JOURNAL OF MATERIALS SCIENCE: MATERIALS IN MEDICINE **15** (2004) 1355–1360

Ki67, HSP70 and TUNEL for the specification of testing of silicone breast implants *in vivo*

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This investigation of capsular tissue adjacent to silicone breast implants concerns the long-term tissue response to the implant environment.

Fifty-three silicone breast implants have been analyzed at the time of explantation. The implant duration ranged from 2 months to 153 months. The reason for explantation was capsular contracture (57%), dissatisfaction with the effect (11%), local inflammation (6%), implant rupture (4%) and exchange of tissue expanders (21%). The cell turnover within the interface of the silicone device and the fibrous capsule was detected by specific antibodies against Ki67 for cell proliferation, by TUNEL for apoptosis, and by DNA strand breaks and heat shock protein 70 (HSP70) for cell stress.

We found a negative correlation between the expression of HSP 70 and the capsular thickness ($p < 0.043$) and decreased levels in specimens obtained from Baker IV implant capsules. Ki67, and TUNEL were significantly positive ($p < 0.001$ for both) and HSP 70 were significantly negative ($p < 0.001$) with signs of inflammation. Both Ki67 and TUNEL indicated decreasing values over time.

Ki67 and TUNEL showed no correlation with clinical signs of implant failure, such as the Baker score. The expression of HSP70, on the other hand, was connected with structural changes of the implant capsule, in terms of capsular thickness and the Baker score.

1. Introduction

Silicone breast implants continue to be the focus of many studies that seek to correlate implant failure with clinical and pathological factors. Earlier studies have emphasized the outcome analysis in silicone breast implants [1–6]. In addition to the risks of systemic side effects, there is concern that local complications may be the main problem in the use of silicone breast implants [7–12]. To date, little is known about the long-term biocompatibility of silicone implants and their modifications in humans. The actual significance of the cellular events in the interface between the biomaterial and the host tissue is unconfirmed, in particular with reference to the clinical outcome in the long term [13, 14].

Our objective was to define the cellular activity in the immediate vicinity of the implant shell. We searched, on the one hand, for patterns of cellular activity or damage that would define the reaction of the environment to the biomaterial and, on the other hand for a correlation between any recognizable immunohistochemical characteristic and the patient history data. Cellular response to silicone implants was examined using antibodies against Ki67 to test proliferation activity, apoptosis and DNA strand break (TUNEL) and cellular stress response (HSP 70). To date, there has been no study that demonstrates the reaction of Ki67, TUNEL and the induction of HSP 70 in the environment of silicone breast implants. Such a study would be a major contribution to the evaluation of the biocompatible function of breast implants *in vivo* [15]. Cumulatively, the relevant parameters could form a supplement to conventional histology and morphometry.

2. Materials and methods

Over a three-year period (1998–2001), a total of 53 silicone breast implants and surrounding fibrous implant capsules were obtained at the Department of Obstetrics and Gynecology. The mean age of the patients was 51.1($\pm$11.5) years. Silicone devices were explanted from subglandular (35) and subpectoral (18) implant sites. A total of 23 (43.4%) smooth and 30 (56.6%) textured silicone devices were obtained. All the smooth devices and 18 of the textured implants were gel-filled while twelve textured devices were saline-filled. Polyurethane-coated implants were excluded. We

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selected the cases on the basis of the availability of sufficient capsule material and clinical data for purposes of this study. Full clinical data on the implantation and the reason for explantation were obtained from 43 patients. In the cases of capsular contracture, the Baker score was determined before explantation in order to quantify the symptoms of pain and the firmness of the capsule.

The complete capsules and the implants were sent to the Department of Pathology.

Morphological study: Breast implants and adjacent tissue of the fibrous capsule were examined in the fresh state as received immediately from the operating theatre. All capsular specimens were studied by means of light microscopy. Tissue samples from representative areas in the lower front of the implant were sliced into 0.3×1 cm pieces and embedded in paraffin. In addition, visibly conspicuous areas, such as folds or node formations, were investigated. Between 10 and 15 sections of 5 μm thickness were stained with hematoxylin and eosin (H&E), periodic-acid Schiff plus diastase and Elastic van Gieson.

Immunohistochemistry: Light microscopy was checked by immunohistochemistry that was performed on the material embedded in paraffin using the avidin-biotin complex method with diaminobenzidine as a chromogen. The procedure was repeated twice for each sample at different points in time.

Antibodies: Antibodies used in this study included polyclonal rabbit anti-heat shock protein (HSP) 70 A500, 1:200 (DAKO, Hamburg, Germany) and monoclonal anti-HSP70/HSC70 SPA-820, 1:200 (BIOMOL, Hamburg, Germany) as markers for the cell stress response as well as monoclonal human Ki67 (=MIB1) dia 505, 1:10 (DIANOVA, Hamburg, Germany) as marker for cell proliferation.

TUNEL: Tunel histochemistry was performed by an *in situ* apoptosis detection kit (APOPTAG®, ONCOR, Cat. No. S7100, Germany). Briefly, 4 μm thick, paraffin embedded sections were taken from the each specimen, affixed to slides by heating at 60 °C, deparaffinized, and rehydrated. After digestion with 0.02% trypsin in phosphate-buffered saline (PBS) at room temperature and washes in PBS, the sections were first incubated in buffer A (200 mmol/l potassium cacodylate, 0.025 mmol/l Tris, 0.25 mg/ml bovine serum albumin at pH 6.6) for 5 min. The sections were then incubated with a labeling solution containing TdT, biotinylated-16-dUTP, 1.5 mmol/l cobalt chloride in buffer A at 37 °C for 60 min. The reactions were terminated by rinsing in a stop/wash buffer (300 mmol/l sodium chloride and 30 mmol/l sodium citrate at pH 7). The sections were then washed in PBS three times for 5 min. For light microscopy, the labeled DNA fragments were visualized by incubating the sections with streptavidin-conjugated alkaline phosphatase followed by reaction with a medium containing fast red as chromogen. The slides were then washed, counterstained with methyl green, and mounted in Permount medium.

Morphometry: The morphometric evaluation consisted of a quantitative analysis of the cell response. The cells were counted with a grid of 10 points in the interface capsule/implant tissues (0–150 μm, 400×, area 625 μm^2) in 10 fields/slide.

Statistics: The data obtained was examined and processed by one of the authors, a biostatistician (U.K.). The influence of the clinical data on the cellular response was tested for significance by performing an ANOVA with LSD-modification according to Bonferroni. The Pearson's correlation coefficient was calculated as an indicator for the association between clinical and immunohistological data. Multivariate analysis was used to verify independent effects for indication for implantation, patient age, implant duration, implant position, capsular thickness and the Baker Score before implantation. Statistical significance was considered at $p < 0.05$. Statistical tests were performed using SPSS (Statistical Package for the Social Sciences, Chicago, Ill., USA).

3. Results

A total of 53 silicone breast implants including the adjacent fibrous capsule were examined. The implants came from 43 patients with an age range from 24 to 75 years. The reason for augmentation was breast reconstruction following breast cancer in 30 cases (56.6%) and cosmetic augmentation in 20 cases (37.7%).

The main indication for explantation in the 30 breast reconstruction cases (56.6%) was capsular contracture with symptoms such as pain, breast hardening and deformation of the breast. The median implant duration in cases of contracted capsules was 153.4 months. In 6 cases, (11.3%), dissatisfaction, after 20 months, with the effect of the implantation was the reason for explantation. Local inflammation of the implant plane occurred in 3 cases (5.6%), after 2 months, and rupture of the implant wall in 2 cases (3.7%) with a median implant duration of 10.5 months. In 11 cases (20.8%) the explantation of an inflatable implant was part of an anticipated two-stage reconstruction. The mean duration of these devices was 7.1 months. These details refer to the number of implants, not to the number of patients.

3.1. Macroscopic and histological findings

The macroscopic evaluation of the silicone devices revealed consistently the presence of a fibrous capsule that varied in thickness, adjacent to the outer implant shell. In cases in which capsule formation was more intense, it resulted in considerable shrinkage and folding of the implant surface, indicating the constricting nature of fibrous implant capsules. The fibrosis was the histological equivalent of a chronic persistent reaction to a foreign body that results in various histological indications related to the duration of the implant. A predominant foreign body reaction of the early implant period are giant cells passing into a synovial-like metaplasia opposite the implant surface, as seen in the environment of tissue expanders. The long-term foreign body reaction was reflected by the heterogeneity in the number and compactness, and the content of the collagen within the fibrous capsule. Collagen fibers within the capsule tended to be oriented parallel to the surface. Within the capsule, we found histiocytic cells and rounded empty spaces containing silicone gel on the inner side of the

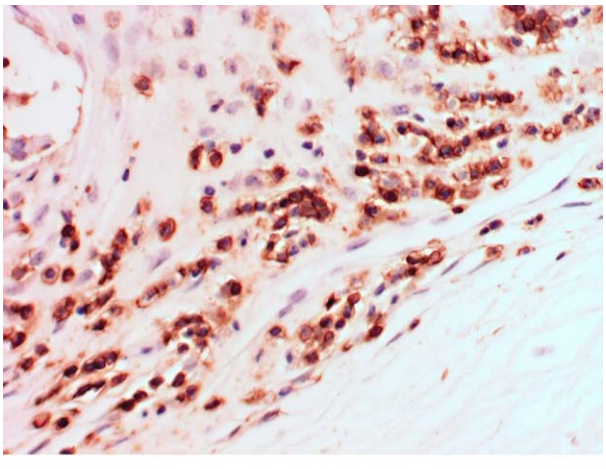
fibrous capsules. A differentiated analysis of breast tissue in the direct environment of the implant was not possible, since it was largely scarred when identified on the external aspect of the capsule.

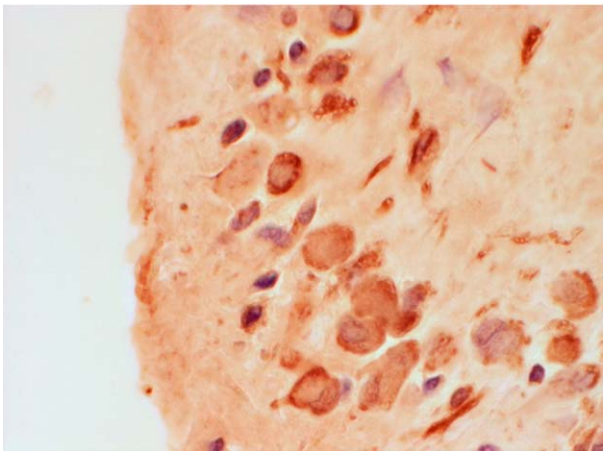
3.2. Cellular response and clinical correlation

The cellular reaction in the fibrous implant capsule surrounding breast implants has been analyzed with an antibody/biomarker for proliferation (Ki67), DNA-damage and apoptosis (TUNEL), and cellular stress (HSP 70). We analyzed cellular layers in direct contact with silicone implants with different surface properties, implant position, and varying implant indications, expecting to find higher rates of cellular activity because of an assumed higher rate of reorganization in this area (Figs. 1–5).

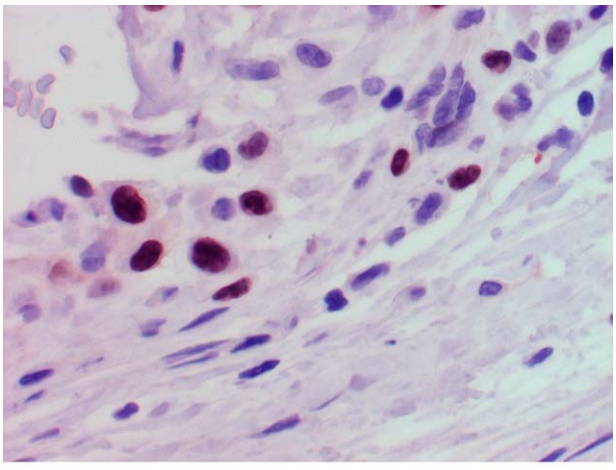
We found no significant correlation between the induction of Ki67, TUNEL and HSP 70 and the age of the patient at the time of implantation, the implant duration, the initial indication for the implantation (breast reconstruction or breast augmentation), the plane of implantation (submuscular or subpectoral) and the surface properties of the implants (smooth or textured). HSP70 showed a minor but insignificant decrease in capsules removed for Baker IV (Fig. 9). The various types of implant (temporary tissue expanders, saline-filled implants, silicone gel implants, and different surface properties) did not result in any characteristic pattern of correlation with our biomarkers.

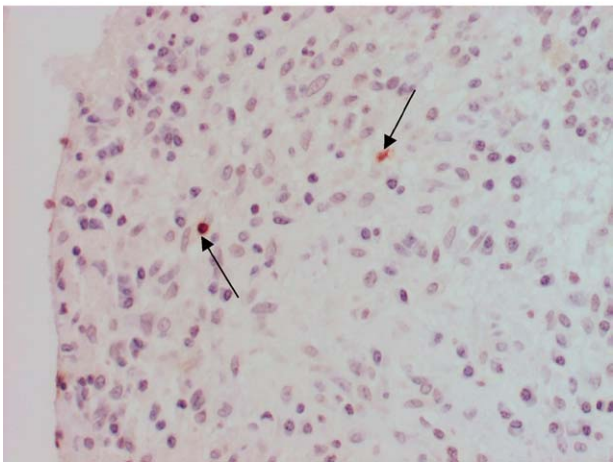
When searching for correlation with histological results of the fibrous implant capsule, we found a significant negative correlation between the expression of HSP 70 and the capsular thickness ($p < 0.043$) (Figs. 6





Figures (1 and 2) Cell response to silicone breast implant removed for capsular contracture (Baker IV): Specific intracytoplasmatic staining with antibodies against HSP70. Multinucleated giant cells and macrophages in the fibrous implant capsule surrounding a silicone breast implant.





Figures (3 and 4) Implant capsule, proliferating macrophages with a specific intranuclear staining with antibodies against Ki67. Silicone breast implant removed for painful capsular contracture (Baker III).

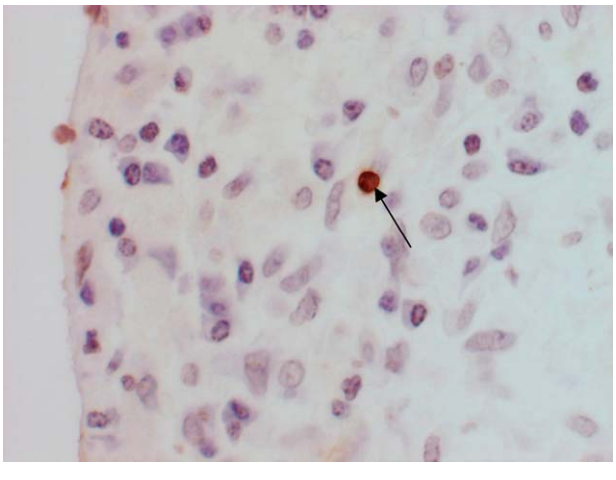


Figure 5 Apoptotic cells in the implant capsule, stained with antibodies against TUNEL.

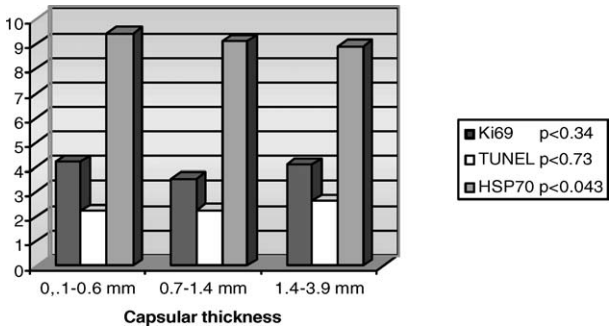


Figure 6 Correlation between values of HSP70 (/10), TUNEL and Ki67 in percent and capsular thickness in mm.

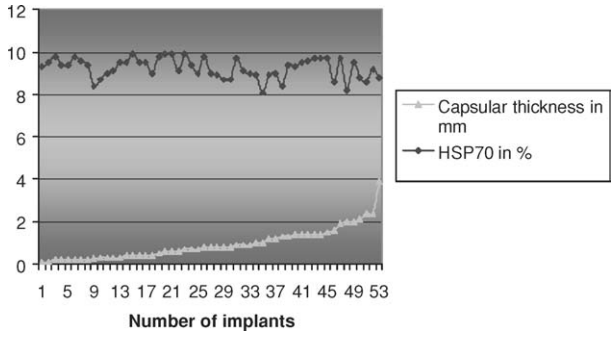


Figure 7 Relation between the expression of HSP 70 (/10) and capsular thickness of the fibrous implant capsule.

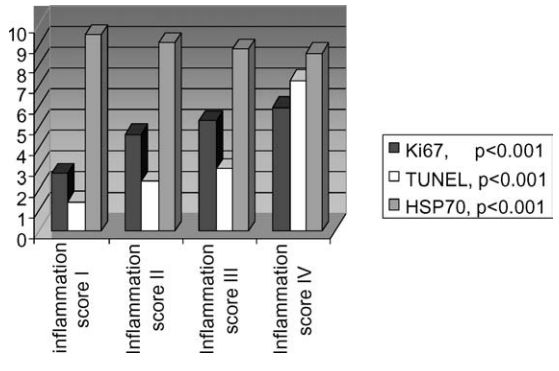


Figure 8 Direct correlation between the degree of cellular inflammation and the percentage of Ki67 and TUNEL. Inverse correlation between inflammation and HSP70.

and 7). We have to be cautious, however, in interpreting the biological significance. The histological presence of inflammation showed a significant correlation with Ki67, and TUNEL ($p < 0.001$ for both). HSP 70 was negatively correlated ($p < 0.001$) with histological signs of inflammation (Fig. 8). We found no

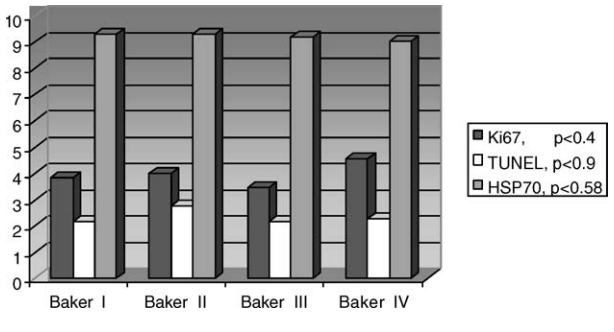


Figure 9 Correlation between the Baker score and the percentage of Ki67, TUNEL and HSP 70 (/10).

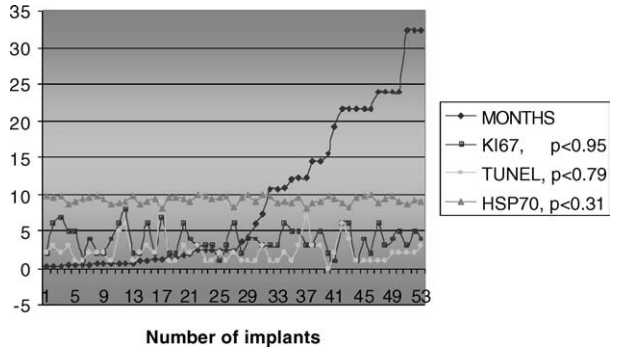


Figure 10 Values of HSP70 (/10), TUNEL and Ki67 in % in relation to implant duration in months (/10).

correlation with the presence of silicone leakage in the fibrous capsule, calcification of the capsule, or evidence of synovial-like metaplasia.

There was evidence of a slight, but continuous, decline of the expression of TUNEL and Ki 67 and the release of HSP 70 with the implant duration (Fig. 10). This observation is not significant. The definitive values are presented in Tables I and II.

4. Discussion

Modern silicone breast implants have proved suitable for breast augmentation and reconstruction. Today, there appears to be ample evidence of the safety of silicone gel-filled breast implants [1, 16]. However,

TABLE I Mean values of Ki67, TUNEL and HSP70 in % corresponding to different degrees of capsular thickness

Capsular thickness In (mm)	*n*	Ki67 in % (Standard-deviation)	TUNEL in % (Standard-deviation)	HSP70 in % (Standard-deviation)
0.1–0.6	22	4.23 (1.9)	2.23 (1.3)	94.1 (4.01)
0.7–1.4	22	3.52 (1.6)	2.28 (1.6)	91.33 (4.4)
1.5–3.9	9	4.12 (1.7)	2.62 (2.4)	89.25 (4.7)

TABLE II Mean values of Ki67, TUNEL and HSP70 in % corresponding to the Baker score and the degree of inflammation

Baker score/ Inflammation score	*n*	Ki67 in % (Standard-deviation)	TUNEL in % (Standard-deviation)	HSP70 in % (Standard-deviation)
Baker I	17	3.82 (2.12)	2.17 (1.19)	92.7 (4.1)
Baker II	10	4.0 (2.0)	2.77 (2.5)	92.9 (4.5)
Baker III	14	3.46 (1.3)	2.15 (1.7)	92.46 (5.63)
Baker IV	12	4.54 (1.37)	2.27 (1.8)	89.9 (3.1)
Inflammation score I	27	2.77 (1.13)	1.41 (0.68)	95.0 (3.17)
Inflammation score II	13	4.66 (1.79)	2.41 (1.03)	91.5 (3.66)
Inflammation score III	10	5.33 (0.94)	3.0 (1.3)	88.55 (3.83)
Inflammation score IV	3	6.0 (0.5)	7.3 (1.5)	86.0 (4.0)

local complications may occur, such as capsular contracture and silicone bleeding through the membrane of the prosthesis into the surrounding soft tissue [17–19]. The most common clinical reasons for explantation are pain and increased hardening of the breast, loss of implant integrity and local infection of the implant plane [7, 12]. Specimens of the fibrous implant capsule obtained when the implant is removed after different implantation times provide insight into the behavior of the close environment of these implants and give a general impression of the biocompatibility of these devices.

Surprisingly little data on the long-term biocompatibility of silicone breast implants is available, despite the fact that these implants have been in clinical use for four decades. In addition to surgical meshes, silicone breast prosthesis represents the group of alloplastic implants most frequently used in modern medicine. Between 1999 and 2001, we systematically collected and analyzed different types of breast implants at the Department of Obstetrics and Gynecology. By 2001, fifty-three different implants had been collected.

The main question to be answered is whether cell response changed during the time of implant duration and whether these results could be correlated with the clinical reason for the removal of the implant. An integral part of current biocompatibility testing involves the demonstration of cell proliferation. It is taken as a sign of diminished acceptance of the implant when the materials sustain or promote cell proliferation. The immunophenotypic studies of breast capsules conducted to date have not been able to demonstrate the presence of any specific pattern of lymphocyte and histiocyte response that would suggest an immune or sensitivity process [13].

We investigated the tissue response on silicone implants using a biomarker for the detection of proliferating processes (Ki 67), cellular stress response (HSP 70) and Apoptosis and DNA strand break (TUNEL). Tissues were tested in the immediate vicinity of the implant, with the expectation of higher rates of cellular proliferation and damage. Assuming silicone to be an inert biomaterial, we were expecting an adaptive stress response in the implant vicinity. The molecular rationale responsible for an adaptive cell response to stress is assigned by the expression of a variety of intracellular stress proteins responsible for cellular homeostasis. As an acute stress response, heat shock is characterized by reversible cellular changes at the cell metabolism level, which allows the adaptation to non-physiologic conditions. This defense mechanism is known to be triggered by miscellaneous injurious agents such as ischemia, heavy metals, proinflammatory mediators and hyperthermia. It has been shown that polymers are capable of inducing HSP 70 [15, 20].

Our study did not show any specific pattern of cellular reaction concerning clinical data such as: the reason for the implantation, the length of implantation, the operative procedure, the implant plane or the kind of silicone device. We were not able to produce any significant evidence of activation or damaging of capsular tissue in correlation to the incidence and severity of the symptoms at the time of implant failure. In contrast to the previously reported differences in the severity and quantity of the inflammatory reaction [21], the local cell response indicates a uniform reaction to silicone breast implants with low rates of proliferation ranging between 3 and 4% and low rates of apoptosis between 2 and 3%. These data suggest an insignificant cellular turnover in the capsular tissue surrounding silicone breast implants in the long term.

We found a significant inverse correlation between HSP70 and capsular thickness and a slight but insignificant decrease of HSP 70 in specimen from Baker IV implant capsules. We concluded that this relationship was an impaired cellular adaptation to stress in the presence of increasing capsular thickness, although the actual measured values were, in all cases, relatively small. Caution is necessary, therefore, in interpreting the biological significance. The intrinsic impact of HSP 70 is anti-inflammatory. In an earlier study, we found a direct correlation between a chronic persistent inflammation and increasing capsular thickness [21]. Based on these results, we expected significantly higher levels of Ki67 and TUNEL in specimens with high values for capsular thickness and the Baker score but the evidence showed only a slight trend (Figs. 6 and 9). This suggests that, in the long term, HSP70 can play an important role in the definition of the biocompatibility of silicone breast implants. HSP70 could be a suitable biomarker for the examination of innovative polymer surfaces that are used in future implants. The very surprising result was the little difference found in the amount of apoptosis and proliferation in the implant environment in breast implants removed for clinical failure. This points to higher rates of proliferation or apoptosis not being part of the mechanisms that lead to implant failure.

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Received 15 October 2003
and accepted 27 April 2004