microbiological procedures. Gram-positive cocci were characterized by biochemical methods. Catalase-positive and coagulase-positive colonies were identified as *Staphylococcus aureus*; catalase-positive and coagulase-negative colonies were identified as coagulase-negative staphylococci. Gram-negative bacilli were characterized with Vitek 2 software (VT2-R04.02, bioMérieux, Inc., Durham, North Carolina). Fungi were characterized following their macroscopic appearance and microscopic morphology.

Rabbit skin. A total of 54 contact plates were pressed to shaved dorsal skin surfaces (18 brain-heart agar, 18 mannitol salt agar, and 18 Sabouraud agar). The brain-heart and mannitol salt contact plates were incubated for three days at 28°C; the Sabouraud contact plates were incubated for seven days at 28°C. Bacterial and fungal colonies were counted and reported as colony-forming units per square centimeter. For the identification of the bacteria and fungi grown, the same methods listed above were applied.

Capsules and tissue expanders. Excised implants and representative capsule samples were incubated at 37°C for three days in brain-heart agar plates and examined daily; changes in turbidity of the broth media were considered positive and were subcultured in solid agar media.

Characterization of microbial isolates followed the above-described procedures.

Histological Assessment

Capsule specimens were fixed with 10% buffered formalin and embedded in paraffin. Sections were stained with hematoxylin and eosin and histologically evaluated for tissue inflammation and capsular thickness. The type and intensity of the inflammatory infiltrate were analyzed. Inflammation was grouped into three categories: mononuclear/chronic (lymphocytes, plasmocytes, and histiocytes), mixed/subacute (mononuclear cells and eosinophils), and polymorph/acute (eosinophils and heterophils). Inflammatory infiltrate intensity was categorized according to the following criteria: absent (−), mild (+), moderate (++), and severe (+++).[25]

Samples were stained with Masson trichrome[44] to characterize the collagen (loose, slightly dense [≤ 25%] or more dense [> 25%]), the organization of collagen fibers (parallel or haphazard), the angiogenesis (absent, mild, moderate, or high), and the fibroblast density (mild, moderate, or high). Histological sections were reviewed and graded by a pathologist blinded to the protocol.

Statistical Analysis

Data were grouped according to the type of product applied to the tissue expander: none (control), blood (blood), Tissucol/Tisseel (fibrin), and FloSeal (thrombin). Each was analyzed for the rabbits euthanized at two and four weeks after surgery, as well as for all 18 rabbits. One-way analysis of variance was applied to compare the intraexpander pressure before insertion. A two-tailed paired *t*-test and the nonparametric alternative Wilcoxon signed rank test were applied to determine whether continuous variables (intracapsular pressure and histologically measured thickness) were significantly different between the control and experimental groups. Categorical variables were evaluated by chi-square statistics and by phi, Cramer V, and contingency coefficients. Statistical significance was presumed at $p \leq .05$. Major trends within each group were further examined by the chi-square automatic interaction detection (CHAID) method,[45] based on the likelihood ratio chi-square statistic as growing criteria, along with a Bonferroni 0.05 adjustment of probabilities. All analyses were carried out with SPSS version 16 (SPSS, Inc., Chicago, Illinois).

RESULTS

Intracapsular Pressure

No significant differences were observed in the pressure-volume curves between the control and experimental groups at baseline (tissue expander introduction) or at two weeks. At four weeks, rupture was observed during pressure measurement with six capsules in the control group, five capsules in the blood group, and one capsule in thrombin group; no capsule ruptures in the fibrin group were noted. To avoid reducing the sample size, the ruptured capsules were not excluded from statistical analyses, but it is important to note that the pressure levels measured before capsule rupture were maintained after additional saline was added. At four weeks, significantly decreased intracapsular pressures were registered in the fibrin group ($p \leq .0006$) and thrombin group ($p \leq .003$) (Figure 1).

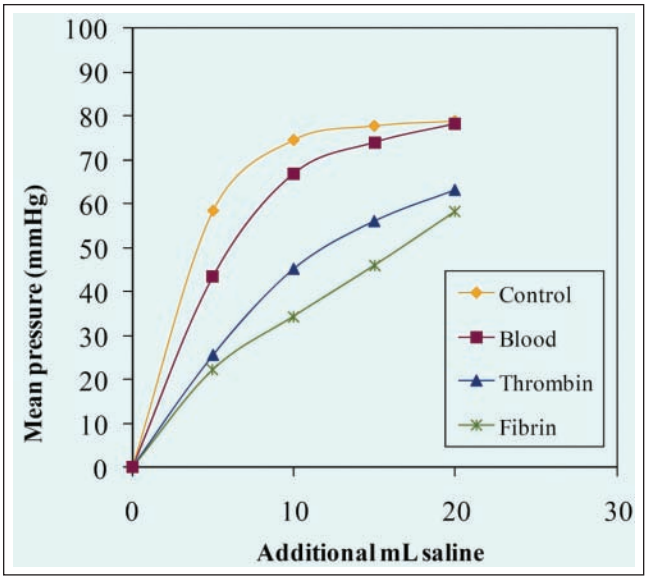

Figure 1. Pressure-volume curves at four weeks. There was a significant difference in intracapsular pressure in the thrombin (FloSeal) and fibrin (Tissucol/Tisseel) experimental groups.

Histology

The average capsular thicknesses were similar among all groups at two and four weeks (Table 1). At two weeks, mixed types of inflammatory cells were predominant in the capsules, and no statistically significant differences were found among the groups. At four weeks, mononuclear cells were predominant in the control, blood, and thrombin groups; in the fibrin group, mixed cells were predominant. However, these differences were not statistically significant. At both two and four weeks, trends in the intensity of inflammation showed no significant difference (Table 2).

Fibrosis developed in all capsules at two and four weeks. No significant differences were observed regarding the organization of collagen fibers between the control and experimental groups. At two weeks, more dense collagen (> 25%) was found in the control group, whereas loose and slightly dense collagen (≤ 25%) was found in the blood group ($p = .023$). At both two and four weeks,

Table 1. Average Capsular Thickness of Control Versus Experimental Groups

Group	Two Weeks, mm	Four Weeks, mm
Control	0.83 ± 0.085	0.64 ± 0.078
Blood	1.02 ± 0.207	0.78 ± 0.572
Fibrin: Tissucol/Tisseel	0.89 ± 0.082	0.72 ± 0.083
Thrombin: FloSeal	0.90 ± 0.064	0.71 ± 0.105

increased angiogenesis was observed in the control group (moderate/ high) versus the blood group (negative/mild) (p = .018). At four weeks, significant differences were found in the fibroblast density between the control and blood groups (p = .047)—mild in the control group and moderate in the blood group.

Microbiology

At two and four weeks, bacteria were isolated in 53% of the capsules (38 of 72) and 47% of the tissue expanders (34 of 72). The specimens included coagulase-negative staphylococci (41%), *Escherichia coli* (10%), *Staphylococcus aureus* (8%), *Pseudomonas* spp (0.7%), and other gram-negative bacilli (0.7%). In the capsules, the predominant isolated bacteria was coagulase-negative staphylococci, identified in 53% at two weeks (19 of 36), decreasing to 33% at four weeks (12 of 36). In tissue expanders, coagulase-negative staphylococci was identified in 44% at two weeks (16 of 36), decreasing to 22% at four weeks (eight of 36). Capsules yielded a single isolate in 43% (31) and more than one isolate in 10% (seven); tissue expanders yielded a single isolate in 32% (23) and more than one isolate in 15% (15). No fungi were recovered from the removed capsules or tissue expanders in any rabbits.

Similar bacterial isolates were cultured from rabbit skin. The predominant isolated bacteria was coagulase-negative staphylococci, in 16 of 18 euthanized rabbits (89%). A single skin sample was culture-negative, whereas two samples yielded more than one bacteria. Isolated bacteria from rabbit skin were not different from those removed from the capsules and tissue expanders. Coagulase-negative staphylococci were also isolated from all air samples. Other isolates included gram-positive bacilli and *Staphylococcus aureus* (although these were found much less frequently). Several species, such as *Penicillium spp.*, *Aspergillus niger*, and *zygomycetes*, were recovered from the operating room air.

Statistical analyses revealed no significant differences in the frequency of culture positivity and the type of bacteria among all the groups and no significant correlation between the microbial presence and the histological characteristics.

CHAID Modeling Associations

At four weeks, statistical analysis with CHAID modeling demonstrated association with intracapsular pressure at 20 mL for the control and fibrin groups. The determining factor for intracapsular pressure at four weeks was the type of inflammatory cells (Figure 2). The CHAID analysis showed in both trees that mixed inflammation was related to decreased intracapsular pressure and that mononuclear inflammation was related to increased intracapsular pressure. In the control tree, moderate inflammation was related to decreased pressure in the capsules with mononuclear

Table 2. Outcomes for Capsule Inflammation of Control Versus Experimental Groups

Group	Type of Inflammatory Cells	Two Weeks, %	Four Weeks, %	Intensity	Two Weeks, %	Four Weeks, %
Control	Mononuclear: chronic	22.2	55.6	Mild	11.1	55.6
	Polymorph: acute	0.0	0.0	Moderate	77.8	44.4
	Mixed: active chronic	77.8	44.4	High	11.1	0.0
Blood	Mononuclear: chronic	33.3	55.6	Mild	33.3	33.3
	Polymorph: acute	0.0	0.0	Moderate	66.7	66.7
	Mixed: active chronic	66.7	44.4	High	0.0	0.0
Fibrin: Tissucol/Tisseel	Mononuclear: chronic	11.1	22.2	Mild	0.0	22.2
	Polymorph: acute	0.0	0.0	Moderate	66.7	33.3
	Mixed: active chronic	88.9	77.8	High	33.3	44.4
Thrombin: FloSeal	Mononuclear: chronic	22.2	77.8	Mild	11.1	66.7
	Polymorph: acute	0.0	0.0	Moderate	77.8	33.3
	Mixed: active chronic	77.8	22.2	High	11.1	0.0

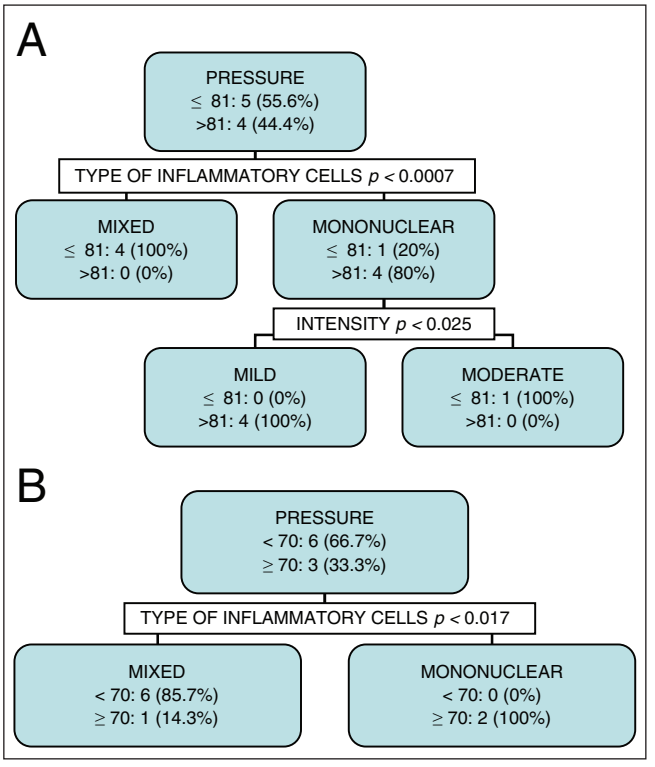

Figure 2. Decision tree by CHAID algorithm for histology data at four weeks. (A) control group; (B) experimental fibrin group (Tissucol/Tisseel).

inflammatory cells, whereas capsules with mild inflammation had increased pressure.

CHAID classification analyses with intracapsular pressure at 20 mL for the fibrin and thrombin groups showed that the determining factor for intracapsular pressure at four weeks was the kind of bacteria isolated from tissue expanders (Figure 3). Cultures showing an organism other than *Staphylococcus* (*E. coli*, *Pseudomonas* spp) and negative cultures (those with no contamination) were correlated with decreased intracapsular pressure. Coagulase-negative staphylococci and *Staphylococcus aureus* were correlated with increased intracapsular pressure.

DISCUSSION

The major findings of our study were observed on capsules and tissue expanders in the rabbits euthanized at four weeks. Compared to the control group, the fibrin and thrombin groups showed significantly decreased intracapsular pressures. The fibrin group was the only group with no capsular ruptures during pressure measurement. For the control and fibrin groups, mixed inflammation was associated with decreased intracapsular pressures, whereas mononuclear inflammation was associated with increased intracapsular pressures. For the fibrin and thrombin groups, cultures with bacteria other than staphylococci and negative cultures were associated with decreased intracapsular pressure, whereas staphylococci cultures were associated with increased intracapsular pressure. In the blood group, increased fibroblast densities were observed as compared to the control group. Increased angiogenesis was observed in the control group compared to the blood group. Average capsular thicknesses, the type and intensity of the inflammatory infiltrate, and collagen density and organization were similar among all groups. Also, the isolated bacteria in capsules, tissue expanders, and rabbit skin were similar among the groups. In capsules, tissue expanders, and rabbit skin, the predominant isolated bacteria was coagulase-negative staphylococci, which was also isolated from all air samples. No fungi were recovered from capsules, tissue expanders, or rabbit skin, but they were isolated from all air samples.

Of note, this study was performed with tissue expanders to measure the capsule pressure directly[46] to achieve more accurate results. Similar capsules and increased pressure levels were observed in both the control group and the blood group. On the basis of wound-healing principles,[47] we can conclude that increased pressure levels and capsule rupture rates correlate with contracture. That increased angiogenesis is associated with fibrosis has been documented,[31,46,48] supporting the major trends observed in CC development in the control group of this study.

FloSeal requires blood for activity; therefore, given the thrombin group results, we may conclude that an active hemostasis is indispensable to preventing CC, although it is unnecessary with a hemostatic commercial product. However, the fibrin group demonstrated mixed inflammation, which correlated with decreased intracapsular pressures as compared with the control group. This is consistent with other reports observing that the activation of fibrosis in the early implant period may be the major mechanism for CC development.[25]

In our study, inflammation was not significantly correlated with capsular thickness, which is consistent with the results reported by Siggelkow et al.[25] Sead et al studied fibrin sealant prepared from a Tisseel kit without aprotinin and observed a reduction in the extracellular matrix and TGF-β1, especially from adhesion fibroblasts, which may indicate a role in the reduction of postoperative adhesion development.[49] It is well known that fibrosis is associated with excessive collagen extracellular matrix formation, cell proliferation, and activation of myofibroblasts. In this context, macrophages and mast cells have been implicated as important participants in the inflammatory process involving fibrosis.[1] Macrophages contribute to this process by the production of TGF-β1 and IL-6.[50]

In a study by Ruiz-de-Erenchun et al,[51] TGF-β1 inhibitor peptide applied in a matrix with tetraglycerol dipalmitate was significantly effective in achieving a reduction in periprosthetic fibrosis after placement of silicone implants. Interestingly, in our fibrin group, mixed inflammation was correlated with decreased intracapsular pressure, but intracapsular pressure increased in the presence of *Staphylococcus* infection. Our results suggest that fibrin plays a role in preventing CC, that the bacterial colonization of mammary implants may be partially responsible for CC, and that

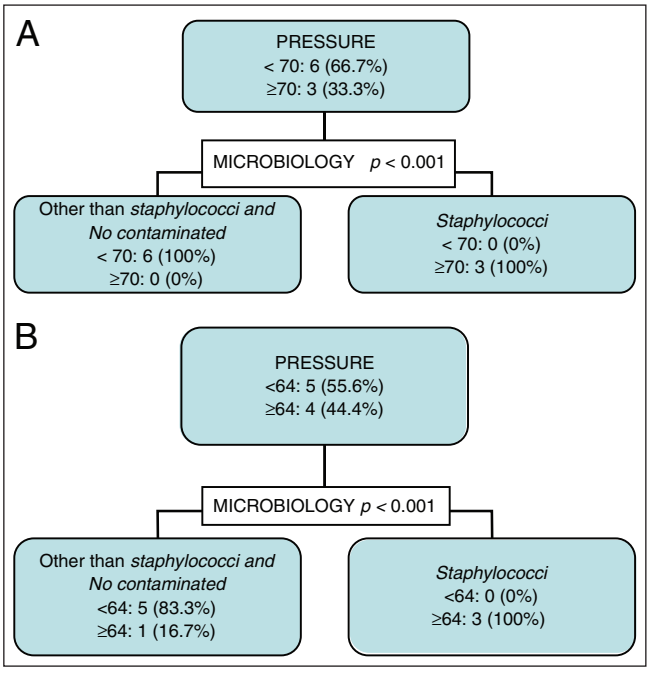

Figure 3. Classification tree by CHAID algorithm for microbiology data at four weeks. (A) experimental fibrin group (Tissucol/Tisseel); (B) experimental thrombin group (FloSeal). Bacteria other than *Staphylococcus* (*Escherichia coli*, *Pseudomonas* spp) and those with no contamination were considered negative cultures; *Staphylococcus* contamination included coagulase-negative staphylococci or *Staphylococcus aureus*.

coagulase-negative staphylococci may play a large role.[52-66] As reported in the literature, infection of implanted medical devices is commonly mediated by formation of bacterial biofilms.[67-70] However, Pajkos et al[13] reported that biofilm was found with scanning electron microscopy in a single culture-negative sample. It is interesting that extensive amorphous biological deposits were observed with scanning electron microscopy, even in the absence of bacterial structures. Moreover, because of the low pathogenicity of coagulase-negative staphylococci and the existence of microorganisms in a dormant phase within the biofilm around the implant, CC does not usually clinically manifest until some remote time after placement of mammary implants.[13,67-70] For all of these reasons, we did not consider biofilm investigation in this preclinical study. All the methods for biofilm investigation are expensive, not routinely used, and require a longer follow-up period.

We euthanized the rabbits at two or four weeks to study capsule formation and wound healing.[47] Our study demonstrated wound-healing results at two weeks among all groups that were similar to those from a report by Adams et al.[26] Our results differed in that intracapsular pressure decreased with fibrin glue application, whereas their results showed increased pressure.[26] This may be explained in part by the fact that our data were collected at four weeks, whereas that study focused on capsules at eight weeks. Also, the Adams et al study utilized an autologous fibrin glue of unknown fibrin concentration, whereas our study utilized a commercial fibrin product widely studied and used in clinical practice (Tissucol/Tisseel) to reduce polypropylene mesh adhesions.[2,3] To the best of our knowledge, this is the first report examining CC with a commercially-available fibrin product. In addition, this study is the first to include an examination of bacterial contamination from rabbit skin and operating room air. Furthermore, our study (which is an extension of the Adams study) placed expanders in New Zealand white rabbits rather than mice, given that the rabbits had the capacity to support all four expanders. The data in porcine models are limited.

One limitation of this study was the use of tissue expanders instead of commercial silicone breast implants, which are not available in an appropriate size for the rabbit model. One strength of our study was the statistical analyses among the four groups with the CHAID method, a sophisticated algorithm widely used in other disciplines because it models a single variable among multiple variables. Future studies may expand upon our results by extending the follow-up period, by inserting breast implants, instead of tissue expanders, sprayed with fibrin, or by focusing on fibrosis that may influence or modulate CC.

CONCLUSIONS

Fibrin applied in the breast implant pocket may reduce CC. With its relatively-well-documented safety profile, fibrin-containing compounds can be considered an attractive adjunct in breast implant surgeries. Clinical strategies for preventing bacterial contamination during surgery are crucial, given that *Staphylococcus* (mainly, coagulase-negative staphylococci) may promote CC even with fibrin.

Disclosures

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Publication IV

Effects of Coagulase-Negative Staphylococci and Fibrin on Breast Capsule Formation in a Rabbit Model

Marisa Marques, Spencer A. Brown, Natália D. S. Cordeiro, Pedro Rodrigues-Pereira, M. Luís Cobrado, Aliuska Morales-Helguera, Lara Queirós, André Luís, Rui Freitas, Acácio Gonçalves-Rodrigues and José Amarante

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Effects of Coagulase-Negative Staphylococci and Fibrin on Breast Capsule Formation in a Rabbit Model

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Abstract

Background: The etiology and ideal clinical treatment of capsular contracture (CC) remain unresolved. Bacteria, especially coagulase-negative staphylococci, have been previously shown to accelerate the onset of CC. The role of fibrin in capsule formation has also been controversial.
Objective: The authors investigate whether fibrin and coagulase-negative staphylococci (CoNS) modulate the histological, microbiological, and clinical outcomes of breast implant capsule formation in a rabbit model and evaluate contamination during the surgical procedure.
Methods: Thirty-one New Zealand white female rabbits were each implanted with one tissue expander and two breast implants. The rabbits received (1) untreated implants and expanders (control; n = 10), (2) two implants sprayed with 2 mL of fibrin and one expander sprayed with 0.5 mL of fibrin (fibrin; n = 11), or (3) two implants inoculated with 100 µL of a CoNS suspension (10^8CFU/mL–0.5 density on the McFarland scale) and one expander inoculated with a CoNS suspension of 2.5×10^7 CFU/mL (CoNS; n = 10). Pressure/volume curves and histological and microbiological evaluations were performed. Operating room air samples and contact skin samples were collected for microbiological evaluation. The rabbits were euthanized at four weeks.
Results: In the fibrin group, significantly decreased intracapsular pressures, thinner capsules, loose/dense (<25%) connective tissue, and negative/mild angiogenesis were observed. In the CoNS group, increased capsular thicknesses and polymorph-type inflammatory cells were the most common findings. Similar bacteria in capsules, implants, and skin were cultured from all the study groups. One Baker grade IV contracture was observed in an implant infected with *Micrococcus* spp.
Conclusions: Fibrin was associated with reduced capsule formation in this preclinical animal model, which makes fibrin an attractive potential therapeutic agent in women undergoing breast augmentation procedures. Clinical strategies for preventing bacterial contamination during surgery are crucial, as low pathogenic agents may promote CC.

Keywords

capsule, tissue expander, breast implants, histology, microbiology, coagulase-negative staphylococci, fibrin

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The investigation of various possible mechanisms for capsular contracture (CC) has been complicated by the lack of standardized animal models. Multiple reports have described the effects of bacteria on CC, and one report exists on the possible role of fibrin, but no single study has compared these parameters at one experimental time point.

There is evidence that bacterial colonization of breast implants is partially responsible for CC, and coagulase-negative staphylococci (CoNS; particularly *Staphylococcus epidermidis*) have been largely implicated.[1-15] Adams et al[16,17] showed that *S. epidermidis* colonization of breast implants was more likely to result from bacterial contamination at the time of implantation than from ongoing contamination from the adjacent ductal system. Because of the low pathogenicity of CoNS and the existence of microorganisms in a dormant phase within the biofilm formed around the implant, CC does not usually clinically

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manifest until some remote time after placement of breast implants.[18-22]

Fibrin glue consists of two components, a fibrinogen solution and a thrombin solution rich in calcium. Fibrin serves as a binding reservoir for several growth factors, such as vascular endothelial growth factor (VEGF),[23] transforming growth factor–β1,[24] and basic fibroblastic growth factor (bFGF).[25] Fibrin glue has been studied for decades for its applications both in a surgical setting and as an hemostatic and sealant agent. It is routinely used in gastrointestinal anastomosis, breast surgery, facelifts, abdominoplasty, nerve repairs, graft securing, neurosurgery, and ophthalmology.[26-36] More recently, it has also gained attention as a possible delivery mechanism for drug therapies.[37] For example, in a study by Zhibo and Miaobo,[38] release of lidocaine through fibrin glue was tested for pain reduction in breast augmentation patients. Patients who received fibrin glue with lidocaine in the subpectoral pocket experienced less pain than those who received the same amount of lidocaine or fibrin glue alone. In another study,[39] we applied an autologous fibrin to the implant pocket as a contracture-inducing agent and compared the results to a control group. The degree of fibrosis was greater in the fibrin-exposed groups in both the rabbit and human components of the study. Importantly, there was a significant increase in intracapsular pressure in the fibrin-exposed group. However, in another preclinical study[40] with fibrin (Tisseel/Tissucol; Baxter US, Deerfield, Illinois) sprayed onto the tissue expander and capsule pocket, a significant decrease in intracapsular pressures was found in the experimental fibrin group as compared to a control group at four weeks. For both the control and fibrin groups, mixed inflammation was correlated with decreased intracapsular pressure, whereas mononuclear inflammation was correlated with increased intracapsular pressure. The predominant isolate in capsules, tissue expanders, and rabbit skin was CoNS.

The purpose of this study was to perform a comprehensive evaluation, in a New Zealand white rabbit model, of the relationships among intracapsular pressure (recorded directly by a tissue expander), histological characteristics, and infection of breast implants. Microbiological analysis of rabbit skin and operating room air was performed to account for contamination during the surgical procedure. Our aim was to provide research data that could be translated into clinical practice. Therefore, we elected to (1) apply a commercial fibrin product (Tisseel/Tissucol) into the breast implant pocket to clarify the effect on capsule formation and (2) assess implants contaminated directly with CoNS, which were previously reported as CC-inducing agents. At present, there are no reports examining these two variables with a preclinical animal protocol for direct comparison.

METHODS

Thirty-one New Zealand white female rabbits (3-4 kg) were each implanted with one 20-cc textured tissue expander (Allergan, Inc., Santa Barbara, California) and two textured breast implants (90 cc; Allergan, Inc., Santa Barbara, California), according to approved institutional animal care protocol. Prior to surgery, the skin of each rabbit was washed with Betadine surgical scrub (Purdue Pharma LP, Stamford, Connecticut), which contains 7.5% povidone-iodine, and their skin was disinfected with Betadine solution, which contains 10% povidone-iodine. The surgical procedure was performed in an animal operating theater following aseptic rules. Penicillin G 40,000 U/kg intramuscularly (IM) was administered intraoperatively. Talc-free gloves were used at all times during the procedure.

Implant pockets were developed in the subpanniculus carnosis along the back region, with atraumatic dissection. Under direct vision, particular attention was paid to hemostasis, avoiding blunt instrumentation; there was no obvious bleeding. A sterile dressing was placed over the skin around the incision before the tissue expander and the implants were inserted to eliminate contact with the skin.[41] A new pair of talc-free gloves was worn when inserting the tissue expander and the implants.

The rabbits were divided into three groups: (1) those that received untreated implants and expanders (control; n = 10), (2) those that received two implants sprayed with 2 mL of fibrin and one expander sprayed with 0.5 mL of fibrin (fibrin; n = 11), and (3) those that received two implants inoculated with 100 µL of a CoNS suspension (10^8 CFU/mL—0.5 density on the McFarland scale) and one expander inoculated with a CoNS suspension of 2.5×10^7 CFU/mL (CoNS; n = 10).

All rabbits were sacrificed at four weeks. Prior to sacrifice, each animal was anesthetized, and the dorsal back area was shaved. A pressure-measuring device (Stryker Instruments, Kalamazoo, Michigan) was connected to the tissue expander port, and intracapsular pressures were recorded in 5-mL increments prior to any incision in the capsule. All capsule samples were submitted for histological and microbiological evaluation. All implants and expander devices were also submitted for microbiological evaluation.

Microbiological Assessments

Air. Operating room air samples (n = 36) were collected as described in our previous study[40] with a MAS 100-Eco air sampler (EMD Chemicals, Inc., Gibbstown, New Jersey) at a flow rate of 100 L per minute. Identification of bacterial and fungal isolates followed standard microbiological procedures. Gram-positive cocci were characterized by biochemical methods. Catalase-positive and coagulase-positive colonies were identified as *Staphylococcus aureus;* catalase-positive and coagulase-negative colonies were identified as coagulase-negative staphylococci. Gram-negative bacilli were characterized with Vitek 2 software (VT2-R04.02, bioMérieux, Inc., Durham, North Carolina). Fungi were characterized following their macroscopic appearance and microscopic morphology.

Rabbit skin. A total of 93 contact plates (31 brain-heart agar, 31 mannitol salt agar, and 31 Sabouraud agar) were pressed to the shaved dorsal skin surfaces, also as described in our previous study.[40] Brain-heart and mannitol salt agar plates were incubated for three days at 28°C; Sabouraud plates were incubated for seven days at 28°C. Bacterial and fungal colonies were counted and reported as cfu/cm^2. The identification of the bacteria and fungi followed the procedures reported above.

Capsules/implants/tissue expanders. Excised implants, tissue expanders, and representative capsule samples were incubated at 37°C for three days in brain-heart broth and examined daily; changes in turbidity of the broth media were considered positive and were subcultured in solid agar media. Characterization of microbial isolates followed the procedures described in the section on skin testing.

Histological Assessment

Capsule specimens were fixed with 10% buffered formalin and embedded in paraffin. Sections were stained with hematoxylin and eosin and histologically evaluated for tissue inflammation and capsular thickness. The type and intensity of the inflammatory infiltrate were analyzed. Inflammation was grouped into three categories: mononuclear/chronic (lymphocytes, plasmocytes, and histiocytes), mixed/subacute (mononuclear cells and eosinophils), and polymorph/acute (eosinophils and heterophils). Inflammatory infiltrate intensity was categorized according to the following criteria: absent (−), mild (+), moderate (++), and severe (+++).[40,42]

Samples were stained with Masson's trichrome to characterize the connective tissue (loose or dense), organization of the collagen fibers (arranged in a parallel array or haphazard), angiogenesis (absent, mild, moderate, or high), and fusiform cell density (mild, moderate, or high). The density of the connective tissue was semiquantitatively separated into one of four groups: (1) less than 25%, (2) 25% to 50%, (3) 50% to 75%, and (4) >75%.

Histological sections were reviewed and graded by a pathologist blinded to the protocol.

Statistical Analysis

Data were grouped according to the type of product applied to the breast implants: control (none; n = 10 rabbits, 20 implants), CoNS (n = 10 rabbits, 20 implants), or fibrin (n = 11 rabbits, 22 implants). One-way analysis of variance test—either parametric or nonparametric (Kruskal-Wallis *H* test)—was performed to determine whether the continuous variables (intracapsular pressure and histologically measured thickness) were equal, followed by post hoc range tests to identify homogeneous subsets across groups. Two-tailed independent paired *t* tests were used, along with the nonparametric alternative Mann-Whitney *U* tests. Categorical variables were evaluated by chi-square statistics and by phi, Cramer's V, and contingency coefficients. Statistical significance was calculated at $p \leq .05$. Major trends within each group were further examined with the Chi-squared Automatic Interaction Detection (CHAID) method,[43] using the likelihood ratio chi-square statistic as growing criteria, along with a Bonferroni 0.05 adjustment of probabilities. All analyses were carried out with the Statistical Package for Social Sciences Version 16 (SPSS, Inc., an IBM Company, Chicago, Illinois).

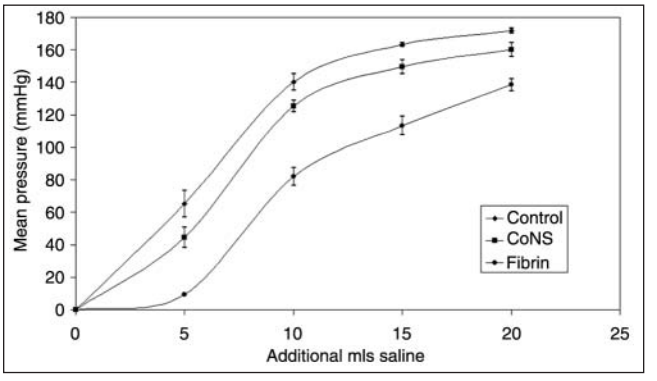

Figure 1. Pressure-volume curves. Note the significant difference in intracapsular pressure in the fibrin group. CoNS, coagulase-negative staphylococci.

RESULTS

Statistical analyses revealed no significant differences in histological and microbiological results between breast implants and tissue expanders. Because no differences were found, these data are not shown.

Intracapsular Pressure

During pressure measurements, five (50%) capsules ruptured in the control group, and five (50%) capsules ruptured in the CoNS group. To avoid a prohibitively small sample size, the ruptured capsules were not excluded from our statistical analyses, but the pressure value measured before rupture was maintained after further additional milliliters of saline were added. Significantly decreased intracapsular pressures were registered for the fibrin group as compared to the control and the CoNS groups ($p \leq .001$ and $p \leq .05$, respectively; Figure 1). Statistical analyses revealed no significant differences between the CoNS and the control groups.

Histology

Average capsular thicknesses were 0.81 ± 0.21 mm, 0.47 ± 0.13 mm, and 1.06 ± 0.29 mm in the control, fibrin, and CoNS groups, respectively. Capsular thickness was not

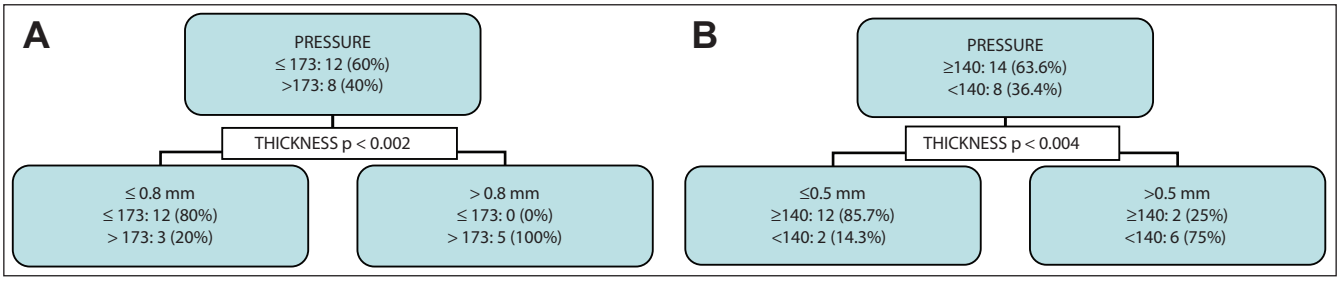

Figure 2. Decision tree by Chi-squared Automatic Interaction Detection (CHAID) algorithm for pressure and thickness. (a) Control group and (b) fibrin group.

statistically homogeneous across the three groups ($p \leq .001$). Three subsets of similar means were found after applying post hoc range tests: the first one comprised the fibrin group (with the thinnest capsule), the second comprised the control group, and the third comprised the CoNS group (with the thickest capsule). CHAID statistical modeling showed a correlation between intracapsular pressure measured at 20 mL and thickness for the control and the fibrin groups (Figure 2). Decreased intracapsular pressure was associated with thinner capsules for both groups, and the converse was also true.

A mixed type of inflammatory cells was the most common finding in the control and fibrin groups, but in the CoNS group, the polymorph type of inflammatory cells was predominant (Table 1). For types of cells, significant differences were observed between the control and CoNS groups ($p = .0001$), as well as between the CoNS and fibrin groups ($p = .0009$), but not between the control and the fibrin groups. Intensity of inflammation was moderate in the control and fibrin groups and mild in the CoNS group (Table 1). Significant differences were also found with regard to inflammatory intensity between the control and CoNS groups ($p = .011$), as well as between the CoNS and the fibrin groups ($p = .0058$), but not between the control and the fibrin groups. Significant correlations between the intensity of inflammation and the type of inflammatory cells were observed for the control ($p = .005$) and the fibrin ($p = .006$) groups.

Fibrosis was detected in all capsules; no significant differences regarding the fusiform cell density were observed in any of the groups. With regard to connective tissue, significant differences were found between the control and fibrin groups ($p = .005$) and between the CoNS and fibrin groups ($p = .0007$), with dense (>25%) connective tissue in the control and the CoNS groups and ≤25% connective tissue in the fibrin group.

Significant differences in the organization of the collagen fibers were observed between the control and fibrin groups ($p = .019$) and between the CoNS and fibrin groups ($p = .0039$), with haphazard collagen fibers in the control and CoNS groups and fibers arrayed in parallel in the fibrin group. Significant differences in angiogenesis were found between the control and fibrin groups ($p = .003$) and between the CoNS and the fibrin groups ($p = .016$), with moderate/high in the control and CoNS groups and negative/mild in the fibrin group.

Table 1. Outcomes for Capsule Inflammation in Control vs Experimental Groups

Group	Type of Inflammatory Cells	%	Intensity	%
Control	Mononuclear	25.0	Mild	30.0
	Polymorph	0	Moderate	70.0
	Mixed	75.0	High	0
Fibrin	Mononuclear	13.6	Mild	31.8
	Polymorph	13.6	Moderate	59.1
	Mixed	72.8	High	9.1
CoNS	Mononuclear	35.0	Mild	70.0
	Polymorph	50.0	Moderate	30.0
	Mixed	15.0	High	0

CoNS, coagulase-negative Staphylococci.

Microbiology

Bacteria were isolated in 31% (19 of 62) of the removed capsules and in 84% (56 of 62) of the removed implants (Table 2). The predominant isolates were CoNS, which were found in 16% of all culture-positive capsules (10 of 62) and in 60% of culture-positive implants (37 of 62). Overall, 97% of culture-positive capsules and 90% of culture-positive implants yielded a single isolate, whereas 3% and 10% (respectively) yielded two. No bacteria were detected on 69% of the removed capsules and on 16% of the removed implants. No fungi were recovered from the removed capsules or implants among all groups.

Statistical analysis revealed no significant differences in the type of bacteria or in the frequency of culture positivity among the study groups. Also, there was no significant association between microbial presence and histological data.

Table 2. Bacteria Isolated From Capsule and Implant Samples Removed From All Sacrificed Rabbits[a]

		Number (%) of Positive Cultures	
Bacteria	Group[b]	Capsules	Implants
Coagulase-negative staphylococci (CoNS)	Control	2 (10)	13 (65)
	Fibrin	2 (9)	9 (41)
	CoNS	6 (30)	15 (75)
Staphylococcus aureus	Control	2 (10)	2 (10)
	Fibrin	1 (5)	7 (32)
	CoNS	0 (0)	2 (10)
Bacillus gram-positive	Control	1 (15)	1 (5)
	Fibrin	3 (14)	4 (18)
	CoNS	0 (0)	2 (10)
Micrococcus spp.	Control	0 (0)	0 (0)
	Fibrin	0 (0)	0 (0)
	CoNS	2 (10)	1 (5)

[a]Sixty-two capsule samples and 62 implant samples were obtained from 31 rabbits.
[b]Control (10 rabbits; 20 capsules and 20 implants), fibrin (11 rabbits; 22 capsules and 22 implants), and CoNS (10 rabbits; 20 capsules and 20 implants).

Similar bacteria were isolated from the rabbit skin. The predominant isolates were CoNS, which was found in 37 of all 45 sacrificed rabbits (82%), followed by gram-positive bacilli (60%), *S. aureus* (33%), and *Micrococcus* spp. (9%). Other isolates found were *Enterococcus hermannii* and *Proteus mirabilis*, although all occurred much less frequently than the others listed previously. No skin sample was culture negative; 35 samples yielded more than one isolate. The bacterial isolates from rabbit skin were similar to those from the removed capsules and implants. Finally, CoNS were also cultured from all of the air samples; other airborne isolates were gram-positive and gram-negative bacilli such as *Micrococcus* spp., *Cryptococcus laurentii*, *Acinetobacter lwoffii*, and *Enterococcus agglomerans*. Fungal species such as *Penicillium* spp., *Aspergillus niger*, *Aspergillus flavus*, and *Aspergillus fumigatus* were recovered from the operating room air, with *Penicillium* being the most common fungal isolate.

In the CoNS group, one animal developed a Baker grade IV contracture[44] in one breast implant (Figure 3a). The capsular thickness measured 1.70 mm and was the largest among all capsules (Figure 3b). The type of inflammatory cells was polymorphous, with moderate intensity. Histological evaluation of fibrosis revealed 25% to 50% connective tissue density, haphazard collagen fibers, and moderate angiogenesis. The capsule and breast implant were both infected with a *Micrococcus* spp. isolate; no other bacteria or fungi were detected.

DISCUSSION

Significant results were demonstrated in each of our experimental groups. In the fibrin group, the data showed significantly decreased intracapsular pressures and capsular thicknesses without any capsule rupture, as compared to the control and CoNS groups. For the fibrin and control groups, decreased intracapsular pressures were correlated with thinner capsules. In terms of inflammation, mixed-type inflammatory cells were the most common finding for both fibrin and control groups. In the fibrin group, ≤25% connective tissue density was observed, as compared to the control and the CoNS groups, which had >25% connective tissue density. In the fibrin group, negative/mild angiogenesis was observed; the control and the CoNS groups had moderate/high angiogenesis. No significant differences

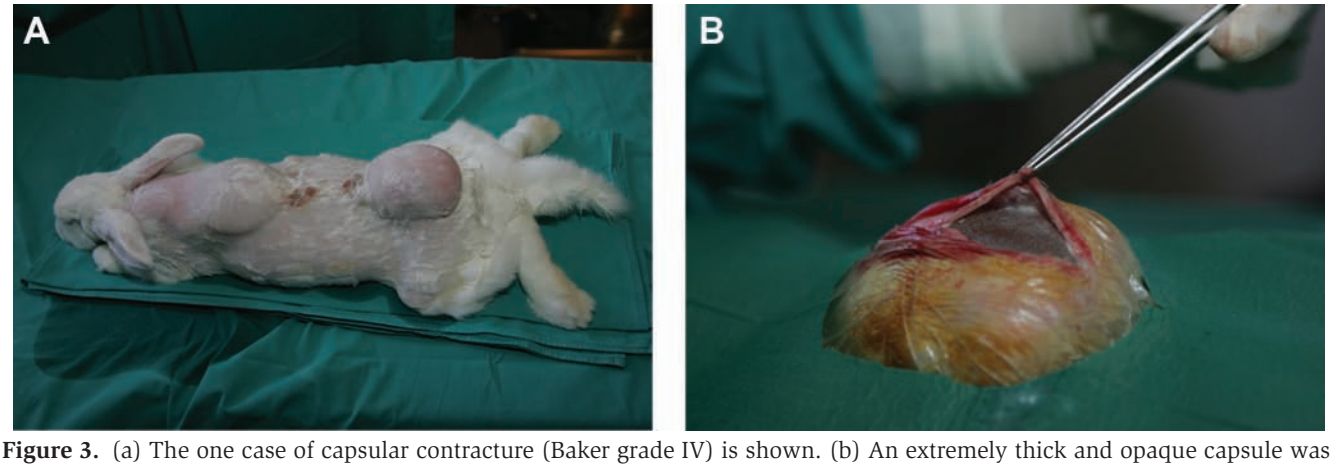

Figure 3. (a) The one case of capsular contracture (Baker grade IV) is shown. (b) An extremely thick and opaque capsule was evident in the implant associated with the contracture.

regarding fusiform cell density were observed between the fibrin and control groups.

In the CoNS experimental group, capsular thickness was increased as compared to the control. Polymorph-type inflammatory cells were the most common observation in the CoNS group, which was significantly different from the control group. Regarding fusiform cell density, connective tissue characteristics, organization of the collagen fibers, and angiogenesis, similar results were observed for both CoNS and control groups.

Similar bacterial isolates were observed among all the study groups for both implants and capsules. Implants were 2.7 times more frequently infected than capsules. The predominant isolates were CoNS, which were present 3.8 times more frequently in implants than in capsules. There was no significant association between microbial presence and histological data. Bacteria isolates from rabbit skin were similar to those isolated from capsules and implants. As expected, the predominant isolate in rabbit skin, as in implants and capsules, was CoNS. Unexpectedly, *Micrococcus* spp. was isolated from rabbit skin specimens, operating room air samples, and one rabbit; in that specific rabbit, *Micrococcus* spp. was detected on the capsule but not on the implant surface, and this capsule did not develop CC. Interestingly, on the contralateral implant in the same rabbit, a *Micrococcus* spp. isolate was detected on both the implant surface and the capsule, which demonstrated a Baker grade IV contracture.[44] To the best of our knowledge, this is the first report that shows a direct association between the presence of *Micrococcus* spp. and clinical CC in a rabbit model. Fungi were isolated from the operating room air samples but not from the rabbit skin, capsules, or implants. As far as we know, this is also the first report examining microbial cross-contamination among air, rabbit skin, capsules, and implants.

Our results support the probable role of fibrin as an agent that may modify capsule formation and mitigate subsequent CC since it is associated with decreased capsule thickness and pressure, ≤25% connective tissue density, and negative/mild angiogenesis. The decreased intracapsular pressure and thinner capsules were also consistent with other clinical reports of CC.[42,45-47] The relationship of CC to dense collagen and increased angiogenesis has already been demonstrated in other reports[45,48,49]; this was also found in our control and CoNS groups. The relationship between the organization of the collagen fibers (parallel or haphazard) and CC is controversial; our results are similar to a study from Karaçal et al.[47]

The cytokine-transforming growth factor beta 1 (TGF-β1) is a central mediator of fibrosis.[50-52] Some reports have focused on fibrin's properties for enhanced wound healing through the reduction of collagen extracellular matrix and decreased TGF-β1.[53-56] The TGF-β1-inhibitor peptide was shown to be significantly effective in achieving a reduction in fibrosis in silicone breast implants.[57] The use of fibrin-containing preparations (Tisseel and Vi-Guard, Melville Biologics, Inc, Melville, New York) allows for the closure of dead space and approximation of the skin flaps, and it has been argued that fibrin-containing tissue adhesives produce a dense architecture that inhibits angiogenesis and vascular ingrowth.[58] To the best of our knowledge, this is the first preclinical study with a commercial fibrin compound (Tissucol/Tisseel) applied to a textured silicone breast implant.

According to our results, bacterial infection of breast implants was more common than capsule infection, and the predominant isolates were CoNS. This is consistent with the fact that CoNS, a commensal bacteria of the skin, is the predominant cause of biomaterial-associated infection, commonly mediated by formation of biofilms.[18-21,59,60] The major pathogenicity is related to extensive biofilm formation on solid surfaces, which is extremely difficult to treat with antibiotics, thereby necessitating invasive procedures to remove the infected tissue or devices.[61-63] A strong correlation between the presence of biofilm (particularly by *S. epidermidis*) and significant CC was reported by Pajkos et al.[22] They assumed that biofilm on the outer surface of the implant, once established, acts as a focus of irritation and chronic inflammation, leading to accelerated CC.[22] However, our results are contradictory to that report.[22] In the Pajkos et al[22] study, the rate of recovery from bacteria from the implant surface was lower than the rate of recovery from the capsule surface, but the authors explain that there was a greater sensitivity in detecting bacterial growth on capsules.

The Baker grade IV contracture in our study, which occurred in the implant with the thickest capsule, was unusual in that contracture developed quickly with an acute inflammation. Unexpectedly, both the capsule and implant were infected only with *Micrococcus* spp., a low pathogenic agent. As far as we know, there are few reports concluding that *Micrococcus* spp. may have a true etiologic role in infection[64] or that it is mediated by formation of bacterial biofilms.[65,66]

Our fibrin results are contradictory to one of our previous reports[39] but consistent with our previous preclinical study.[40] This may be explained by the product we applied. In the first study,[39] we applied an autologous fibrin glue of unknown fibrin concentration into the implant pocket; in the current experimental design and the previous preclinical study,[40] we sprayed a commercial fibrin product widely studied and used in clinical practice in Europe and the United States to reduce polypropylene meshes adhesions,[67,68] the incidence of posterior spinal epidural adhesion formation,[30] and the recurrence rate of pterygium after surgery.[36] Another explanation may lie in the application mechanism (manual with a syringe in the first study vs sprayed in the latter two). A previous study found that a thin layer of glue is preferable to a thick one[69]; a thin layer of fibrin glue (such as would occur with a spray) may support the healing process, whereas a thick layer of adhesive inhibits skin graft healing.[70] Also, in this study, capsule pressure was measured directly in the tissue expanders to achieve more accurate results.[47]

Fibrin glue has been shown to act as an hemostatic agent,[71] an agent for enhanced wound healing by the reduction of collagen extracellular matrix and decreased TGF-β1 (a mediator of fibrosis),[53-56] an agent for adhesion

prevention,[67,68] a widely used ophthalmology tool,[31-36] and a drug delivery system for antibiotics.[72] Fibrin glue was also specifically tested in a clinical model as a drug delivery system following breast augmentation.[38] Our preclinical animal model results also show it to be a promising agent for the prevention of CC.

The limitation of this study was the insertion of only one tissue expander per rabbit, which allowed for direct intercapsular pressure measurement through the port. To correlate intracapsular pressure from tissue expanders with histological and microbiological results from breast implants, we performed statistical analyses that revealed no significant differences in histological and microbiological results between breast implants and tissue expanders. It would have been better to measure the pressure directly through 90-cc silicone breast implants with ports to achieve more accurate results, but these are not commercially available. One strength of this study was the statistical analyses of the data among the three groups using the CHAID method, a sophisticated algorithm used in many other disciplines that allows the investigator to adjust for the probability of a single variable among multiple variables.

Future studies include a prospective clinical study comparing a female control group with an experimental group that had Tissucol/Tisseel sprayed on the implant or pocket, with a follow-up period longer than 42 months.[73] A preclinical study analyzing *S. epidermidis* and *Micrococcus* spp. biofilm development in silicone breast implants where the ports have been sprayed with Tissucol/Tisseel and infected with bacteria would also be helpful.

CONCLUSIONS

The results from this preclinical rabbit model suggest that fibrin applied to the breast implant pocket may reduce capsular contracture. Since their relatively safe profile has been well established, fibrin-containing compounds are therefore an attractive adjunct for use in women undergoing breast augmentation. Clinical strategies for preventing bacterial contamination during surgery are crucial, as low pathogenic agents may promote capsular contracture.

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Publication V

Animal Model of Implant Capsular Contracture : Effects of Chitosan

Marisa Marques, Spencer A. Brown, Pedro Rodrigues-Pereira, M. Natália, D. S. Cordeiro, Aliuska Morales-Helguera, Luís Cobrado, Lara Queirós, Rui Freitas, João Fernandes, Inês Correia-Sá, Acácio Gonçalves Rodrigues and José Amarante

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Animal Model of Implant Capsular Contracture: Effects of Chitosan

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Abstract

Background: The mechanism(s) responsible for breast capsular contracture (CC) remain unknown, but inflammatory pathways play a role. Various molecules have been attached to implant shells in the hope of modifying or preventing CC. The intrinsic antibacterial and antifungal activities of chitosan and related oligochitosan molecules lend themselves well to the study of the infectious hypothesis; chitosan's ability to bind to growth factors, its hemostatic action, and its ability to activate macrophages, cause cytokine stimulation, and increase the production of transforming growth factor (TGF)–β1 allow study of the hypertrophic scar hypothesis.

Objective: The authors perform a comprehensive evaluation, in a rabbit model, of the relationship between CC and histological, microbiological, and immunological characteristics in the presence of a chitooligosaccharide (COS) mixture and a low molecular weight chitosan (LMWC).

Methods: Eleven adult New Zealand rabbits were each implanted with three silicone implants: a control implant, one impregnated with COS, and one impregnated with LMWC. At four-week sacrifice, microdialysates were obtained in the capsule-implant interfaces for tumor necrosis factor alpha (TNF-α) and interleukin-8 (IL-8) level assessment. Histological and microbiological analyses were performed.

Results: Baker grade III/IV contractures were observed in the LMWC group, with thick capsules, dense connective tissue, and decreased IL-8 levels ($p < .05$) compared to control and COS groups. Capsule tissue bacterial types and microdialysate TNF-α levels were similar among all groups.

Conclusions: Chitosan-associated silicone implantation in a rabbit model resulted in Baker grade III/IV CC. This preclinical study may provide a model to test various mechanistic hypotheses of breast capsule formation and subsequent CC.

Keywords

breast implants, capsular contracture, chitosan, microdialysis, histology, microbiology, immunology

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The true etiology of breast capsular contracture (CC) associated with implant devices, along with the most appropriate course of treatment, remains elusive despite extensive study. Two prevailing theories have emerged in the literature[1-18]: the infectious hypothesis and the hypertrophic scar hypothesis. As a solution, various molecules have been applied to implant shells in the hope of modifying or preventing CC. Chitin, the polymer D-glucosamine in β (1,4) linkage, is the major component of the exoskeletons of crustaceous and cell wall fungi.[19] Chitosan is a deacetylated product of chitin. In the literature, the term *chitosan* is used to describe chitosan polymers with different molecular weights (50-2000 kDa), viscosities, and degrees of deacetylation (40%-98%).[20] Material with lower levels of deacetylation degrades more rapidly.[21-23] Chitosan has been a better-researched version of the biopolymer because of its ready solubility in dilute acids, which makes it more accessible for utilization and chemical reactions.[24] Chitooligosaccharides (COS) are degraded products of chitosan, or the deacetylated and degraded products of chitin, by chemical and enzymatic hydrolysis.

Chitosan and related oligochitosan molecules have intrinsic antibacterial and antifungal activities[25-28] that lend themselves well to the study of the infectious hypothesis. Furthermore, chitosan's ability to bind to growth factors,[29,30] its hemostatic action,[31] and its ability to activate macrophages, cause cytokine stimulation,[31] and increase the production of transforming growth factor (TGF)–β1[32] permit study of the hypertrophic scar hypothesis.

Data from a study by Khor and Lim,[24] which included cell cultures and an animal model, indicated that chitin and chitosan processed in different shapes and in combination with different materials were noncytotoxic. The authors suggested that inclusion of these materials might yield tissue-engineered implants that would be biocompatible and viable. These attributes make chitosan a promising biopolymer for modulating wound healing (full-thickness skin defects and dermal burns)[26,29,33] and for use in orthopedics (cartilage, anterior cruciate ligament, intervertebral disk, bone, osteomyelitis)[28,34] and otologic diseases (tympanoplasty).[35]

Fibrosis is a major global health problem, but its etiology, pathogenesis, diagnosis, and therapy have yet to be addressed. Fibrosis can occur as a consequence of many pathologic conditions: (1) spontaneously (keloids, Dupuytren's contracture), (2) from tissue damage (postoperative adhesions, burns, alcoholic and postinfection liver fibrosis, silica dust, asbestos, antibiotic bleomycin), (3) as a result of inflammatory disease (infections, scleroderma), (4) in response to foreign implants (breast implant capsular contracture, cardiac pacemakers), and (5) from tumors (fibromas, neurofibromatosis). The early stages of fibrotic conditions are characterized by a perivascular infiltration of mononuclear cells and the subsequent imbalance of anti- and profibrotic cytokine profiles. One of the most prominent activators of mononuclear cells and fibroblasts is hyaluron fragments, which not only induce the expression of various cytokines (interleukin [IL]–1, IL-12, and tumor necrosis factor alpha [TNF-α]), chemokines (MPI-1A, MCP-1, IL-8), and inducible nitric oxide synthase (iNOS) but also trigger the expression and secretion of macrophage-derived matrix metalloproteinases (MMP), enzymes essential for extracellular matrix (ECM) cleavage.[36] IL-8 is a neutrophil chemoattractant factor. Levels of IL-8 are increased in scleroderma skin biopsy specimens.[37] Cultured scleroderma dermal fibroblasts make more IL-8 than normal fibroblasts. Studies in animal models of pulmonary fibrosis have shown the importance of chemokines in promoting angiogenesis, which is necessary for the development of pulmonary fibrosis.

Clues about the potential role of IL-8 in fibrosis come from studies of patients with idiopathic pulmonary fibrosis.[38] A low concentration of TNF-α increases fibroblast proliferation, whereas high TNF-α concentration decreases fibroblast proliferation.[39] However, TNF-α levels are markedly elevated in liver fibrosis, considered a profibrinogenic cytokine such as TGF-β1.[40] The immune inflammatory response and macrophage release of IL-8 and TNF-α induced by phagocytosis of periprosthetic wear debris stimulate bone reabsorption at implant or cement-bone interface. These cytokines directly induce fibroblast proliferation and tissue necrosis. Increased concentrations of IL-8 and TNF-α in the peripheral circulation of patients with large joint prostheses would indicate aseptic loosening.[41] As far as we know, there are no reports correlating capsule formation or CC with TNF-α and IL-8 levels. For all of these reasons, we believe that studying the role of these markers in capsule formation is important to the literature.

Microdialysis enables measurement of the molecules in the extracellular fluid around the capsule. Originally initiated more than 30 years ago,[42] microdialysis studies in humans have been mainly limited to head injury,[43-46] subarachnoid hemorrhage,[47] epilepsy,[48] and cerebral tumors.[49,50] IL-8 and TNF-α are known major biomarkers for inflammation,[51-53] which can be examined through microdialysis. To date, no preclinical model has been reported to assess possible environmental challenges that may prevent or modulate the wound-healing response with chitosan and related oligochitosan molecules associated with silicone implants. Therefore, we performed a comprehensive evaluation, in a rabbit model, of the relationships among CC rates and histological, microbiological, and immunological characteristics in the presence of COS mixtures and low molecular weight chitosan (LMWC). To monitor levels of inflammatory biomarkers in the breast capsule extracellular fluid, IL-8 and TNF-α were determined with the microdialysis technique.

METHODS

Eleven New Zealand white female rabbits (3-4 kg) were each implanted with three different textured breast implants, according to an approved institutional animal care protocol. The implants were each 90 cc and were provided by Allergan, Inc. (Santa Barbara, CA). Prior to surgery, the skin of each rabbit was washed with Betadine

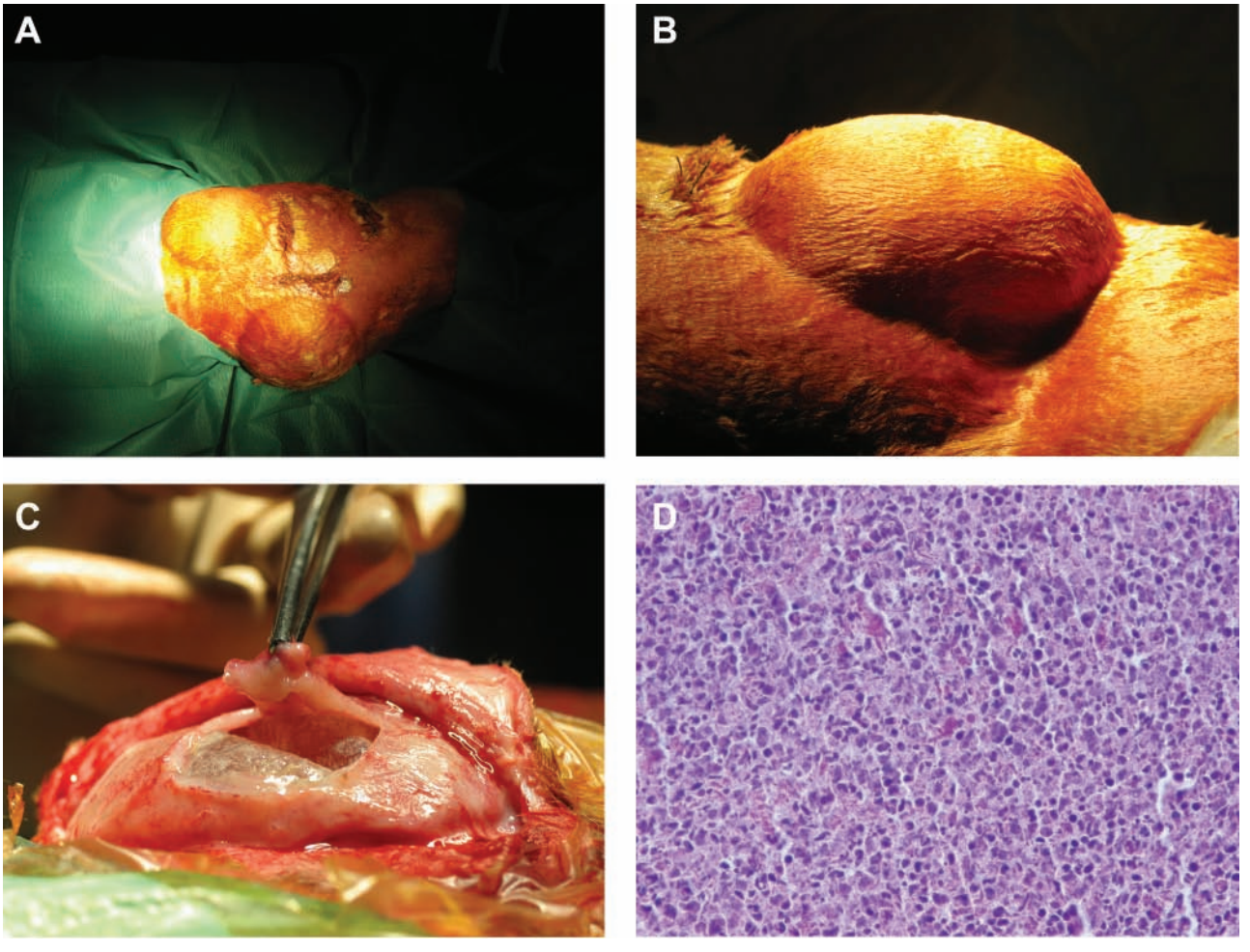

Figure 1. (A) Rabbit is shown with control implant, chitooligosaccharide (COS) implant, and chitosan implant (contracture grade IV). (B) The chitosan implant is pictured. Baker Grade IV contracture is evident. (C) The chitosan implant's extremely thick, dense, and opaque capsule can be seen. (D) Hematoxylin and eosin stain of the chitosan implant, at × 100 magnification, with apoptotic cells. These cells have hyperchromatic and fragmented nuclei.

surgical scrub (Purdue Pharma LP, Stamford, Connecticut) that contained 7.5% povidone-iodine, and their skin was disinfected with Betadine solution that contained 10% povidone-iodine. The surgical procedure was performed in an animal operating theater following aseptic rules. Penicillin G 40,000 U/kg intramuscularly (IM) was administered intraoperatively. Talc-free gloves were used at all times during the procedure.

Implant pockets were developed in the subpanniculus carnosis along the back region, with atraumatic dissection. Under direct vision, particular attention was paid to hemostasis, avoiding blunt instrumentation; there was no obvious bleeding. A sterile dressing was placed over the skin around the incision before the tissue expander and the implants were inserted to eliminate contact with the skin.[54] A new pair of talc-free gloves was worn when inserting the tissue expander and implants.

Each implant was placed beneath the panniculus carnosis along the back (Figure 1A). Each rabbit received an untreated implant (control), an implant impregnated with COS (molecular weight [MW] 1.4 kDa; Nicechem, Shanghai, China), and an implant impregnated with LMWC (MW 107 kDa; Sigma-Aldrich, Sintra, Portugal). Both chitosan mixtures possessed a deacetylation degree in the range of 80% to 85%. Implants were prepared by immersion in either COS (20.0 mg/mL) or LMWC (10.0 mg/mL) solutions with pH adjusted to 5.8 to 5.9 for two hours. Implants were incubated at 37 °C in a flow chamber for two days, then packed and sterilized by ethylene oxide.

Rabbits were sacrificed at four weeks. Prior to sacrifice, each animal was anesthetized, and a 5-mm incision was made directly over the implant, through the skin, panniculus carnosus, and capsule. A 100,000-MW cutoff microdialysis probe (CMA Microdialysis, Stockholm, Sweden) was placed by the capsule-implant interface, and microdialysates were collected with sterile, normal saline solution (6 µL/min) for one hour. Whole blood was obtained by venipuncture,

and serum was collected after centrifugation (2000 g min^{-1}, 4 °C). Capsule samples were submitted to histological and microbiological evaluations.

Microbiological Assessments

Air. Operating room air samples (n = 20) were collected during all surgical procedures with the MAS 100-Eco air sampler (EMD Chemicals, Inc., Gibbstown, New Jersey) at a flow rate of 100 L/min. Identification of bacterial and fungal isolates followed standard microbiological procedures. Gram-positive cocci were characterized by biochemical methods. Catalase-positive and coagulase-positive isolates were reported as *Staphylococcus aureus*; catalase-positive and coagulase-negative isolates were reported as coagulase-negative staphylococci. Gram-negative bacilli were characterized with Vitek 2 software (VT2-R04.02; bioMérieux, Inc., Durham, North Carolina). Fungi (molds) were characterized according to their macroscopic and microscopic morphology.

Rabbit skin. A total of 33 contact plates (11 brain-heart agar, 11 mannitol salt agar, and 11 Sabouraud agar contact plates) were pressed to the shaved dorsal skin surfaces. Brain-heart and mannitol salt agar plates were incubated for three days at 28°C; Sabouraud plates were incubated for seven days at 28°C. Bacterial and fungal colonies were counted and reported as cfu/cm^2. The identification of the bacteria and fungi followed the procedures reported above.

Capsules and implants. Excised implants and representative capsule samples were incubated at 37°C for three days in brain-heart broth and examined daily; changes in turbidity of the broth media were considered positive and were subcultured in solid agar media. Characterization of microbial isolates followed the procedures described above.

Histological Assessment

Capsule specimens were fixed with 10% buffered formalin and embedded in paraffin. Sections were stained with hematoxylin and eosin and evaluated histologically for tissue inflammation and capsular thickness. Inflammatory cells were grouped into three categories by type: (1) mononuclear (lymphocytes, plasmocytes, and histiocytes), (2) mixed (mononuclear cells and eosinophils), or (3) polymorph (eosinophils and heterophils/neutrophils). Inflammatory infiltrate intensity was categorized according to the following criteria: absent (−), mild (+), moderate (++), or severe (+++).[55]

Samples were stained with Masson's trichrome[56,57] to characterize the organization of the collagen fibers (arranged in a parallel array or haphazard), angiogenesis (absent, mild, moderate, or high), and fusiform cell density (mild, moderate, or high). The dense connective tissue was semiquantitatively analyzed as (a) less than 25%, with thick collagen bundles less than 25%; (b) 25% to 50%; (c) 51% to 75%; or (d) more than 75%.

Histological sections were reviewed and graded by a pathologist blinded to the protocol.

Microdialysis Assessment

TNF-α levels were determined with the manufacturer's instructions from a commercial kit (Invitrogen, Hu TNF-α cat. no. KHC3014:1; Life Technologies, Inc., Carlsbad, California). The assay was a solid-phase sandwich enzyme-linked immunosorbent assay (ELISA) in which 100 µL of microdialysis fluid was pipetted into each well. The protocol for IL-8 was performed with the BioSource Hu IL-8 US kit (cat. no. KHC0083/KHC0084; Life Technologies, Inc.).

Statistical Analysis

Data were grouped according to the type of product applied to the implant: control (none), COS, and LMWC (chitosan). Group data were also analyzed separately for the 11 sacrificed rabbits at four weeks after surgery. A two-tailed paired *t*-test and the nonparametric alternative Wilcoxon signed rank tests were applied to determine whether continuous variables (histologically measured thickness and dialysate levels of IL-8 and TNF-α) were significantly different among control and experimental groups. Categorical variables were evaluated by chi-square statistics and by phi, Cramer's *V*, and contingency coefficient tests. Statistical significance was presumed at $p \leq .05$. Major trends within each group were further examined by the chi-squared automatic interaction detection (CHAID) method,[58] using the likelihood ratio chi-square statistic as growing criteria, along with a Bonferroni 0.05 adjustment of probabilities. All analyses were carried out with the Statistical Package for Social Sciences Version 17 software (SPSS, Inc., an IBM Company, Chicago, Illinois).

RESULTS

Clinical

In the control group, one of the 11 implants was ulcerated; none had developed clinical CC. In the COS group, three of the 11 implants were ulcerated, and no cases of CC were observed. The chitosan group had one ulcerated implant, and all 11 implants had developed Baker grade III/IV capsular contracture (Figure 1B). All chitosan group capsules were extremely thick, opaque, stiff, and resistant to cutting (Figure 1C). They were constricted, and surface folding was observed.

Histology

The average capsular thickness was 0.418 ± 0.160 mm in the control group, 0.6364 ± 0.216 mm in the COS group, and 2.746 ± 0.817 mm in the chitosan group. Capsular thicknesses were found to be statistically different among the three groups: capsular thicknesses from the control

Table 1. Outcomes for Capsule Inflammation of Control Versus Experimental Groups

Group	Type of Inflammatory Cells	%	Intensity	%
Control	Mononuclear	9.1	Mild	72.7
	Polymorph	36.4	Moderate	27.3
	Mixed	54.5	High	0.0
Chitooligosaccharide	Mononuclear	9.1	Mild	54.5
	Polymorph	27.3	Moderate	45.5
	Mixed	63.6	High	0.0
Chitosan	Mononuclear	0.0	Mild	36.4
	Polymorph	45.5	Moderate	63.6
	Mixed	54.5	High	0.0

Table 2. Bacteria Isolated From Capsule and Implant Samples Removed From All Sacrificed Rabbits

		Number (%) of Positive Cultures	
Bacteria		Capsules	Implants
Coagulase-negative staphylococci	Control	4 (36.4)	9 (81.8)
	COS	5 (45.5)	8 (72.7)
	Chitosan	2 (18.2)	3 (27.3)
Staphylococcus aureus	Control	0 (0)	0 (0)
	COS	0 (0)	1 (9.1)
	Chitosan	0 (0)	0 (0)
Bacillus gram-negative	Control	0 (0)	2 (18.2)
	COS	0 (0)	1 (9.1)
	Chitosan	0 (0)	1 (9.1)
Enterococcus	Control	0 (0)	1 (9.1)
	COS	1 (9.1)	0 (0)
	Chitosan	0 (10)	0 (0)

COS, chitooligosaccharide.

group were different from both the COS group ($p = .035$) and the chitosan group ($p = .003$); capsular thicknesses were also different between the COS and chitosan groups ($p = .003$). No significant differences were observed regarding the type of inflammatory cells or the intensity of capsule inflammation among the groups (Table 1).

Apoptotic cells and necrosis (Figure 1D) were observed strongly in the chitosan group. Fibrosis was a component of all capsules, and no significant difference was found regarding the organization of collagen fibers, fusiform cell density, or angiogenesis among all groups. Regarding the characteristics of connective tissues (either loose or dense), significant differences were found between the control and the chitosan groups ($p = .001$). The control group had less than 25% density of connective tissue, and the chitosan group had more than 25% dense connective tissue.

Microbiology

Bacteria were isolated from 36.4% (12 of 33) of the capsules and from 78.8% (26 of 33) of the implants. The organisms cultured (Table 2) included coagulase-negative staphylococci, *S. aureus*, gram-negative bacilli, and *Enterococcus* spp. Among capsules that yielded bacteria, 11 of 12 harbored coagulase-negative staphylococci (91.7%); enterococci were associated with one capsule (8.3%). The same trend was observed in excised implants. In 20 of 26 implants that yielded bacteria, coagulase-negative staphylococci were cultured from 76.9%, and *Enterococcus* spp. was associated with one capsule (3.8%). In contrast to the capsules, four of 26 bacteria-contaminated implants harbored gram-negative bacilli (15.4%), and one of 26 demonstrated evidence of *S. aureus* (3.8%).

Overall, 39.4% (13 of 33) and 63.6% (21 of 33) of culture-positive capsules and implants, respectively, yielded a single isolate; 0% (zero of 33) and 9.1% (three of 33) yielded more than one. No fungi were recovered from either capsules or implants.

No significant differences in the frequency of culture positivity or the type of bacterial isolates were observed among all study groups, nor was any significant association between microbial presence and histological data observed.

With regard to skin isolates, the predominant isolate was again coagulase-negative staphylococci, which were formed in all rabbits. Bacterial isolates from skin were similar to those from capsules and implants. Coagulase-negative staphylococci and gram-positive bacilli were isolated from all operating room air samples, along with *Penicillium* spp. and *Aspergillus* spp.

Immunology

Interstitial fluid of IL-8 levels decreased to the following: 89.4 ± 26.7 mg/mL in the control group, 78.3 ± 32.7 mg/mL in the COS group, and 66.8 ± 17.9 mg/mL in the chitosan group. Significant differences were observed in IL-8 levels between the control and chitosan groups ($p = .028$).

Levels of TNF-α decreased to the following: 143.9 ± 123.8 mg/mL in the control group, 96.8 ± 38.5 mg/mL in the COS group, and 81.5 ± 31.8 mg/mL in the chitosan group. Statistical analysis revealed no significant differences in the dialysate levels of TNF-α among all groups. There was a correlation between IL-8 and TNF-α in the

control group ($p < .001$) but not in the COS group ($p = .073$) or the chitosan group ($p = .099$).

DISCUSSION

In this study, we report on the development of CC in a rabbit model associated with chitosan. All chitosan group implants demonstrated clinical Baker grade III/IV breast contractures with significantly thicker capsules than non-treated implants. Chitosan-exposed capsules were opaque, stiff, and resistant to cutting, and considerable shrinkage and folding of the implant surfaces were observed. These characteristics may indicate the constricting nature of fibrous implant capsules. Control (untreated) capsules demonstrated thin capsule thicknesses, and the connective tissue had less than 25% dense tissue, compared to the more than 25% dense connective tissue observed with the LMWC-exposed capsules. This is consistent with the fact that the major component of chitosan, glucosamine, forms in cartilage tissue and is also present in tendons and ligaments.[59]

The collagenous layer of granulation tissue is increased with chitosan application; according to this finding, chitosan may stimulate fibroblast proliferation and extracellular matrix production.[60] Chitosan has been shown to induce an accelerated wound-healing process that did increase TGF-β1, which had several proinflammatory regulatory influences such as cell migration, granulation tissue formation, and increased collagen production[32] and was a central mediator of fibrosis.[61]

A mixed/polymorph type of inflammatory cells was the most common finding in all rabbit capsules, and inflammation was moderate/mild in all capsules. This finding was expected, as chitosan is a chemoattractant for neutrophils.[28,62] Chitosan enhanced the function of inflammatory cells such as polymorphonuclear leukocytes (PMN), macrophages, fibroblasts (production of IL-8), angioendothelial cells,[60] and had a systemic effect.[63] Apoptotic cells and necrosis were observed strongly in chitosan implants, consistent with other reports.[64,65]

Statistical analyses revealed no significant differences in the frequency of culture positivity and bacteria type among the groups. Interestingly, no significant associations between microbial presence and histological data were observed in any group. Similar bacterial isolates were cultured from the rabbit skin and air samples, and the predominant isolates were coagulase-negative staphylococci. The antimicrobial activity of chitosan and its derivatives against several bacterial species has been recognized and considered one of the most important properties linked directly to their possible biological applications[25-28]; however, recent studies investigating chitosan as a delivery method for drugs such as antibiotics[66-69] questioned the high efficacy of chitosan alone as an antibacterial agent. This study supports the idea that CC formation is not the result of bacterial infection alone, in contrast to the infectious hypothesis that has been championed and consistently supported by Burkhardt et al.[1,3,70]

To gain insight into the inflammatory process, major biomarkers TNF-α and IL-8 were measured. This is the first report examining extracellular levels of IL-8 and TNF-α in a breast capsule implant environment. Microdialysate levels of IL-8 were decreased ($p < .05$) in the chitosan group as compared to the control group. No significant differences in the microdialysate levels of TNF-α were observed among the groups. In the control group, a correlation between IL-8 and TNF-α was observed; no significant correlation between IL-8 and TNF-α levels was observed in the experimental groups.

We originally hypothesized that serum concentrations of the inflammatory mediators would be significantly increased in the chitosan group due to the expected greater inflammatory response with chitosan, as this molecule promotes the production of IL-8.[60] The actual data results did not support our hypothesis but were consistent with a study from Tilg et al,[71] who reported increased IL-8 and TNF-α levels in bacterial infection and decreased IL-8 and TNF-α levels in acute rejection. Interestingly, we found clinical Baker grade III/IV breast capsule contractures in all rabbits exposed to chitosan associated with acute (polymorph) and subacute (mixed) inflammation, not due to a bacterial infection.

Not all chitosan implants were infected, and IL-8 and TNF-α were decreased in the chitosan group. Molecular regulation of IL-8 production has been studied in vitro, and TNF-α has proven to be a major regulatory molecule. It is not surprising that in vivo IL-8 and TNF-α serum levels were also significantly correlated in the control group. The correlation between IL-8 and TNF-α has been well established in the case of bacterial infection, less pronounced in cytomegalovirus hepatitis, and not apparent in acute cellular liver rejection episodes. Lack of correlation in acute rejection was also associated with low levels of IL-8.[71] This suggests that, in contrast to bacterial infection, countering cytokines may be active in CC (at least promoted by chitosan), downregulating IL-8 transcription and/or translation.

So far, no reports exist on the production and regulation of IL-8 in CC. Recent studies have demonstrated that COS displayed anti-inflammatory properties in immunocytes, including the inhibition of nitric oxide, the downregulation of IL-6 and TNF-α, and the increase of cell viability of neutrophils.[72,73] Additionally, IL-8 was induced by a wide range of stimuli, including lipopolysaccharide (LPS), a component of the outer membrane of gram-negative bacteria and TNF-α. Lund et al[74] concluded that LPS induced IL-8 release in monocytes, whereas TNF-α was a good inductor of IL-8 in PMN. In the chitosan contracture model, we found decreased levels of IL-8, and it was possible to conclude that there was no gram-negative bacteria infection to induce IL-8. On the other hand, chitosan increased the production of TGF-β1,[32] a central mediator of fibrosis; the degree of CC is directly related to an increased level of TGF-β.[55] Even with contradictory studies about the role of TNF-α, Morimoto et al[75] concluded that TNF-α played a pivotal role in the maintenance of hemostasis and tissue repair by inhibiting TGF-β1.

Our data support the theory that chitosan initiates CC response due to a toxic local effect that results in an impaired wound-healing response. An earlier series of pilot studies were performed with much higher levels of chitosan (data not shown). Using a similar experimental protocol in the rabbit model, implants exposed to 25.0-mg/mL levels were implanted. The majority of animals expired within a short time period; surviving animals had decreased weight (15%-25.8%) compared to baseline body weights, with leukocytosis and decreased hemoglobin. At autopsy, fat biopsies were atrophied, and liver specimens had lymphoid infiltration in the portal spaces. We found toxicity with 25.0 mg/mL of implanted LMWC per rabbit. The study design was modified to test decreased chitosan levels that were not systemically toxic to the animals. In the reported data, all animals were clinically healthy. Literature data reporting general toxicity testing for chitosan are limited,[31] and our results are consistent with the few studies about chitosan toxicity.[60,63,76-78]

In several important studies,[15,79] each rabbit received different implants. Darouiche et al,[15] with the objective of examining in vivo the antimicrobial efficacy of minocycline/rifampin–impregnated saline-filled silicone implants, placed four implants in each rabbit (two antimicrobe-impregnated and two control implants). Shah et al,[79] who examined the infectious hypothesis in vivo, gave each rabbit a *Staphylococcus epidermidis*–contaminated implant and a control implant. Despite the fact that this type of protocol is well supported in the literature, due to the systemic influence of chitosan, the use of three different implants in the same rabbit in our study obviously had the potential to confound the results.

To clarify this issue, a control limb study was performed (data not shown) and compared with the control group from this study. Using a similar experimental protocol, 10 rabbits were implanted with two textured breast implants. Interestingly, results from the control group in the main study showed a lower capsular thickness than the control limb group (0.81 ± 0.21 mm; $p = .001$). No significant differences were observed regarding the intensity of inflammation, characteristics of connective tissue (either loose or dense), fusiform cell density, or angiogenesis between the groups. However, significant differences were observed with respect to the type of inflammatory cells, with a mixed type of inflammatory cells found in 54.5% of the control group in this study and mononuclear type of inflammatory cells found in 55.6% of the control limb group ($p = .017$). Significant differences were also observed in the organization of the collagen fibers, which were arrayed in sequence in the control group of this study and haphazard in the control limb group ($p = .007$). Statistical analysis revealed no significant differences in the type or frequency of bacteria between the control group of this study and the control limb group. Decreased levels of IL-8 ($p = .016$) and TNF-α ($p = .001$) were observed in the control group of this study when compared with the control limb group, which proves the systemic influence of chitosan.

In a previous commentary,[70] Burkhard considered that if a rabbit model must be used for research, a more appropriate model was the one reported by Shah et al,[79,80] who used bacterial contamination to produce contracture. In the Shah et al study,[79] 16 New Zealand white rabbits each received a *S. epidermidis*–contaminated implant and a control implant. The capsules were dissected at two, four, six, and eight weeks. Capsules on the contaminated side were two to three times thicker than those on the control side, and they did not change thickness with time. Capsules on the contaminated side consisted of densely packed, longitudinally oriented thick bundles of collagen fibers; there was a large cellular infiltration with leukocytes and macrophages. By contrast, the capsules on the control side were thinner and consisted of loosely organized connective tissue fibers predominantly parallel to the prosthesis surface. Bacteriological cultures on the contaminated side consistently yielded *S. epidermidis* with occasional diphtheroids, whereas the control side showed no bacterial growth.

As reported in the Prantl et al[81] study, we believe that subclinical infection with chronic inflammation represents one of the possible important reasons for the development of CC. We also hypothesize that all possible causes of fibrosis result in the common key factor of pathological response with the development of chronic inflammation. The Prantl et al[81] study included only those implants with high gel cohesiveness (third-generation implants); in these implants, silicone filler presumably does not leak from the shell into the tissue in the case of implant rupture. Surprisingly, in 67% of their specimens, the authors detected vacuolated macrophages with microcystic structures containing silicone. Also, in 54% of the specimens, the capsular tissue contained empty spaces with varying sizes of silicone particles. It remains unclear whether these silicone structures represented friction particles from the surface of the implant or particles from the implant filler. Heppleston and Styles[82] performed in vitro experiments demonstrating that silica damages macrophages, which subsequently produce TGF-β1 and stimulate fibroblasts to produce collagen. However, since the Shah et al[79] study, even with the many publications on infected implants, we were unable to find any translation of the Baker classification into a preclinical model.

An infection-induced contracture limb study was performed (data not shown) and compared with the chitosan group of this study. Using a similar experimental protocol, 10 rabbits were implanted with two textured breast implants, each one with a suspension of 100 µL of coagulase-negative staphylococci (108 CFU/mL; 0.5 density on the McFarland scale). Histologically, the average capsular thickness was 1.065 ± 0.287 mm in the infection-induced contracture limb group (CoNS group) and 2.746 ± 0.817 mm in the chitosan group. Capsular thicknesses were found to be statistically different among the two groups ($p = .00003$). A significant difference was also observed regarding the type of inflammatory cells among the two groups ($p = .021$), with the polymorph type being predominant in the CoNS group and the mixed type being predominant in the chitosan group. No significant differences were found between the two groups regarding the intensity of capsule inflammation. Significant differences in

angiogenesis were found between the CoNS and chitosan groups ($p = .004$)—with absent/mild and moderate/ high being equally present in the CoNS group but only high in the chitosan group—as well as in the synovial metaplasia ($p = .043$), which was always absent in the chitosan group but present in some cases of the CoNS group. However, no significant differences were found between the two groups regarding the characteristics of the connective tissue (loose or dense), the organization of the collagen fibers (parallel or haphazard), or fusiform cell density (mild, moderate, or high). Histologically, the type of CC induced by chitosan was different from that induced by infection, in that (1) the capsule was thicker, (2) the mixed type of inflammatory cells was predominant, (3) angiogenesis was high, and (4) the synovial metaplasia was absent. Our results contribute a preclinical noninfectious model of CC to the literature, but further studies are necessary.

We sacrificed the rabbits at four weeks to study early capsule formation and to understand the possible models of wound healing.[39] A longer term study would be important and is planned. However, long-term differences in capsule structures under these experimental challenges result from different wound-healing trajectories from Day 0. Our strategy was to examine these early differences with methods that were sensitive to detecting histological or biomarker changes. There is no consensus about the length of time necessary in a preclinical model. In a clinical mode, we proposed a follow-up period longer than 42 months.[83] However, it might be expected that the finding of a dense collagenous capsule would increase with time, reflecting a continued stimulus toward a fibroplasia and ultimate collagen remodeling.[84-86]

The weaknesses of this study include the relatively small size and the lack of capsule immunohistochemistry detection of IL-8 and TNF-α in tissue specimens. Nevertheless, the release of IL-8 and TNF-α represented a "spillage" of factors rather than a direct signal driving inflammation and leukocyte recruitment; the use of microdialysis was appropriate for determining tissue concentration of cytokines such as IL-8 and TNF-α. Because of the proximity of the sampling site to the source of the cytokine, microdialysis provided a means of sensitively detecting relative changes of inflammatory mediator concentration with experimental treatments.

Possible future studies would include a model in which one silicone breast implant with ports (to measure the capsule pressure directly) impregnated with LMWC is implanted per rabbit, as well as a model designed for detection of IL-8, TNF-α, TGF-β1, and determination of a fibrosis index. We previously reported complementary studies in which the same protocol is used to analyze silicone breast implants with ports impregnated with LMWC and those sprayed with Tissucol/Tisseel (Baxter International, Deerfield, Illinois).[87,88]

CONCLUSIONS

Baker grade III/IV CC was observed in a rabbit model when implants were impregnated with chitosan; the CC was not due to a bacterial infection. This preclinical study may provide a model to test various mechanistic hypotheses of breast capsule formation and subsequent CC and suggests an approach of studying CC with a preclinical animal model.

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Publication VI

The Impact of Triamcinolone Acetonide in Early Breast Capsule Formation in a Rabbit Model

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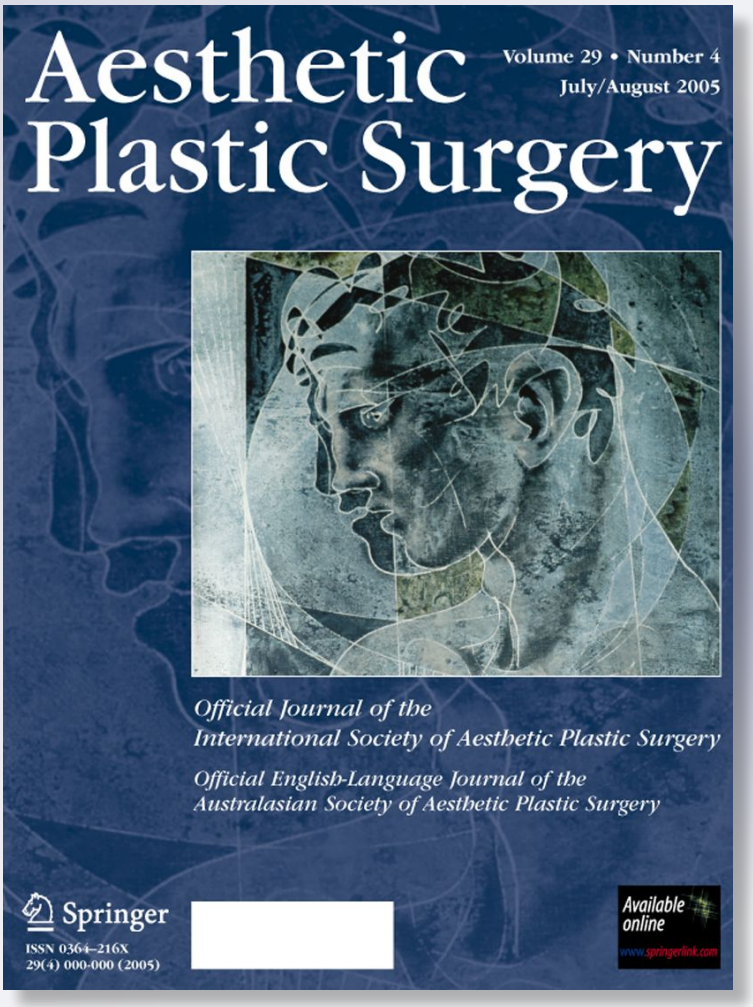

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The Impact of Triamcinolone Acetonide in Early Breast Capsule Formation in a Rabbit Model

Marisa Marques · Spencer Brown · Inês Correia-Sá · M. Natália D. S. Cordeiro · Pedro Rodrigues-Pereira · Acácio Gonçalves-Rodrigues · José Amarante

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Abstract
Background The etiology and clinical treatment of capsular contracture remain unresolved as the causes may be multifactorial. Triamcinolone acetonide applied in the pocket during surgery was reported to be ineffective in prevention of capsular contracture. However, if injected 4–6 weeks after surgery or as a treatment for capsular contracture, decreased applanation tonometry measurements and pain were observed. It was assumed that intraoperative application of triamcinolone was not effective because its effect does not last long enough. However, betadine, antibiotics, and fibrin were found to be effective in preventing capsular contracture with intraoperative applications and are more effective in the early phases of wound healing than in later stages. The role of triamcinolone acetonide in capsule formation is unknown. The purpose of this study was to determine if triamcinolone acetonide modulates breast capsule formation or capsular contracture in the early phases of wound healing in a rabbit model.
Methods Rabbits ($n = 19$) were implanted with one tissue expander and two breast implants and were killed at 4 weeks. Implant pocket groups were (1) Control ($n = 10$) and (2) Triamcinolone ($n = 9$). Pressure/volume curves and histological, immunological, and microbiological evaluations were performed. Operating room air samples and contact skin samples were collected for microbiological evaluation.
Results In the triamcinolone group, a decreased capsular thickness, mild and mononuclear inflammation, and negative or mild angiogenesis were observed. There were no significant differences in intracapsular pressure, fusiform

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cell density, connective tissue, organization of collagen fibers, and microbiological results between the groups. There was no significant difference in the dialysate levels of IL-8 and TNF-α, but correlation between IL-8 and TNF-α was observed.

Conclusion Triamcinolone acetonide during breast implantation influences early capsule formation and may reduce capsular contracture.

Level of Evidence III This journal requires that authors assign a level of evidence to each article. For a full description of these Evidence-Based Medicine ratings, please refer to the Table of Contents or the online Instructions to Authors at www.springer.com/00266.

Keywords Breast capsule · Triamcinolone acetonide · Pressure · Histology · Microbiology · Immunology

Capsule formation is a foreign body reaction that occurs in all patients who have breast implants. Normally not thicker than 1-mm [44], the capsule is part of the normal healing process and may help keeping the implant in place [21, 22]. Capsular contracture (CC) remains the most severe complication with silicone and saline breast implants, with an incidence ranging from 8 to 45 % [8, 23, 29, 33, 41]. The etiology of CC is not completely understood, but it is thought to be multifactorial [4, 34]. Factors related to wound healing [49] and infection [2, 3, 14, 18, 41, 47] are known to influence the development of this clinical condition.

Etiology, prevention, and treatment measures for CC have been extensively discussed, but there is no agreement on a generally accepted therapeutic pathway. All the reported clinical procedures used to minimize points of contamination are crucial, and many plastic surgeons follow the general principles of the "Betadine Era" [2] and the "Post-Betadine Era" [3, 5] to prevent CC. Betadine [2], antibiotics [3, 5], and fibrin [35, 36] are clinically associated with a low incidence of CC and are more effective in the early phases of wound healing. However, even when following all the procedures proven to be effective for diminishing this complication, it is still an important late complication of breast implant surgery [41].

In preclinical studies, treatment with mesna [6], mitomicina C [24], zafirlukast [9, 46], pirfenidone [25], or halofuginone [51] reduced capsule thickness, fibroblast cell proliferation, and collagen deposition. Nevertheless, these drugs are not commonly used in clinical practice, with the exception of the zafirlukast. This drug is currently approved for the treatment of asthma, but its role in the treatment of CC is limited to severe cases due to the possibility of severe side effects [11, 28].

The capsule is known to be composed of a layer of fibrous dense connective tissue [17] and is an integral part of the wound-healing process. Although initially beneficial, the healing process can become pathogenic if it continues unchecked, leading to considerable tissue remodeling and the formation of permanent scar tissue [13], as in CC. Corticosteroids administered during wound healing have been shown to stop the growth of granulation completely, stop the proliferation of fibroblasts, diminish new outgrowths of endothelial buds from blood vessels, and stop the maturation of the fibroblasts already present in connective tissue [7]. Also, when administered early after injury, corticosteroids delay the appearance of inflammatory cells, fibroblasts, the deposition of ground substance, collagen, regeneration of capillaries, contraction, and epithelial migration [20]. These data raised interest in the use of steroids in the treatment and prevention of CC.

The data available in the literature regarding the role of steroids in the prevention and treatment of CC is sparse and contradictory. Perrin [38] reported less than 5 % of significant capsule formation in patients who underwent augmentation mammaplasty with inflatable breast prostheses filled with saline and a cortisone derivative, with no evidence of wound complications attributable to the steroid. These results were reinforced by those of Ksander [31], who, in a preclinical model with rats, showed that saline implants filled with saline solution were harder and surrounded by a thicker capsular membrane than those filled with methylprednisolone sodium succinate at 60 and 120 days.

On the other hand, Caffee et al. [16] reported in a preclinical study that putting triamcinolone in the pocket during surgery was ineffective in the prevention of CC, but if injected 4 and 8 weeks postoperatively (invasive method), the drug was able to completely eliminate CC. Caffee [15] also reported the effectiveness of postoperative injection of triamcinolone in reducing the risk of recurrent contracture in a high-risk group of patients. Sconfienza et al. [43] demonstrated that US-guided injection of 40 mg of triamcinolone acetonide (TA) into the peri-implant pouch of women with augmented or reconstructed breasts affected by Baker grade IV CC was effective in reducing capsular thickness and the patient's discomfort.

Although the data have been presented, the role of steroids in the treatment and prevention of CC is not completely understood. It is not clear whether steroids are effective in preventing CC when placed in the implant pocket, as the data available are inconsistent and contradictory. None of the clinical studies are prospective or randomized. Moreover, none of the studies discussed here established a clearly comprehensive role and mechanism of steroids in the development of CC. Steroids have an important role in the earlier phases of wound healing [20], and the role of those effects on the early phase of breast capsule formation are also not understood nor explored.

The main objective of this study was to perform a comprehensive evaluation of the role of TA in capsule formation in the early phases of wound healing [13] and the histological, microbiological, and immunological characteristics in a rabbit model [4].

Materials and Methods

In an approved institutional animal care protocol, 19 New Zealand white female rabbits were implanted with one textured tissue expander (nonfilled; Allergan, Inc., Santa Barbara, CA, USA) and two textured breast implants (90 ml, Allergan). Prior to surgery, rabbit skin was washed with Betadine® Surgical Scrub, which contains 7.5 % povidone-iodine, followed by Betadine® solution, which contains 10 % povidone-iodine (Purdue Pharma LP, Stamford, CT, USA). The surgical procedure was performed in an animal operating theatre following aseptic rules. Penicillin G 40,000 U/kg was administered intramuscularly intraoperatively. Talc-free gloves were used at all times during the procedure. Two 5 cm incisions and one 2.5 cm incision were made directly over the skin and subpanniculus carnosus to introduce the implants and the expander, respectively. Pockets were developed in the subpanniculus carnosus with atraumatic dissection along the back region. Particular attention was paid to hemostasis under direct vision, avoiding blunt instrumentation, and there was no obvious bleeding. A sterile Op-site dressing was placed over the skin around the incision before inserting the tissue expander and the implant to avoid contact with the skin. Wearing a new pair of talc-free gloves, the surgeon introduced the implants and the tissue expander with intact connecting tube and port. In the experimental group, triamcinolone acetonide (Trigon depot®, Bristol-Myers Squibb, New York, NY, USA) was introduced into the implant and expander pocket. All wounds were closed with two planes of interrupted suture.

The rabbits were divided into two groups: (1) the control group with untreated implants and expander ($n = 10$), and (2) the triamcinolone group which had the introduction of 1 ml (40 mg) of TA into each implant pocket and 0.25 ml (10 mg) of TA into each expander pocket ($n = 9$). No fluid suction was performed to retain the TA (Trigon® depot) in the surgical pocket.

Rabbits were killed at 4 weeks. Before that, each animal was anesthetized and the dorsal back area was shaved. A pressure-measuring device (Stryker Instruments, Kalamazoo, MI, USA) was connected to the tissue expander port and intracapsular pressures were recorded at each 5 ml increment before any incision made to the capsule. Then, a 5-mm incision was made directly over the implant through skin, panniculus carnosus, and capsule. A 100,000 molecular weight cutoff microdialysis probe (CMA Microdialysis, Stockholm, Sweden) was placed near the capsule–implant interface and microdialysates were collected using sterile normal saline solution (6 μl/min) for 1 h. Whole blood was obtained by venipuncture and serum was collected after centrifugation ($2{,}000 \times g$ min^{-1}, 4 °C). All capsule samples underwent histological and microbiological evaluation and all implants and expander devices also underwent microbiological evaluation.

Microbiological Assessments

Air

Operating room air samples ($n = 24$) were collected during all surgical procedures using the MAS 100-Eco air sampler (EMD Chemicals, Inc., Gibbstown, NJ, USA) at a flow rate of 100 l/min. Identification of bacterial and fungal isolates followed standard microbiological procedures. Gram-positive cocci were characterized by biochemical methods. Catalase-positive and coagulase-positive isolates were reported as *Staphylococcus aureus*; catalase-positive and coagulase-negative isolates were reported as coagulase-negative *Staphylococci*. Gram-negative bacilli were characterized with Vitek 2 software (VT2-R04.02, bioMerieux, Inc., Durham, NC, USA). Fungi (molds) were characterized according to macroscopic and microscopic morphology.

Rabbit Skin

A total of 57 contact plates were pressed to the shaved dorsal skin surfaces (19 brain–heart agar, 19 mannitol salt agar, and 19 Sabouraud agar contact plates). Brain–heart and mannitol salt agar plates were incubated for 3 days at 28 °C, and Sabouraud plates were incubated for 7 days at 28 °C. The identification of the bacteria and fungi followed the procedures reported above.

Capsules/Implants/Tissue Expanders

Excised tissue expanders/implants, and representative capsule samples were incubated at 37 °C for 3 days in brain–heart broth and examined daily. Changes in the turbidity of the broth media were considered positive and were subcultured in solid agar media. Characterization of microbial isolates followed the above-described procedures.

Histological Assessment

Capsule specimens were fixed with 10 % buffered formalin and embedded in paraffin. Sections were stained with hematoxylin and eosin and evaluated histologically for tissue inflammation and capsular thickness. Both the type

of inflammatory infiltrate and the intensity were analyzed. The inflammatory cells was grouped into three categories: (1) mononuclear (lymphocytes, plasmocytes, and histiocytes), (2) mixed (mononuclear cells and eosinophils), and (3) polymorph (eosinophils and heterophils/neutrophils). Inflammatory infiltrate intensity was categorized according to the following criteria: absent (−), mild (+), moderate (++), and severe (+++) [44].

Samples were stained with Masson's trichrome to characterize the connective tissue (loose or dense), the organization of the collagen fibers (arranged in a parallel array or haphazardly), angiogenesis (absent, mild, moderate, or high), and fusiform cell density (mild, moderate, or high). The dense connective tissue was semiquantitatively analyzed as (a) ≤25 % with thick collagen bundles less than 25 %, (b) 25–50 %, (c) 50–75 %, and (d) >75 %.

Microdialysis Assessment

TNF-α levels were determined using Invitrogen's Hu TNF-α (catalog No. KHC3014:1; Life Technologies, Inc., Carlsbad, CA, USA). The assay was a solid-phase sandwich enzyme-linked immunosorbent assay (ELISA) in which 100 µl of microdialysis fluid was pipetted into each well. The protocol for IL-8 was performed using the BioSource Hu IL-8 US kit (catalog No. KHC0083/KHC0084; Life Technologies).

Data Analysis

Data were analyzed by groups: Control ($n = 20$) and Triamcinolone ($n = 18$). One-way analysis of variance (parametric or nonparametric) was performed to check whether the several means of continuous variables (histologically measured thickness and dialysate levels of IL-8 and TNF-α) were equal, followed by post hoc range tests to identify homogeneous subsets across groups. A two-tailed independent paired *t*-test and the nonparametric alternative Mann–Whitney *U* test were used to determine whether such continuous variables were likely to show differences between control and experimental groups. Categorical variables were evaluated by χ^2 statistics and by ϕ, Cramer's V, and contingency coefficients. Statistical significance was presumed at $p \leq 0.05$, and all analyses were carried out with SPSS software (SPSS, Inc., Chicago, IL, USA).

Results

Statistical analyses revealed no significant differences in the histological, immunological, and microbiological results between breast implants and tissue expanders (data not shown). The expanders were included in the protocol to determine the pressure–volume curves.

Clinical

In the triamcinolone group (Fig. 1), the capsules were thinner and more transparent than those of the control group.

Pressure

During pressure measurements, five (50 %) capsules ruptured in the control group. To avoid too little sampling, the ruptured capsules were not excluded from statistical analyses; however, in such cases, the pressure value measured before rupturing was maintained after further additional saline was added. Pressure–volume curves were generated for all rabbits that were killed. Statistical analyses revealed no significant differences between the triamcinolone group and the control group (Fig. 2).

Histology

Significant decreased capsular thickness was registered for the triamcinolone group compared with the control group ($p \leq 0.001$) (Table 1). A mixed cells were the most common finding in the control group and mononuclear cells were the most common finding in the triamcinolone group (Table 1). Significant differences were found between the control group and the triamcinolone group ($p = 0.0003$). A significant difference was observed between the triamcinolone and control groups ($p = 0.009$) with respect to the

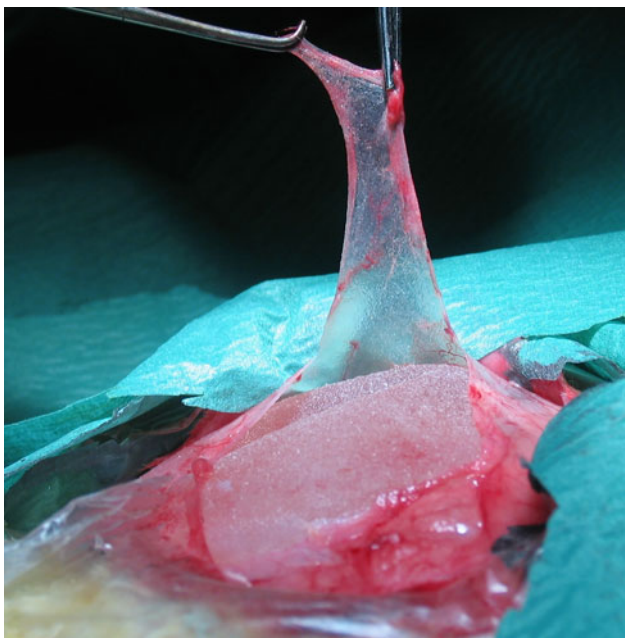

Fig. 1 Capsule in the triamcinolone experimental group

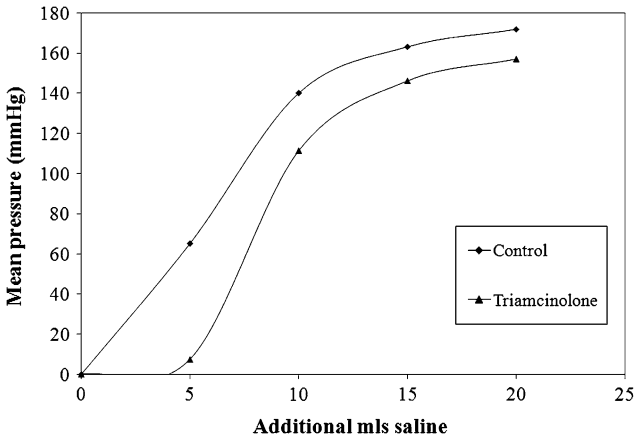

Fig. 2 The pressure–volume curves

intensity of inflammation, which was mild in the triamcinolone group and moderate in the control group (Table 1). No significant differences in the fusiform cell density, connective tissue, and organization of the collagen fibers were observed between the control and triamcinolone groups. Significant differences were found in angiogenesis between the control group, where it was basically moderate or high, and the triamcinolone group ($p = 0.007$), where it was negative or mild.

Microbiology

Statistical analysis revealed no significant difference in the type of bacteria and in the frequency of culture positivity for bacteria between the control and triamcinolone groups with respect to either implants or capsules. Also, there was no significant association between microbial presence and histological data. The predominant isolate was undoubtedly coagulase-negative *Staphylococci*, which was identified predominantly in the removed implants (Table 2). Isolated bacteria from the rabbits' skin and from the operating room air were statistically similar to those from the removed capsules and implants, with coagulase-negative *Staphylococci* prevailing.

No fungi were recovered from the removed capsules, implants, or skin samples of all rabbits. Fungal species, such as *Penicillium* spp. and *Aspergillus*, were recovered from the operating room air.

Immunology

The dialysate levels of IL-8 decreased from 115.56 ± 128.03 mg/ml in the control group to 54.41 ± 31.21 mg/ml in the triamcinolone group. Statistical analysis revealed no significant difference in the dialysate levels of IL-8 between the control group and the triamcinolone group. The dialysate levels of TNF-α decreased from 328.62 ± 307.55 mg/ml in the control group to 148.9177 ± 211.92273 mg/ml in the triamcinolone group. Statistical analysis revealed no significant difference in the dialysate levels of TNF-α between the control group and the triamcinolone group. There is a correlation between IL-8 and TNF-α in the control group ($p < 0.001$) and in the triamcinolone group ($p = 0.036$).

Table 1 Outcomes for capsular thickness and inflammation of control vs. triamcinolone groups

Group	Capsular thickness (mm)	Type of inflammatory cells	(%)	Intensity	(%)
Control	0.81 ± 0.209	Mononuclear (chronic)	25.0	Mild	30.0
		Polymorph (acute)	0	Moderate	70.0
		Mixed (active chronic)	75.0	High	0
Triamcinolone	0.53 ± 0.136	Mononuclear (chronic)	83.3	Mild	72.2
		Polymorph (acute)	0	Moderate	27.8
		Mixed (active chronic)	16.7	High	0

Table 2 Bacteria isolated from capsule and implant samples removed from all sacrificed rabbits

Bacteria	Group	No. of positive cultures	
		Capsules (%)	Implants (%)
Coagulase-negative *Staphylococci*	Control	2 (10)	13 (65)
	Triamcinolone	6 (33)	14 (78)
Staphylococcus aureus	Control	2 (10)	2 (10)
	Triamcinolone	2 (11)	2 (11)
Bacillus gram-positive	Control	1 (15)	1 (5)
	Triamcinolone	2 (11)	2 (11)

Data collected from control (10 rabbits; 20 capsules and 20 implants) and triamcinolone (9 rabbits; 18 capsules and 18 implants) groups

Discussion

The capsule that forms around the breast implant is composed by a layer of fibrous dense connective tissue [17], and is an integral part of the wound-healing process. To understand the formation of this late complication and the potential therapeutic roles of both pharmacological and nonpharmacological treatment approaches, it is crucial to know the physiological mechanisms that are behind the process that causes the formation of capsules.

Wound healing has been divided into three distinct phases: inflammation, proliferation, and maturation [42]. The first phase of wound healing, which begins immediately upon injury through day 4–6, is characterized first by hemostasis, an important event that serves as the initiating step of the healing process; and an inflammatory response. The second phase of wound healing (proliferative phase) is characterized by epithelialization, angiogenesis, and provisional matrix formation and courses from day 4 through 14, overlapping phases 1 and 3. Fibroblasts and endothelial cells are the predominant proliferating cells during this phase. The maturation and remodeling (phase 3), which occurs from day 8 through 1 year, is characterized by the deposition of collagen in an organized and well-mannered network [13].

As seen before, corticosteroids are known to have an important role in wound healing, as they can stop the growth of granulation completely, stop the proliferation of fibroblasts, diminish the new outgrowths of endothelial buds from blood vessels, and stop the maturation of the fibroblasts already present in connective tissue [7]. Also, when administered soon after injury, corticosteroids delay the appearance of inflammatory cells and fibroblasts; the deposition of ground substance, collagen, and regenerating capillaries; contraction; and epithelial migration [20]. Steroids can have an important role in CC formation in both the early and the late phase of fibrous phase formation.

The efficacy of triamcinolone in treating and preventing CC in women has been reported [15, 43]. However, this still represents an off-label practice and further studies are required to validate the efficacy of this approach. Both works have limitations: they were nonrandomized, with no control group, had a limited follow-up period [15, 43], and neither had as an objective the determination of the mechanism of action of TA in capsular contracture formation. A comprehensive understanding of the effects of TA on the mechanisms of capsular formation, the systemic side effects, and the potential adverse events are, in our opinion, crucial for the improvement of TA in clinical activity.

This study is the first to analyze the impact of TA in early capsule formation. We examined the effects of TA on pressure and histological, microbiological, and immunological characteristics of capsules in an animal model to understand the role of this steroid in early capsule formation and its possible role in the prevention of CC. In our study, TA was found to decrease capsular thickness upon macroscopic and microscopic examination when compared to the control group. These findings were also associated with decreased inflammation and angiogenesis, as was expected, as steroids are anti-inflammatory drugs capable of delaying the appearance of inflammatory cells and they diminish the proliferation of endothelium from blood vessels [7] and regeneration of capillaries [20]. Although no significance was found in the intracapsular pressure between the groups, a tendency to lower pressures (and no capsule rupture during the pressure measurement) was observed in the triamcinolone group compared to the control group (Fig. 2). Also, both cytokine markers (IL-8 and TNF-α) were lower in the triamcinolone group, even without statistic significance. No significant differences were observed in fusiform cell densities, connective tissue, or organization of collagen fibers. Taken together, these results suggest that the introduction of TA in the pocket intraoperatively has a role in capsule formation and might prevent CC.

Like Caffee et al. [16], we were not able to observe a significant decreased capsular pressure in the group treated with triamcinolone at the time of implant placement. However, in our study we went further and analyzed not only the pressure, an unquestionable indicator of capsular contracture, but also other characteristics that are related to the formation of this pathology as a continuous process. The breast capsule begins to be formed after implant placement; however, in clinical practice, the contracture is a late complication, and follow-up, as long as 42 months [33], is required to diagnose this entity. In preclinical models, there is no consensus on the timing for sacrifice and for representative stages for capsule formation. We were not able to observe significant differences in pressure between the groups, probably because we killed animals too early. However, we were able to observe the early alterations that are characteristic of capsule formation as thinner and more transparent capsules on macroscopic and microscopic evaluation and decreased inflammation and angiogenesis. It might be expected and is reasonable to assume that a more dense collagenous capsule with increased thickness would be present with longer incubation times, reflecting a continued stimulus toward fibroplasia and ultimately collagen remodeling [10, 12, 45].

Caffee et al. reported in both preclinical [16] and clinical [15] studies that triamcinolone injected postoperatively was able to eliminate CC and prevent the recurrence of this condition. Those findings were confirmed by Sconfienza et al. [43], who were able to demonstrate that US-guided injection of triamcinolone acetonide in the peri-implant pouch of women with augmented or reconstructed breasts

affected by Baker grade IV CC is effective in reducing capsular contracture. Both authors concluded that triamcinolone was effective in the late stages of capsule formation. With our study we were able to observe that triamcinolone is probably not only effective when injected postoperatively, but also has a role in the early phases of the development of capsular contracture.

In a previous report [36] that used the same protocol, the authors were also able to find another compound, fibrin (Tissucol/Tisseel) that was associated with a lower incidence of CC when sprayed in the pocket/implant during surgery and was more effective in the early phases of wound healing than in the later phases. It was found that fibrin [36] was able to decrease intracapsular pressures when compared to control ($p \leq 0.001$, data not shown), and the capsular thickness was decreased (0.47 ± 0.129-mm) ($p \leq 0.001$) as in triamcinolone group.

TNF-α plays an important role in the wound-healing process. It is produced by activated macrophages, platelets, keratinocytes, and other tissues and it stimulates mesenchymal, epithelial, and endothelial cell growth and endothelial cell chemotaxis [27, 32]. During the inflammatory phase, it draws neutrophils into the injured area [39], generates NO [26] from macrophages, and digests damaged extracellular matrix via matrix metalloproteinase [1]. During the second phase, TNF-α upregulates KGF gene expression in fibroblasts; upregulates integrins, a matrix component that serves to anchor cells to the provisional matrix; stimulates epithelial proliferation [32]; and is also a potent promoter of angiogenesis. TNF-α is known to be a growth factor for normal human fibroblasts and promotes the synthesis of collagen and prostaglandin E2. IL-8 enhances neutrophil adherence, chemotaxis, and granule release and enhances epithelialization during wound healing [30, 32]. TNF-α levels were reported to be markedly elevated in fibrotic diseases such as liver fibrosis and is considered a mediator of fibrosis like TGF-β1 [19]. Moritomo et al. [37] concluded that TNF-α played a pivotal role in the maintenance of hemostasis and tissue repair by inhibiting TGF-β1. We were not able to find significant differences in IL-8 and TNF-α levels, although decreased levels were observed in the group treated with triamcinolone, possibly reflecting a role for this drug in the modulation of the wound-healing process and fibrotic response in the presence of the implant. More studies, with longer follow-up and increasing doses of the compound, are needed to confirm these data.

On the other hand, a significant correlation was also found between IL-8 and TNF-α in both groups. This was not unexpected, as correlations between IL-8 and TNF-α with bacterial infections have been reported [48]. We did not find any differences in the microbiology cultures between groups, but further studies are necessary to clarify whether triamcinolone increases the risk of infection.

With fibrin, a significant decrease in TNF-α (140.9 ± 165.9 mg/ml) and IL-8 (23.9 ± 43.4 mg/ml) levels ($p = 0.003$ and $p = 0.048$) was observed, supporting the possible role of this compound in early capsule formation and in reduction of the collagen extracellular matrix. No correlation between IL-8 and TNF-α was observed in the fibrin group, which suggests a possible antibacterial role of fibrin [36].

The main limitations of this study were (1) inappropriate dosage in this model system; rabbits have much faster basal metabolic rates than humans, and, as such, it is presumed that rabbits have shorter drug half-lives [50]; (2) unknown pharmacokinetics of triamcinolone in the capsule pocket and subsequent metabolism, although triamcinolone modeling may be based on systemic steroid modeling [40]; (3) short follow-up, as capsular contracture usually takes more than 4 weeks to develop; and (4) the use of one tissue expander per rabbit to directly measure the internal expander pressures using the port. Silicone breast implants with ports with a 90 ml volume capacity would be optimal to achieve more accurate results but are not commercially available. In addition, the preclinical model would not support the use of multiple large expanders or implants over long time periods. Our data do support future studies examining triamcinolone as a potential agent for preventing CC.

Possible future studies may include (1) a preclinical study using silicone breast implants with ports (to measure the capsule pressure directly) and with introduction of 1.5 ml (60 mg) of triamcinolone acetonide into each implant pocket and sacrifice of the animals at a much longer time point with detection of IL-8, TNF-α, and TGF-β1 and determination of fibrosis index; and (2) with the same protocol, assessment of the effects of saline or other vehicles of triamcinolone acetonide on pressure/volume curves. We believe that in a preclinical study, a higher dose of triamcinolone acetonide introduced into the implant pocket and a longer follow-up time period will support the growing body of evidence that triamcinolone acetonide mitigates capsular contracture.

In summary, our results suggest that triamcinolone has a role in early capsule formation and it may have a role in the prophylactic management of this complication. Obviously, its role is centered on the management of the factors related to wound healing [49], and it is important to exclude a deleterious role in the factors related to infection that are also known to increase CC [2, 3, 14, 18, 41, 47]. The clinical intraoperative use of triamcinolone acetonide may prove to be a reliable and safe way to prevent capsular contracture in women undergoing breast implantation. The ultimate goal is to translate these preclinical results to the clinic, as these findings may help not only patients with breast implants, but all patients with any device in which

capsule contracture around that device may lead to an adverse clinical event.

Conflict of interest The authors have no conflicts of interest or financial ties to disclose.

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ABSTRACTS

Selected Abstracts from The Voice of Europe Session of the 4th Annual Congress of the EASAPS

(*Editorial Coordinator*: Cristino Suárez López de Vergara)

Marketa Duskova · Salvatore Giordano · Asko Salmi · Delmar Henry · Dirk F. Richter · Csaba Viczian · Huba Bajusz · Mario Pelle Ceravolo · Georges J. Ghanimé · Marisa Marques · D. Jianu · M. Filipescu · S. Adetu · Teresa Bernabeu · Selahattin Özmen · Cristino Suárez López de Vergara

Metamorphosis

Marketa Duskova (Department of Plastic Surgery, Charles University, Srobarova 50, 10034 Prague, Czech Republic, email: duskova@fnkv.cz)

The main aim of the aesthetic surgery is to improve quality of life. It is known that less attractive people find it harder to obtain a good personal and professional position in the society. The main point of interest and the most important aspect is the face because human attractiveness is specifically connected with facial appearance. In considering correction of the facial visage with a great change, the surgeon must pay attention, prepare meticulously with analysis of the situation, and choose a suitable approach according to the circumstances as a whole. Then surgery must be performed with perfect surgical technique, and the postoperative care must be carried out in close cooperation with patient, perhaps also with other specialities or even nonmedical experts.

The concrete process is shown in the case of a woman who underwent complete profiloplasty (rhinoplasty, chin reduction), teeth reconstruction, upper and lower blepharoplasty, augmentation of both lips by synthetic implant, and application of injectable fillers into facial wrinkles and rhytides. In addition, the beautician, hairdresser, image consultant, and stylist put the last touches on the outcome.

Only such complex treatment may increase the patient's mental stability, self-confidence, and quality of life. The more perfect the elimination of functional problems and stigmatizing disharmony, the better are the preconditions for patients' success and their assertion in society.

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Capsular Contracture After Cosmetic Breast Augmentation: Do Topical Antibiotics Matter?

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Introduction: Antibacterial lavage with topical antibiotics may reduce the occurrence of capsular contracture in breast implant surgery. A retrospective analysis was performed to investigate this effect.
Materials and Methods: The study participants included 308 women who underwent cosmetic breast augmentation during two different periods: 2004–2008 ($n = 168$, group A) and 2009–2010 ($n = 140$, group B). The same surgeon performed the surgery for all the women using the inframammary approach and the dual-plane pocket. All the patients had McGhan/Allergan 410 form stable textured implants. The group A patients received antibiotics as a single perioperative intravenous dose of cephalothin 1.5 g and cephalexin 750 mg as an oral course twice a day for 1 week after discharge. In the group B, perioperatively, 750 mg of cefuroxime was administrated intravenously. Implants and pockets were irrigated with 10 ml of 10 % povidone–iodine solution mixed with 750 mg of cefuroxime and 40 mg of gentamicin. After discharge, 500 mg of levofloxacin was administered as an oral course once a day for 10 days. The postoperative complications included occurrence of infection, seroma, and capsular contracture. We considered capsular contracture significant when it was graded Baker 3 or 4.
Results: The average postoperative follow-up period was 11 ± 13 months for group A and 3 ± 8 months for group B. No postoperative infections or seroma were detected. Group B had no capsular contraction cases. The capsular contraction rate was significantly higher in group A (5.9 vs. 0 %; $p = 0.003$).
Conclusions: The use of topical antibiotics in cosmetic breast surgery is recommended because a significant increase in capsular contracture was observed in patients not treated with topical antibiotics.

The Middle Third of the Face: Analysis, Techniques, and Indications

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The Aging Process: The aging process of the face acts in many modes including squeletization, ptosis, and desequilibrium of muscular

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balance, with loosened tissues and lack of firmness and structure. This gives modelization of the aging process in three modes: squeletization, ptosis, and fattening. This modelization gives the surgeon the opportunity to propose an adequate association of techniques.
Malar Elevation: The indication of the ptosis mode is elevation of the malar region. In 1994, we described, with F. Trepsat, a technique of low malar suspension with the buccal approach, which allows correction of the ptosis and transfer of volume from low to high. The aging process of the cheekbone is more superficial than deep. To address this, many authors improve the technique with a suspension of the orbicularis oculi by the palpebral approach. The goal for traction of the orbicularis is a superficial lifting of the skin of the cheekbone. But the weak point is a high traction in the palpebral region, which results in a deformation of the glance, with palpebral deformity. To enable correction for the superficial modification of the cheekbone without palpebral deformity, we propose a new technique with medical devices as follows:

- Subperiosteal dissection of the cheekbone using the buccal approach
- Installation of medical devices both superficially and deep
- Palpebral surgery and temporal lifting adapted to the indication.

This technique is called malar isolated positioning (MIP).
Indications: The indications for suspension of the cheekbone depend on the aging lower eyelid and its treatment. Without treatment of the lower eyelid, a buccal technique is recommended. In the situation of a blepharoplasty, an eyelid approach and bone fixation are proposed. The indications relate to the highness of the cheekbone. Indications and results are shown.

What Can Be Achieved Through an Upper-Lid Incision?

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Upper-lid blepharoplasty, one of the most demanded aesthetic procedures, is not just treatment for dermatochalasis. The upper blepharoplasty incision can be used to adjust retro-orbicularis oculi fat and for glabellar myotomy, lateral cantopexy, and browpexy (i.e., brow-lift). The transblepharoplasty brow-lift is suitable for the lateral two thirds of the brow. This technique is less invasive and allows an anchoring of the underlying brow soft tissue to the bone. This permits stabilization or elevation of the eyebrow without an endoscope because the nerves are under direct vision.

Another approach is corrugator supercilii muscle resection through a blepharoplasty incision, which is suitable for patients who have significant corrugator hyperactivity and deep frown lines without eyebrow or forehead ptosis. This procedure can be performed with or without a concomitant blepharoplasty.

Through an upper-lid incision, the blepharoplasty as well as the cantopexy, brow-lift, and resection of the corugator muscle can be performed with a less invasive technique and fewer scars, which leads to a high patient acceptance rate and satisfaction.

The Challenging Lower Eyelid Correction: The Aesthetic Effect of Lateral Orbicular Muscle Tightening

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Introduction: Aesthetic correction of the lower eyelids often is more difficult and challenging than correction of the upper eyelids. The characteristics of facial aging result not only from elastosis and sagging but also from atrophy of soft tissues, particularly the orbital septum and orbital fat. The evolution of orbital fat preservation and the midfacial volumetric concept taught clinicians to treat the lower eyelid with the midface as one aesthetic unit.
Methods: Besides the popular methods (arcus marginalis release, fat medialization, septorhaphy, fat transfer), the authors present their results with additional lateral tightening of the orbicularis oculi muscle.
Discussion and Conclusion: To recreate a youthful appearance of the lower lid, a clear indication for the choice of the correct operating method is needed. Before the procedure, the anatomy around the orbit, the eyelid laxity, the fat pads, the lid–cheek junction, and the position of the midface must be analyzed. The lower lid vectors will show the relationship between the anterior projection of the globe, the lower lid, and the malar bony eminence. The authors give special interest to the moderate elevation effect on the midface created by additional lateral tightening of the orbicularis oculi muscle. The authors present their algorithm, which may help in selecting the correct procedures for the lower eyelid and midface operations.

Animation Deformities by Pectoralis Muscle: The Cinderella of Submuscular Mammaplasty

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Animation deformities are present in almost every patient submitted to subpectoral augmentation mammaplasty. These deformities represent the most common complication related to the reported operation and yet are the least known.

Animation deformities have been studied by the author in more than 1,000 patients and classified according to clinical criteria in six different categories. Many patients treated with the dual-plane technique present with animation deformity despite the ability of this technique to avoid its occurrence.

The physiopathology of the deformity is related to the pulling action of the muscle on the breast mass and not to implant dislocation during the muscle contraction. The author presents his algorithm of different techniques used for submuscular augmentation mammaplasty based on different anatomic preoperative situations.

Preservation of pectoralis muscle costal insertions, medial pectoralis nerve section for muscle denervation, and horizontal muscle splitting are the main maneuvers used to avoid breast dynamic distortion. Horizontal muscle splitting consists of a horizontal incision performed in the pectoralis muscle that splits it in two flaps. The upper flap provides good coverage for the implant, whereas the lower flap may be left attached to the chest to improve the projection of the breast lower pole, or it may be rotated laterally or medially depending on the clinical demand.

Horizontal muscle splitting is a personal technique that the author has used during the last 10 years in more than 350 cases with aesthetically good results and a substantial decrease in the occurrence of animation problems.

Conservative Rhinoplasty

Georges J. Ghanimé (Division of Plastic and Reconstructive Surgery, Lebanese University, Faculty of Medicine, Lebanese Hospital, Getawi, Beirut, Lebanon)

Currently, rhinoplasty is one of the most popular aesthetic surgical procedures. This has led to refinement of the techniques, making them simpler and more reliable and minimizing soft tissue trauma by using the least invasive technique to accomplish the predetermined goals.

Our experience includes more than 4,000 rhinoplasties performed since 1992. The majority of the cases are managed by same-day surgeries performed with the patient under general anesthesia using only the closed approach. Because form and function work together, a septoplasty is performed when there is septal deviation.

Our experience has led us to the conclusion that conservative rhinoplasty is indicated in most cases.

Effects of Fibrin (Tisseel/Tissucol) on Breast Capsule Formation in a Rabbit Model

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Background: The etiology and clinical treatment of capsular contracture remain unresolved because causes may be multifactorial. The previously described environmental challenges that accelerate capsule contracture have been bacteria, especially coagulase-negative staphylococci. The role of fibrin in capsule formation was controversial in various independent studies. Study 1 aimed to influence capsule wound healing with blood, fibrin, and thrombin, and to make a comparison with a control group in a rabbit model implanted with tissue expanders. To clarify the results of this first study, study 2 was performed to determine whether fibrin and coagulase-negative staphylococci modulated capsule formation in a rabbit model implanted with a tissue expander and breast implants.
Methods: Study 1: Each New Zealand white rabbit ($n = 18$) received four different tissue expanders and then was killed at 2 or 4 weeks. The four study groups were the control, fibrin, thrombin, and blood cohorts. Study 2: Rabbits ($n = 31$) were implanted with one tissue expander and two breast implants and then were killed at 4 weeks. The implant pocket groups included the control ($n = 20$), fibrin ($n = 22$), and coagulase-negative staphylococci (CoNS) cohorts ($n = 20$). Pressure and volume curves as well as histologic and microbiologic evaluations were performed. Operating room air samples and contact skin samples were collected for microbiologic evaluation.
Results: Study 1: At 4 weeks, significantly lower intracapsular pressures were measured in the experimental fibrin and thrombin groups than in the control group. For the control and fibrin groups, mixed inflammation was correlated with decreased intracapsular pressures, whereas mononuclear inflammation was correlated with increased intracapsular pressure. The predominant isolates in capsules, tissue expanders, and rabbit skin were coagulase-negative staphylococci. For the fibrin and thrombin groups, cultures other than staphylococci and negative cultures were correlated with decreased intracapsular pressures, whereas staphylococci isolation was correlated with increased intracapsular pressures. Study 2: In the fibrin group, significantly decreased intracapsular pressures, thinner capsules, loose or dense (<25 %) connective tissue, and negative or mild angiogenesis were observed. In the CoNS group, increased capsular thicknesses and a polymorph type of inflammatory cells were the most common findings. Similar bacteria in capsules, implants, and skin were cultured from all the study groups. A Baker grade 4 contracture was observed in an implant infected with *Micrococcuss* spp.
Conclusion: Fibrin (Tisseel/Tissucol) was associated with reduction of capsule formation in our preclinical animal model, which makes fibrin an attractive potential therapeutic agent for women undergoing breast implants. Clinical strategies for preventing bacterial contamination during surgery are crucial because low pathogenic agents may promote capsular contracture.

Face and Neck Rejuvenation Using Combined Techniques: Laser Lipolysis, Fractional Laser, Liposuction, and Lipofilling

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Background: This study assessed the role for the combined use of fractional laser (CO_2 laser) and laser lipolysis (980-nm diode laser) for face and neck rejuvenation.
Methods: From September 2008 to February 2011, 39 subjects underwent laser treatments for facial and neck rejuvenation. The treatment consisted of using laser lipolysis (980-nm diode laser MedArt), sometimes with additional facial fractional laser CO_2 MedArt. Laser lipolysis was performed to restore the jaw line and the mandible–neck angle respectively for laxity of the jaws and the anterior cervical part. After tumescent anesthesia, a 1.5-mm-diameter needle (80 mm long) housing a 600-μm optical fiber was inserted into the subcutaneous fat. The cannula was moved in predetermined lines to obtain a homogeneous distribution in the treated area. The laser settings were 10–11 W in relation to the thickness of the subcutaneous fat and dermis. In some cases, additional fine liposuction and lipofilling were necessary. The settings for the fractional laser used in face rejuvenation usually provided a 10-W, medium-density beam for 4 ms. For eyelids, the settings provided an 8-W, high-density beam for 5 ms.
Results: A total of 108 laser lipolysis procedures were performed for 39 patients. The areas treated were the jaws (9 patients) and the jaws together with the anterior part of the neck (30 patients). The mean cumulative energy was 1,800 J for the jaw area and 3,000 J for the neck. Contour correction and skin retraction were noted after 4–7 days for almost all the patients.
Conclusion: This clinical study demonstrates that removal of fat in small volumes with concurrent subdermal tissue contraction can be performed safely and effectively using a 980-nm diode laser. Additional benefits include excellent patient tolerance and a quick recovery time. The study also confirms that accumulated energy derived from fractional laser combined with laser lipolysis is safe and can improve the contraction and skin regeneration, leading to a better rejuvenation of the face and neck.

Combined Mastopexy and Breast Augmentation

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Background: Combined mastopexy and breast augmentation, first described by Gonzalez Ulloa [1] and Regnaul [2] in 1960, has seen an increase in demand in recent years. Whereas a woman previously was satisfied with a mastopexy alone, currently, the patient herself demands the combination of filling and lifting of the breast in a single procedure with the smallest possible scar. These patients are, without doubt, influenced by the increasingly widespread images of the aesthetic appearance conferred by breast implants and the growing trend to maintain C- or D-cup breasts. Driven by these demands, plastic surgeons have increased the indications for this type of intervention and the frequency of their use. These interventions present difficulties and potential risks and can become absolute disasters [3]. The steps to follow are selection of the patient, selection of the mastopexy technique, glandular resection, and implant selection, with all these steps aimed at achieving the aesthetic objectives while leaving minimal and inconspicuous scars.
Methods: The literature contains different rules [4–7] regarding the selection of mastopexy technique based primarily on the distance from the sternal notch and nipple to the areola and inframammary

fold together with the degree of breast ptosis. These rules are helpful, although they may vary from one procedure to another and become modified over time according to this author's experience. In addition to this, before surgery, there is a degree of uncertainty regarding the choice of technique, which in many cases does not become clear until the breast implant is in place. The method used to achieve a longer-lasting result of mastopexy combines three factors: an anatomic implant with its different projections and heights to help prevent recurrence of ptosis, glandular resection as required, and the subfascial placement of the prosthesis, which produces greater concordance and harmony between the implant and the mammary gland.
Results: The results obtained in the last 2 years with the aforementioned method are aesthetically better and have lower rates of complications than those previously obtained by the author.
Conclusion: Subfascial positioning of anatomic implants with maximum projection and glandular resection as required help to provide greater durability in mastopexy.

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Of Form, Function, and Aesthetics in Nose Surgery

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Traditional rhinoplasty operations depend on cartilage, bone, or both, and sometimes soft tissue resections. In modern nose surgery, however, the function and the aesthetic appearance should be seized together.

Resections could be limited mainly to three cartilaginous areas, with cartilages reconstructed in these areas: lower lateral (alar) cartilages, upper lateral cartilages, and septal cartilage.
Nasal Tip Region: Providing a natural-appearing nasal tip contour has always been a key component of a successful rhinoplasty. One prerequisite for a successful rhinoplasty is nasal tip support and its influence on nasal tip projection. Alar cartilages are the chief providers of structural support to the tip of both the nose and the external nasal valve.

To reshape the nasal tip, we use the sliding alar cartilage (SAC) flap, a novel technique for nasal tip contouring and support. The SAC technique [1]

- Provides an aesthetically acceptable and naturally good-looking nasal tip and alar contour
- Supplies effective nasal tip support and could be used for "pinch nose" deformity
- Does not require any cartilage graft and thus results in no donor-site morbidity
- Involves minimal or no risk for malposition, distortion, or resorption because it is a flap secured with sutures
- Results in a nonpalpable cartilage graft, in contrast to other cartilage grafts, because it is prepared from the original alar cartilage and placed under the caudal alar cartilage
- Produces no unwanted effect on the external nasal valve function because the connection between the upper lateral cartilages and the alar cartilages is not broken
- Reserves the cranial parts of the alar cartilages, allowing for their use in the future whenever there is a need (e.g., septal perforations)
- Uses a flap with a double-layered alar cartilage, which can supply more resistance against the thick tip in some noses that poses a real challenge.

Middle Vault: Another point is the resection of the upper lateral cartilages. Upper lateral cartilages are attached to the septum in an obtuse angle forming a T shape. Dorsal hump reduction during rhinoplasty almost always breaks this connection and can create both functional and aesthetic problems if performed incorrectly. We preserve the upper lateral cartilages using the upper lateral cartilage fold-in flap technique [2]. This technique has a combined spreader or splay graft effect without cartilage grafts.

The upper lateral cartilage fold-in flap technique might be applicable for almost all primary rhinoplasty patients because the previous physiologic structure is reconstructed. It also is suitable for patients who have not undergone previous dorsal hump removal.

To have a splay effect, only mucoperichondrial sutures should be used, and at least a 1–2-mm middle nasal vault reduction is necessary. For narrow noses, to prevent a very wide appearance in the middle nasal vault, transcartilaginous mattress sutures should be used. Suturing the mucoperichondrium over the cartilages could supply a smoother dorsum at the middle vault.

Although it is possible to use this technique with closed rhinoplasty approaches, it is easier with the open approach. This technique is not suitable for secondary rhinoplasty cases, in which upper lateral cartilage resection has already been performed. In these cases, spreader or splay grafts might be applied.
Nasal Septum: Septoplasty: Excessive resection of the septal cartilage or bone leaving an L-strut is the technique most surgeons prefer. But the weakened septum could collapse in a relatively minor trauma.

In most septal deviations, the problem is mostly related to the bony septum, including the maxillary crest and the perpendicular plate of the ethmoid bone or vomer. The cartilage usually is not broken, only bent in an anteroposterior or craniocaudal direction. In most cases, just releasing these bonding factors by removing deviated bones and bone spurs leads to a relaxation in the cartilage, with cartilaginous resection unnecessary or minimal.

On the other hand, the cartilage should be excised or reconstructed if there is a fracture or cartilage excess.

Consequently, septal deviations should be corrected very meticulously, and septal cartilages and bones should not be excised when it is not necessary. They should be reconstructed in-site and in an extracorporeal fashion whenever needed.

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