Investigating mechanisms of breast implant failure and the role of radiotherapy

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Author's Declaration Statement

I, Louise Jane Magill, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Signed

Acknowledgements

I would like to thank my supervisors Dr Gavin Jell, Prof Keshtgar and Prof Mosahebi for their support and guidance throughout my project and write up. I would also like to thank Dr Kate Ricketts and Arnold Darbyshire for their support.

Dedication

For my husband Francis and daughter Sophie.

Abstract

Breast implants have a role in both aesthetic and reconstructive surgery. They are however, associated with long-term complications including capsular contracture (a fibrotic encapsulation of the implant), implant rupture and leakage often necessitating further corrective surgery. The mechanisms driving these complications are not fully understood. Indications for post mastectomy radiation therapy are expanding leading to more patients with implant based breast reconstructions receiving it. The aim of this thesis was to investigate failure mechanisms of breast implants and the role of radiation therapy in its pathogenesis.

Meta-analysis was performed investigating the clinical outcomes of PMRT directly upon the permanent implant in patients undergoing breast reconstruction. Retrieved breast implants and the corresponding capsular tissue from patients were collected and their material characteristics and histology studied. Un-implanted (control) Silicone breast implant shells were submitted to treatment dose radiation therapy and their material characteristics evaluated and compared to those of casted PCU and POSS-PCU.

Meta-analysis demonstrated increased surgical complication rates and poorer patient satisfaction and cosmetic outcome in the PMRT group. Retrieved breast implants demonstrated a significant reduction in mechanical strength properties with increasing duration of implantation but there was no correlation with thickness of the corresponding retrieved fibrotic capsule. Treatment dose radiation to un-implanted silicone breast implant shells had no overall significant effect on its material characteristics or *in vitro* cellular response. This was in keeping with the response to PMRT of PCU and POSS-PCU, however POSS-PCU demonstrated different mechanical properties in comparison to silicone.

These results indicate that although radiation therapy is significantly associated with poorer clinical outcomes for patients with implant based reconstruction, it is not due to alterations in the mechanical strength and surface chemical properties of the

silicone implant shells. Therefore further study evaluating the tissue response to the implant in the setting of radiation therapy is required.

Thesis Impact Statement

The aim of this thesis was to investigate mechanisms of breast implant failure and examine the role of radiotherapy. As a result of the systematic review and meta-analysis performed in Chapter 3, this thesis has shown that post mastectomy radiation therapy (PMRT) onto the permanent implant in patients with implant based breast reconstruction leads to increased rates of capsular contracture, revisional surgery and reconstructive failure (as defined by removal or replacement of implant). In addition, we have shown that PMRT on to the definitive implant is associated with poorer patient satisfaction and cosmetic outcome. This is the first meta-analysis examining the effect of PMRT on to the permanent implant in the literature to date and is a valuable for medical professionals when deciding on which breast reconstruction approach to take when patients are undergoing PMRT making informed decisions.

Further work in this thesis examined the mechanical strength and surface chemical properties of retrieved implants from patients attending our unit. In keeping with previous work, mechanical strength properties fell with increasing duration of implantation. However, a key finding was the change in the micro-mechanical properties across the cross section of the implant determined by atomic force microscopy. This work led to form the basis of a subsequent Masters student research project.

Furthermore, a key finding was the effect of radiotherapy treatment upon the silicone breast implant shells. Analysis showed there was no change in tensile, tear or Young's modulus between the fully irradiated and non-irradiated shells however there was a fall in the elongation at break suggesting that the shells become less flexible following radiation. Wettability and ATR-FTIR analysis did not show significant changes and therefore this work forms the basis of the potential future work assessing the effect of radiation delivered to the cell and the breast implant shells in combination to gain a deeper understanding of the pathogenesis of breast implant failure in the setting of radiotherapy.

In addition, further work tested the effect of radiation therapy upon a newer material, the nanocomposite POSS-PCU which has been demonstrated to have different bulk mechanical and surface chemical properties to that of silicone breast implant shells and do not change their properties in response to treatment dose radiation therapy. This work provides the basis for further investigation of this material as a potential alternative to silicone in designing future breast implants.

List of Abbreviations

α-SMA	Alpha-smooth muscle actin
ADM	Acellular dermal matrix
AFM	Atomic force microscopy
ALCL	Anaplastic large cell lymphoma
ATR-FTIR	Attenuated total reflectance-Fourier transform infrared spectroscopy
BCA	Bicinchoninic acid assay
BIA-ALCL	Breast implant associated-anaplastic large cell lymphoma
BSA	Bovine serum albumin
CC	Capsular contracture
CMC	Carboxy-methyl-cellulose
CT	Computed tomography
DAPI	4',6-diamidino-2-phenylindole
DMAC	N,N'-Dimethylacetamide
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethyl sulfoxide
EDMS	Electronic medical database records
GB	Gel bleed
FDA	Food and Drug Administration
FBS	Foetal bovine serum
H & E	Haematoxylin & eosin
HDFa	Human dermal fibroblast
IBBR	Implant based breast reconstruction
IL-8	Interleukin 8
MDA	Medical Device Agency
MPa	Mega Pascal
MRI	Magnetic resonance imaging
MWT	Molecular weight
PBS	Phosphate buffered saline
PCU	Poly(carbonate urea)urethane
PDMS	Polydimethylsiloxane
PenStrep	Penicillin-streptomysin
PIP	Poly Implant Prosthèse
PMRT	Post mastectomy radiation therapy
POSS	Polyhedral oligomeric silsesquioxane
POSS-PCU	Polyhedral oligomeric silsesquioxane Poly(carbonate urea)urethane
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-Analyses
PVP	Polyvinylpyrrolidone
SIBS	Poly(styrene-isobutylene-styrene)
SEM	Scanning electron microscopy
SEM-EDX	Scanning electron microscopy-energy dispersive X-Ray spectroscopy
SMA	Smooth muscle actin
TCP	Tissue culture plastic
TDA	2,4-toluene diamine
TNFα	Tumour necrosis factor alpha

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1.1 Introduction

Breast implants are used for both reconstructive and aesthetic purposes. According to the Breast and Cosmetic Implant Registry, over 8 500 patients in England underwent breast implant surgery between October 2016 and 2017 [3]. Breast cancer is the commonest cancer in women accounting for over 1.5 million newly diagnosed cases worldwide in 2010 [4]. In the UK it accounted for 15% of total cancers diagnosed in 2011 [5]. Of all diagnosed breast cancer patients approximately 40-45% will undergo mastectomy [6,7]. The breast shape is synonymous with femininity, attractiveness and reproduction and thus breast reconstruction is key to improve body image, emotional and sexual well-being particularly in younger patients following partial or total mastectomy [8] however, some studies have suggested breast reconstruction offers no difference in the psychological outcome and quality of life for this group of patients [9]. The current guidelines are that all suitable patients undergoing mastectomy should be offered breast reconstruction. The National Mastectomy and Breast Reconstruction Audit published in 2011 showed that over half (52%) of breast reconstructions performed between 2008-2009 were implant based [10]. There is also a clinical need for breast implants to achieve normal breast contours in patients with congenital disorders of the breast such as hypoplasia (Poland's syndrome) and tuberous breasts. From the aesthetic viewpoint, the breast augmentation industry is thriving. Breast augmentation remains the most popular cosmetic surgical procedure in both the UK and US. In 2016, 7 769 procedures were performed in the UK [11] and 290 467 procedures were performed in the US [12].

Since the inception of the silicone elastomer breast implant shell containing silicone gel in the 1962, silicone breast implants have undergone five generations of manufacturing changes in response to implant related complications. Despite these modifications, all breast implants will inevitably fail due to fibrotic encapsulation thickening, inflammation and contraction, leading to implant distortion, leakage and rupture.

1.1.1 Clinical Need for Improved Breast Implants

Since the inception of the silicone elastomer shell containing silicone gel in the 1962 [13], silicone breast implants have undergone five generations of manufacturing changes in response to implant-related complications. Despite significant advances in

the design and development of silicone breast implants over the past fifty years, long-term post-operative complications such as fibrotic capsular contraction leading to pain, firmness and distortion of breast shape are reported to occur in 9.2-26.8% at 10 years after implantation dependent on the indication and as well as reported implant rupture rates of 9.7% at 10 years [2] . A large meta-analysis by Marotta et al [14] examining implant data from 42 studies and 9774 implants, demonstrated that the implant rupture rate varied from 26% to 69% over a implantation time ranging from 3.9 to 17.8 years. These complications can necessitate further corrective operative intervention with reoperation rates reported up to 28.3% at 8 years follow up in the literature [15,16].

1.2 Evolution of the Breast Implant

It is over one hundred years since Gersuny first injected paraffin into breast tissue in an attempt to augment the breast [17]. This was followed by Czerny in the late 19th century who pioneered the technique of autologous fat transfer as a method of breast reconstruction by excising a lipoma from a patient's flank and inserting this into a mastectomy site. In the following years, a variety of materials including paraffin, ivory, glass, beeswax, soybean oils and injectable silicones were experimented with in the early 20th Century often with disastrous results culminating in serious complications including skin erosion, infection, silicone granulomata, tissue necrosis and soft tissue loss and therefore were abandoned [17,18]. The advent of the silicone implant in 1962 by Cronin and Gerow, composed of a silicone elastomer shell containing a filler material, marked a new era in breast augmentation and reconstruction surgery. Over the past half a century however, despite retaining the original design of a silicone elastomer shell and a filler material, the silicone breast implant has undergone five generations of manufacturing changes in response to complications which have overall reduced complications and improved their safety [1] (Figure 1.2).

1.2.1 The Structure of the Silicone Gel Implant

The breast implant is composed of a silicone elastomer outer envelope containing silicone gel in an attempt to recreate the natural breast mound. Silicone (polydimethylsiloxane, PDMS) is a polymer composed of a Si-O backbone with two

methyl groups bonded to each silicon atom (PDMS :$(CH_3)_2$ SiO) [1] as shown in Figure 1.1.

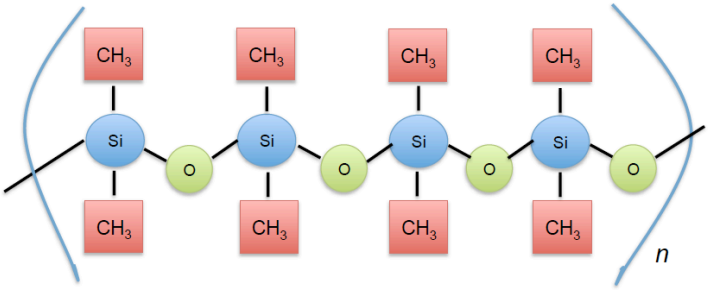


Figure 1.1: Structure of polydimethylsiloxane (PMDS)

Silicone gels are made from PDMS chains, lightly cross-linked through vinyl hydrogen bonds. The silicone elastomer shells are composed of 3-dimensional cross linked PDMS chains blended with amorphous silica (SiO_2) for reinforcement [19] . This is achieved using heat energy in a process called curing or vulcanization to achieve an elastomer. In breast implants, to prevent leakage of PDMS fluids from the silicone gel through the elastomer shell to surrounding tissues, a barrier layer of fluorosiloxane is incorporated into the shell to reduce the incidence 'gel bleed' as it is not as soluble to PDMS [1,20]. The cohesiveness of the silicone gel is dictated by the intensity of cross-linking between chains. Newer fifth generations gel implants feature a highly-cohesive gel filler which allows the shaped-implants to be less likely to disrupt and fold despite changes in breast position and chronic stress loading [21].

1.2.2 The History of the Breast Implant -1962 to present

The first generation silicone implant consisted of a thick smooth-surfaced silicone shell with a thick, viscous gel filling featuring a Dacron patch posteriorly for adherence to the chest wall [22]. However, it was discovered that these implants were associated with significant rates of capsular contracture, leakage and aesthetically were firm to touch. A retrieved breast implant study by Peters et al. [23] of over 400 silicone breast implants, demonstrated that all 28 first generation implants with a mean implantation duration of 17.6 years were intact and demonstrated capsular calcification. Second generation implants were introduced in the early 1970's featuring a thinner, but slightly more permeable shell, containing thinner gel in order mimic the desired natural feel. These, however, were associated with increased rates

of implant rupture and it was discovered that intact implants could 'bleed' silicone into the surrounding tissues from histological examination by Thomsen et al. [24] of breast tissue capsules from 55 patients who had silicone gel breast implants. It was this generation of breast implants which would become a major source of litigation in the 1990's. A retrieved implant study by Collis et al. [25] of implants inserted between 1971 and 1997 reported highest incidence of implant rupture occurred with second generation implants (71 of 110 implants: 65%). Furthermore, Peters et al. [26] showed that 77% second generation implants explanted between 1991 and 1995 had either ruptured or leaked.

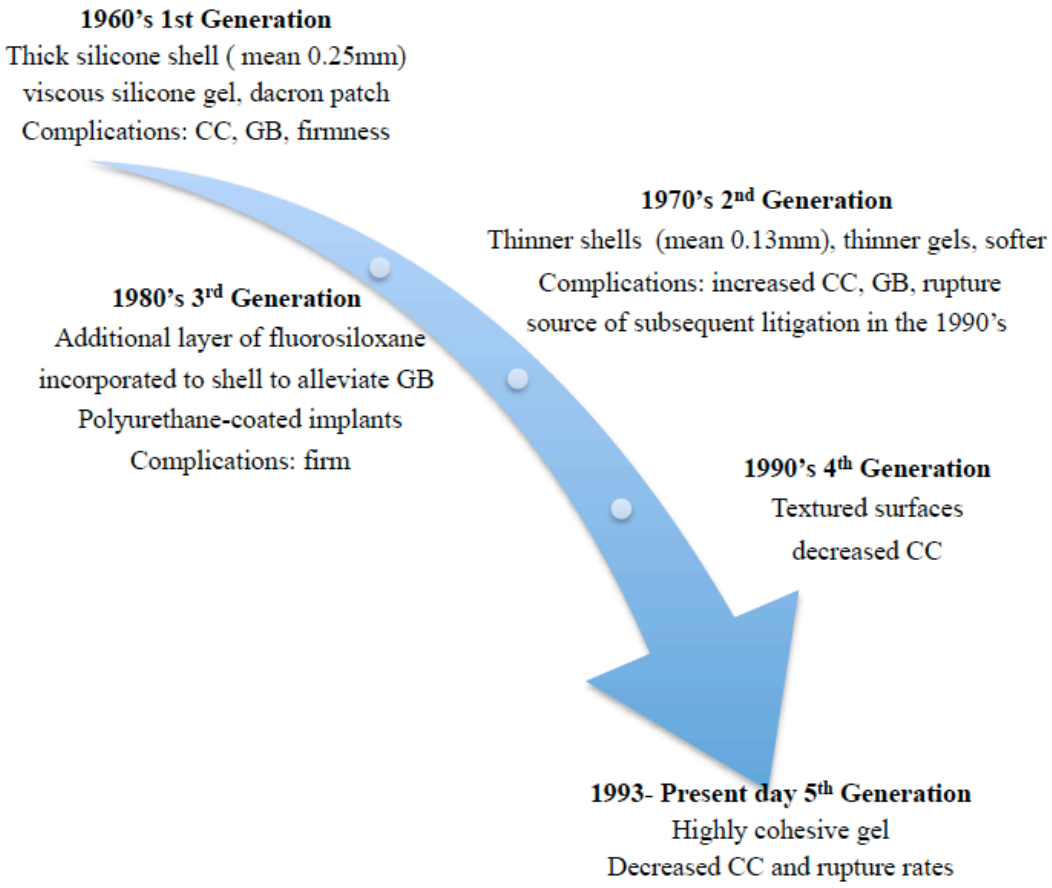


Figure 1.2: Evolution of the silicone breast implant since it's inception in 1962 – (modified from Berry et al. [1]) (CC – capsular contracture, GB- gel bleed)

The beginning of the 1980's heralded the introduction of third generation implants. These featured a viscous gel and a shell containing an extra fluorosiloxane barrier

layer (elastomer) to reduce the rate of implant rupture. Peters et al. [26] reported in their series of explanted implants 27 of 28 retrieved third generation implants were intact. Despite these implants providing durability, they were firmer to the touch. To overcome this, they were required to be surgically placed with adequate soft tissue cover to minimize visibility and palpability. In the 1970s further implant surface modification by coating the silicone breast implant shells with a 1mm thick polyurethane foam layer was pioneered by Ashley et al. [27]. Handel et al. reported in a prospective study of 719 patients receiving breast implants, only 20% of patients who received polyurethane-coated implants developed capsular contracture (Baker Grade 3-4) at 8 years compared to 35% who received textured implants and 50% who had smooth implants [27]. Polyurethane coated implant was shown to reduce the contracture rate ($p< 0.0009$) compared to textured implants [27]. In 1991, the US manufacturing company of these coated implant shells withdrew their product amidst reports that the degradation of polyurethane in vitro produced the carcinogenic by-product toluene diamine (2,4 TDA) [27,28]. However further published data in 1997 by Hester et al. [29] who performed a risk assessment after examining for free 2,4 toluene diamine in urine and serum samples from 60 women with polyurethane coated breast implants reported insignificant levels of 2, 4 TDA in the urine and the FDA reported the risk of developing cancer was negligible. A recent long term study of 382 patients with polyurethane coated implants over a 30 year period reported an overall capsular contracture rate of 2.4% and demonstrated an inverse correlation between amount of polyurethane coating found on the implant and severity of capsular contracture [30].

In the 1990's, fourth generation implants were created featuring textured outer surfaces in response to the reduced capsular contraction rates witnessed with the polyurethane-coated implants. Fifth generation implants were created using a highly cohesive, 'form-stable' gel more solid in nature allowing maintenance of its anatomical shape. This is achieved by increasing the ratio of crosslinking of the silicone gel allowing the gel to maintain its shape despite effects of gravity [21,31].

1.2.3 Litigation in Breast Implant Surgery

In April 1992, the Food and Drug Association (FDA) placed a moratorium on the commercial use of silicone gel implants in the USA. This was a direct result of the increasing litigation, particularly resulting from shell rupture and the available

evidence at the time of a causal link with the development of autoimmune connective tissue diseases [32]. This was largely based upon a series of case reports and a further meta-analysis including nine cohort studies published in 2000 [33] refuted the link. The FDA lifted the ban on the use of silicone breast implants in 2006 [1].

1.2.4 Breast Implant Fillers

The most popular breast implant filler is silicone gel. Silicone is composed of the chains of PDMS of varying lengths with its viscosity dictated by the average length of the polymer chains and the ratio of cross-linking occurring between them. Following the FDA ban on silicone gel implants in the early 1990's, saline-filled implants remained the only alternative. Cunningham et al. performed a retrospective study of 450 patients with saline filled implants commissioned by the FDA with a minimum 10 year follow up. They reported an overall complication rate of 20.2% including capsular contracture and deflation and a propensity for visible rippling and palpable edges, however 93% of patients were satisfied or highly satisfied with their breast implants [34].

During this time period, alternative fillers to silicone were rushed onto the market. The Trilucent™ implant, filled with medical grade soya bean oil encased in a silicone elastomeric shell, was introduced in 1995 [35]. These implants were associated with a high complication rate reported by Rizkalla et al. [35] who studied 29 patients (50 implants) over 3 years and reported an overall reoperation rate of 20% and deflation rate of 10%. It was discovered the rapid degradation of the shell led to implant rupture or leak and the subsequent perioxidation of the oil released genotoxic products [36,37] . The UK Medical Device Agency (MDA) withdrew the implant from the market in 1999 and the following year advised all patients to have their implants removed [35].

Hydrogels, a further class of filler, are polymeric macromolecules that retain water without dissolution [1]. Carboxy-methyl-cellulose gel (CMC) hydrogel implants have been reported to have acceptable patient satisfaction outcomes and allow improved mammographic imaging of the breast due to their translucency [38]. Polyvinylpyrrolidone-hydrogel (PVP)-hydrogel filled breast implants (Misti Gold™ prostheses) were shown to be advantageous initially as they demonstrated improved radiolucency allowing for detection on mammography of breast tissue pathology. However, these were associated with volume expansion and in one retrospective

review 59% of such implants had been removed at 4 years due to complications of volume expansion and capsular contraction and were withdrawn from the market in 2000 [39]. The NovaGold™ implant with a hybrid PVP-guar gum gel was introduced in 1996. These were withdrawn from the market in 2000 due to high reported rates of rupture as well as the propensity of the filler to induce a vigorous subcutaneous inflammatory reaction necessitating surgical intervention [40].
Most notably in recent years, the withdrawal from the market by the French Medical Regulatory Authority of Poly Implant Prothesis™ (PIP) silicone implants in 2010 attracted vast media attention [41]. The filler used in the implant manufacturing process was discovered to be industrial grade silicone and as a result these implants were associated with significantly higher rates of rupture [42]. A study comparing explanted PIP and medical grade silicone implants demonstrated reduced tensile strength and increased degradation of explanted PIP implants shells compared to those of explanted medical grade silicone implants [43]. However, PIP implant rupture rates have been reported to be similar to other silicone implant types in long term studies [44,45].

1.3 Complications Related to Breast Implants

Breast implant surgery is associated with complications often necessitating further corrective surgery most commonly, capsular contracture leading to pain, distortion and firmness of breast, implant rupture and implant leakage. Complications can be further classified into early, occurring within the first 30 days following surgery and late. The available literature regarding the risks and complication rates of breast implants is derived from core manufacturers long term studies. Table 1.1 outlines the incidence of late complications related to 5th generation implants in Allergan's 10 year follow-up core study of Allergan Natrelle 410 anatomical shaped implants in 492 patients undergoing primary augmentation.

Complications occurring in primary augmentations after 10 years in patients with Allergan Natrelle shaped breast implants	Re-operation 29.7% Capsular contracture, Baker Grade III/IV 9.2% Implant rupture (17.7% MRI cohort) Implant malposition 4.7% Breast pain 4.5% Infection 1.7% Seroma 1.6% Haematoma 1.3% Visible rippling of implant/wrinkling 0.9% Asymmetry/Ptosis 1.2% Hypertrophic scar/poor scar formation 1.4% Palpable implant 0.3%

Table 1.1: Reported rates of complications of Allergan Natrelle 410 anatomical shaped implants after 10 years in primary augmentations n=492 (adapted from Maxwell et al [2])

1.3.1 Early Complications

Early complications associated with breast implants include haematoma, infection and seroma formation [46] have been reported to occur in up to 10% of patients in the reconstructive setting [46]. Meticulous haemostasis at time of surgery and use of antibiotic irrigation of the breast implant pocket are routinely incorporated into breast implant surgery to reduce risk of contamination with breast ductal flora [47]. Infection is most commonly caused by Staphylococcus aureus and coagulase negative Staphylococci [48]. Literature reports a 1.8% risk of implant associated infection in patients undergoing augmentation [49] and a higher risk of 5.8% (range 0-29%) reconstructive procedures [50], This may be explained by increased area of surgical dissection, scarring and operative time required to perform reconstructive surgery. Risk factors for infection and skin necrosis include smoking, obesity, diabetes mellitus, renal disease, radiation therapy, steroid use, use of an acellular dermal matrix, operations lasting more than 2 hours and placement of a drain [48,51].

1.3.2 Long Term Complications of Breast Implants

1.3.2.1 Capsular Contracture

Capsular contracture, an abnormal fibrotic encapsulation is the most common adverse complication of breast implants leading to pain, firmness, distortion of the breast and asymmetry and shall be discussed further in the coming section.

1.3.2.2 Implant Rupture

Implant rupture can present as pain, asymmetry or deflation of the breast, presence of silicone granuloma or on radiological imaging. There is no standardised reporting method of breast implant rupture and published literature on rupture rates is derived from large follow-up studies from implant manufacturers performed to meet FDA approval. Implant ruptures can be clinically silent with less than 30% of magnetic resonance imaging (MRI) detected ruptures evident on clinical examination [52] thus the true incidence of ruptures may be underestimated. Allergan's Natrelle round implant 10 year core study reported in their MRI screening group an implant rupture sik of 9.3% for augmentations, 5.4% in revision augmentations and 35.4% in reconstructions [53]. Allergan's Natrelle 410 anatomical shaped implant study at 10 years reported in the MRI screened group a rupture risk of 17.7% for augmentations, 14.7% in revision augmentations and 12.4% for reconstructions [2].

The exact mechanism of breast implant rupture is unknown. Postulated mechanisms of rupture include iatrogenic damage to implant at time of initial surgery [54] and excessive handling of implant, trauma, excessive compressive forces exerted during mammography, the presence of folds or wrinkling of the implant and presence of surrounding capsular contracture [55]. In addition, reduced mechanical properties of the silicone implant shells has been reported in several retrieved implant studies as outlined in Table 1.2 [14,43,56–60].

Postulated mechanisms include the effects of in vivo aging [43,57,58,60] which reduce the silicone shell's mechanical properties accounting for the increased rupture and leakage rates with increasing duration of implantation.

Brandon et al [59] examined the mechanical properties of explanted breast implants from a range of manufacturers and demonstrated that increasing swelling of the

silicone elastomer shell caused by diffusion of low molecular weight silicones from the encapsulated gel into the elastomer shell was associated with reduced mechanical properties of the shells [59]. Furthermore, Neechi et al [61] studied 100 explants and found a significant difference in mechanical properties between ruptured and intact implants and ruptured implants shells. With increased shell swelling evident in ruptured implants shells [61]. In addition, diffusion of lipids into the elastomer shell has been postulated to contribute to mechanical weakening of the breast implant shell [62]. Furthermore, change in mechanical properties of the shell could be attributed to other factors such as potential trauma to the implant at time of surgical handling and implantation described in a cadaver model [54], trauma to the breast in vivo (mammography, trauma, breast tissue biopsy) and by forces exerted on implant at time of explantation.

Author/Year	Retrieved Implants	Implant Type	Duration of implantation	Outcome
Greenwald et al. 1996[63]	25 explants	Smooth-surfaced silicone gel	23 - 216 months (Mean =117, SEM =12.9)	Significantly reduced shell strength, shell toughness and elasticity with increasing implantation time
Philips et al. 1996[58]	29 explants	Silicone gel	4 - 240 months	Reduced material strength with increasing implantation time
Marotta et al. 2002[14]	51 explants	Silicone gel	12 to 228 months (Mean-18.8 months)	Reduced tensile and tear and reduced % elongation compared to un-implanted controls No correlation with duration of implantation.
Brandon et al 2003[59]	42 explants 51 control	Single lumen silicone gel	Controls Explants: 3 - 384 months	Increased extraction of non-cross-linked silicones from shell correlated with reduced tensile and elongation strengths. - effect of shell swelling
Neechi et al 2011[56]	100 explants	Silicone gel	6 – 132 months (Mean 58.3, SD 37)	Intact implants demonstrated significantly higher mechanical strength properties than ruptured implants. Ruptured implants shells demonstrated higher extraction fraction of low molecular weight silicones.
Yildrimer et al 2013[43]	22 explants	4 silicone gel 18 PIP	12 – 150 months (Median = 126)	Reduced mechanical properties comparing silicone to PIP implants PIP implants reduced mechanical properties with increasing implantation time
Bodin et al. 2015[60]	21 implants 11 5th generation 10 4th generation	Silicone gel	3 – 130 months	Reduced breaking strength with increasing implantation times.

Table 1.2: Studies investigating mechanical properties of retrieved breast implants

1.3.2.3 Re-operation

The most common indications for revisional surgery following breast implant augmentation include request for size change, capsular contracture, implant malposition, suspected rupture and following reconstruction most commonly capsular contracture, scarring and suspected rupture [2,64]. Re-operation rates following implant augmentation surgery have been reported up to 29.7% at 10 years [2,49,65].

1.3.2.4 Silicone Granuloma

Silicone granuloma can present as a palpable breast mass, breast pain, mastitis or palpable lymphadenopathy. Its presence indicates leakage of the silicone breast implant and is an indication for revisional surgery [66].

1.3.2.5 Late Seroma

The development of late seroma (peri-implant fluid collection occurring >1 year after implantation) is reported to occur in <0.1% of patients and is often managed conservatively with aspiration [67]. The aeitology of late seromas is unknown however, the development of synovial metaplasia and shear-stress micro-movement exerted by the implant on the surrounding tissues is postulated to have a role.

1.3.2.6 Chronic Infection

Chronic infection can lead to biofilm formation, increased risk of capsular contracture and osteomyelitis of the ribs and can range from mild, subclinical infection to severe leading to wound breakdown and sepsis requiring implant removal and autologous reconstruction [68]. The role of bacteria in capsular contraction is not fully understood however it is thought to cause chronic stimulation of the inflammatory response surrounding the implant. It has been shown that presence of biofilms, detected by sonification on explanted breast implants is directly correlated with increased rates of capsular contracture [69]. Furthermore, a higher rate of capsular contracture has been reported in those patients undergoing sub-glandular placement of the implant in comparison to sub-muscular [70] and a possible hypothesis to explain may be sub-glandular implants exposure to mammary duct bacterial flora. Local irrigation with anti bacterial agents at time of breast augmentation surgery using inflatable prosthesis in 124 patients showed significantly reduced early post operative capsular contracture [71]. The presence of chronic biofilm infection in breast implants may increase the likelihood of developing BIA-ALCL [72]

1.3.2.7 Breast Implant Associated – Anaplastic Large Cell Lymphoma (BIA-ALCL)

Breast implant associated-anaplastic large cell lymphoma (BIA-ALCL) is a rare type of T-cell non-Hodgkin's lymphoma reported to occur in patients with textured surface implants [73]. It is characterized by being CD30 positive and ALK negative and arising from either the effusion surrounding the implant or the peri-implant capsule tissue. Patients with breast implants presenting with a late onset seroma must have cytology performed to exclude this rare disease. First reported in 1997 the incidence has been reported to be higher in patients with higher surface area textured breast implants, with the highest associated with Biocell implants (1 in 3 817 implants) [74]. The pathogenesis of this disease is not fully understood but inflammation and the presence of chronic biofilm infection may increase the likelihood of developing BIA-ALCL [72]. Higher surface area textured implants may allow increased load of biofilm and ingrowth of bacteria thereby further activating immune process and cancer development.

1.4 Capsular Contracture

Capsular contracture has been reported by the manufacturer Allergan's core studies to occur in up to 9.2%–18.9% of primary augmentations and in up to 14.5%-24.6% of primary reconstructive cases at 10 year follow-up [2,53] and is higher for revision augmentations and reconstructions [2]. The aetiology of capsular contracture is poorly understood and is thought to be multi-factorial. It is characterised by a pathological fibrotic encapsulation of the breast implant that can lead to firmness, pain, distortion and asymmetry of the breast. It is classified clinically using the Baker Classification system I-IV. Grade I is a soft, non-visible implant, Grade II feature a mild firmness to the breast, Grade III a moderate firmness with visual distortion of the breast and Grade IV is when the capsular contracture is associated with pain or severe distortion of the breast, is firm with palpable implant.

1.4.1 The Host Response to the Silicone Breast Implant.

The interaction of the silicone breast implant and the host tissue is poorly understood. The insertion of the implant initiates the host 'foreign body response' with tissue injury, blood/material interaction, the acute inflammatory response and recruitment of

neutrophils, acute phase proteins, followed by the chronic inflammatory response with recruitment of macrophages and fibroblasts, collagen deposition and formation of foreign body giant cells leading to the development of a fibrotic encapsulation of the breast implant. This is necessary to support the implant and prevent its unnecessary movement within the breast. However the in the development of capsular contracture, the inflammatory foreign body response is propagated by a pathological trigger(s) which further stimulates the fibroblasts and myo-fibroblasts. It is the parallel alignment of collagen fibres produced which then myo-fibroblast contract upon which produces a tight collagen capsule surrounding the implant ultimately leading to distortion of the breast shape with firmness, discomfort and pain [75,76]. Histological examination of samples from capsules retrieved at time of implant removal by Bui et al. [77] demonstrated thicker capsules, increased myo-fibroblast alpha smooth muscle actin (α-SMA) expression at the implant- capsule surface and overall greater acellular content in those patients with Baker III and IV contracted capsules compared to Baker I and II uncontracted capsules ($p<0.05$) [77].

1.4.2. Cell Material Interactions

The exact patho-aeitiology of capsular contracture is unknown, however it is thought to be multi-factorial and many factors have been implicated including haematoma or seroma at time of implantation, implant surface texture, bacterial colonisation, radiotherapy and shear-forces exerted upon the tissues [78,79]. The surface of the material or substrate can influence the host responses. Hydrophobicity of the breast implant can influence the host response as it influences protein adsorbtion to its surface, producing conformational changes in the protein, altering cell binding receptor sites and triggering inflammatory responses [80] ultimately influencing macrophage and fibroblast behavior. The substrate stiffness, the ability of a material to resist deformation, can also direct cell behavior with cells ‘pulling’ on cell adhesions made via their cytoskeleton and relaying information back the nucleus. Previous studies have demonstrated that substrate stiffness influences fibroblast behavior, attachment and migration [81] with fibroblasts moving toward stiff substrates [81–83].

1.4.3 Textured vs Smooth Implants

Many studies have examined the rate of capsular contracture in smooth compared to textured implants. A recent meta-analysis [84] of 16 randomised control trials and 2 retrospective studies showed that overall textured implants were associated with reduced rates of capsular contracture compared to smooth [84]. The uneven topography of the implant surface in textured implants disrupts the fibroblast cell orientation, leading to random alignment of collagen fibres, reducing their ability to optimally contract and thereby reducing the incidence of capsular contracture. Silicone breast implants are textured by either 'salt-loss' techniques as seen with Allergan Biocell surfaces (dipping the silicone coated mandrel in salt prior to the curing process) or by indenting the outer silicone layer into a polyurethane foam before curing as in Mentor Siltex implants [85]. Further research by Valencia-Lazcano et al. [86] to explain the reduced rates of capsular contracture associated with textured implants found that there was greater fibroblast cell adhesion to textured as opposed to smooth implants which may suggest textured implants provide an improved cell surface interface and reduced micro-movement possibly due to increased surface area [86]. Furthermore, coating the breast implants with extra-cellular matrix proteins collagen I and fibronectin have been shown in in vitro models to demonstrate greater breast fibroblast cell adhesion as demonstrated by resistance to detachment by trypsin [86] Current thinking accredits this reduction in micro-movement and shearing motion at the host-implant interface will reduce overproduction of collagen fibres and thus capsular contracture [87].

1.4.4 Role of Acellular Dermal Matrixes

Acellular dermal matrixes (ADMs) are human, porcine or bovine derived decellularised extra celluar matrix grafts which have been associated with reduced incidence of capsular contracture in breast reconstruction [88] . The hypothesized mechanism is that the ADM closely mimics the host extracellular matrix thereby reducing the inflammatory response, ultimately reducing the development of capsular contracture. This has been strengthened by a study of capsule biopsies taken at time of implant exchange and found histological reduced evidence of inflammation at ADM tissue compared to the host breast capsule [89]. Furthermore, there was found to be reduced levels of inflammatory markers [90].

1.4.5 Site of Surgical Incision

The choice of incision has been shown to contribute to capsular contracture formation with infra-mammary incisions associated with lower rates compared to peri-areolar or trans-axillary approaches [91,92].

1.5 Impact of Radiotherapy

Immediate two-stage breast reconstruction using a tissue expander followed by permanent implant is the most common form of breast reconstruction performed post mastectomy for breast cancer [93]. Offering immediate breast reconstruction provides replacement of the breast mound as well as significant psychological and emotional advantages for the patient [94]. Implant based reconstruction following mastectomy can be performed immediately as a one (direct to permanent implant) or two stage procedure (tissue expander followed by permanent implant) or as a delayed procedure after several months. Post-mastectomy radiation therapy (PMRT) is a known to prevent local recurrence and improve disease free and overall patient survival [95]. Since the publication of the Danish and Canadian trials in 1997, the numbers of patients eligible for post mastectomy radiotherapy are increasing [96,97]. This is further supported by a subsequent study by Tendulkar et al. who reported a significant reduction (12%) in loco-regional recurrence in those patients receiving post mastectomy radiation therapy (PMRT) with only 1-3 positive axillary nodes [98]. Following the publication of a meta-analysis of 22 randomised clinical trials in 2014 [99], guidelines now recommend PMRT should be offered to patients with T1-2 disease and 1-3 positive lymph nodes as well as patients with T1-2 tumours with a positive node on sentinel node biopsy who do not undergo further axillary clearance [100].

Radiotherapy has been shown in several retrospective patient studies to increase rate of capsular contracture as well as the failure rate as defined as necessitating surgical removal of the implant [95,101–103] however the underlying mechanism is yet unknown. A recent study investigating the effect of post mastectomy radiation therapy on silicone breast implants shells showed an increase in low molecular fragments on the surface of the treated implant shells using surface spectroscopy methods which may be responsible for the increase the rate of adverse events in these patients [104]. However, studies investigating the effect of gamma radiation upon

PDMS based materials have shown no significant changes in their material characteristics [105,106].

1.6 Properties of an Ideal Breast Implant

In designing newer breast implants, the material used to form the implant shell should possess the following desirable properties: being non-toxic, non carcinogenic, non-immunogenic, biocompatible (able to perform its function and elicit an appropriate host response), impermeable to leakage of its inner contents, the ability to withstand sterilisation, resistant to damage by radiation therapy and possess the mechanical properties desired to function and regulate the cellular response to the material. Implant rupture and failure is associated with reduced mechanical tensile properties in studies of explanted implants therefore creating breast implants using materials with increased mechanical properties is desired. Substrate stiffness has been proven to influence cell movement, adhesion, proliferation and differentiation [81,82,107]. Since the reduced rates of capsular contracture were seen in patients with polyurethane coated breast implants, textured breast implant surfaces have been employed with a recent meta-analysis [84] showing reduced overall rates of capsular contracture compared to smooth surfaces. The surface nano-topography as well as micro-topography of a material surface has been shown to influence cellular response [108] and cellular adhesion [109]. Thus, research is required to discover the optimal surface topography of future breast implants to direct an appropriate host response.

1.7 Future perspectives: Potential Application of Alternative Biomaterials in the Development of Future Breast Implants

Polymers are widely used in medical applications to replace or augment tissues following injury and disease and for congenital and cancer reconstructive purposes. In addition, research and applications of polymers is rapidly expanding in the field of tissue engineering, drug deliver and medical diagnostics. The requirement of a polymer in biomedical applications is to mimic the natural tissue mechanical properties and function but most also posses the ability to not evoke a host inflammatory response, be able withstand sterilisation, non-toxic, resistant to

degradation and non-carcinogenic [110]. Silicones are widely used for medical applications for possessing the aforementioned qualities and excellent biocompatibility. They have been used in the manufacturing breast implants over the past 50 years as well as other types of aesthetic implants, hydrocephalic shunts, cardiac pacemakers, valves, catheters, small joint replacements and bariatric surgery [110].

Only one in vivo study to our knowledge has investigated the use of nanostructured thermoplastic polymers (linear triblock poly(styrene-b-isobutylene-b-styrene) as an alternative to silicone as breast implants in a rabbit model. Following 2 weeks implantation, the SIBS–type polymers demonstrated superior mechanical properties compared to silicone following explantation with no difference in inflammatory response between all tested materials [111]. This research is still ongoing and further long-term study is required before translating this into clinical practice.

Our lab has developed and patented a nano-composite polymer called polyhedral oligomeric silsequioxane poly (carbonate-urea) urethane (POSS-PCU). Initially, vascular grafts composed of the polymer poly-carbonate urethane (PCU) were discovered to resistant to degradation in in-vivo studies [112]. This led to further studies using the nano-composite polymer POSS-PCU composed of a poly-carbonate urethane (PCU) soft segment incorporating a silica nanocage in the form of polyhedral oligomeric silsesquioxanes (POSS) hard segment. The incorporation of the silica nanoparticle (POSS) cage, measuring less than 1.5nm in diameter and regarded as the smallest achievable silica particles, into the polymer significantly changes the mechanical properties of the polymer [113]. This nano-composite polymer has been studied in comparison to silicone-containing polyurethanes in an in-vivo ovine model demonstrating reduced capsule formation at 36 months [114]. It has also been investigated in the formation of a lacrimal duct [115], cardiovascular grafts [116] and ear reconstruction [117] as well as human tracheal formation [118].

Further research is required in the potential application of other biomaterials in the development of new breast implants to provide ‘tailor-made’ implants and improved outcomes for patients. By optimising the host inflammatory response, controlling fibroblast behavior by adjusting the substrate stiffness, surface topography tailoring the mechanical properties to withstand shear stress forces and implant rupture, this nano-composite may be a promising alternative to silicone breast implant.

1.8 Conclusions

Since their conception in the early 1960's silicone breast implants have revolutionized breast augmentation and subsequently breast reconstruction. They have gone through an array of manufacturing modifications and refinements involving five generations of implant through trial and error to create the current fifth generation form stable implant. Unfortunately, despite these efforts the complications of infection, capsular contracture and implant rupture remain which often necessitate further corrective surgical intervention. Future research should be aimed to uncover the exact mechanisms of why breast implants fail and at exploring alternative materials, which could be employed in order to provide an improved, implant for this cohort of patients.

1.9 Hypotheses of this MD thesis

The hypotheses of the MD thesis were that radiation therapy is associated with poorer clinical outcomes for patients undergoing implant based breast reconstruction. In addition, mechanical properties of retrieved implants would fall with longer duration of implantations and that radiation therapy directly to the silicone breast implant shells would change their mechanical and surface chemical properties.

1.10 Aims of this MD thesis

Radiotherapy has been reported to be associated with adverse outcome for patients with implant based breast reconstruction. With the indications for post mastectomy radiotherapy increasing, it is imperative to understand the effect of post mastectomy radiotherapy upon the breast implant and this shall form part of the focus of this thesis. This shall be done by firstly performing a systematic review and meta-analysis of the current literature regarding outcome of PMRT onto the permanent implant. This shall be followed by examining mechanical, chemical and cellular as well as histological response to retrieved breast implants in our clinical study. Finally the effect of radiotherapy treatment upon the silicone breast implant shells shall be analysed as well as to the nano-composite POSS-PCU to explore its potential as an alternative biomaterial in the development of breast implants to improve the outcome for patients.

Chapter 2: Materials and Methods

2.1 Introduction

The overall aim of this thesis to further understand and elucidate the relationship between the material (breast implants, polymers) and the host's (patient) response to the material to gain a deeper understanding of why breast implants fail with particular focus on the role of radiation therapy. This chapter describes the detailed methods used to perform the systematic review of current literature with respect to the effect of post-mastectomy radiation therapy delivered to the permanent implant in patients undergoing implant based breast reconstruction, how the clinical study was performed (retrieval of explanted breast implants and samples of the surrounding fibrotic capsule), the methods used to characterize the mechanical and chemical properties of the materials as well as to assess their histological and in-vitro response. Experiments specific to a particular chapter described in detail in the respective chapter.

2.2 Systematic Review and Meta-analysis

The systematic review and meta-analysis was performed according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement [119] The study protocol was registered with the University of York Centre for Reviews and Dissemination international prospective register of systematic review (2015:CRD42015026061). Ovid MEDLINE and Embase databases were searched up to and including the second week of October 2016 using the following search algorithm: ((breast reconstruction.mp. or Mammaplasty/) OR ((breast$ adj3 (reconstruction or implant)).mp.)) AND ((Radiotherapy, High-Energy/ or Radiotherapy, Intensity-Modulated/ or Radiotherapy/ or Radiotherapy, Computer-Assisted/ or Radiotherapy, Image-Guided/ or Radiotherapy, Adjuvant/ or Radiotherapy Dosage/) OR (radiotherapy.mp. or Radiotherapy/)). The literature was searched for studies comparing patients who received radiotherapy post mastectomy directly to a permanent implant to those patients who received no radiation therapy. Exclusion criteria included patients undergoing delayed reconstruction post mastectomy, combined autologous reconstruction, radiation delivered to the tissue expander prior to implant exchange for the permanent implant and patients with a prior history of radiotherapy. Primary outcomes were implant loss, capsular

contracture and revisional surgery. Secondary outcomes were cosmesis and patient satisfaction.

A manual search was also performed to search for relevant studies. Publications were excluded if not relevant to the topic, review articles, autologous breast reconstruction articles, letters, comments and conference abstracts.

All patients undergoing immediate one or two stage implant breast reconstruction were included in the study. Articles had to define that PMRT was delivered to the permanent implant or following tissue expander exchange to the definitive implant to be included. Patients who received PMRT to the tissue expander prior to exchange to PI and those patients who had combined implant autologous or autologous breast reconstruction were excluded. A time limit of the studies published in the last 20 years was chosen to reflect the improvements in breast implant technology and design as well as improvements in surgical and radiation techniques to limit bias.

Primary outcomes were defined as capsular contracture (as defined as Baker Grade III or IV), revisional surgery and reconstructive failure (as defined as removal or replacement of the implant).

Secondary outcomes were defined as patient satisfaction and cosmetic outcomes. Patient satisfaction outcomes varied between studies and a good outcome was accepted as 'partially to fully satisfied', 'medium to good' and 'satisfied' for the purpose of our review. Cosmetic outcomes were similarly varied but defined by the operating surgeon.

2.2.1 Statistical Analysis

All primary and secondary endpoints were entered into and analysed using Revman5® software (The Nordic Cochrane Centre, Copenhagen, Denmark) using a random effects DerSimonian-Laird model and results were reported with 95% confidence intervals. Heterogeneity was assessed using τ^2, χ^2, and I^2 measures and was deemed significant if $p<0.10$ or I^2 was greater than 30%.

2.3 Retrieval of Breast Implants and Surrounding Capsule

2.3.1 Ethical Approval and Consent

Local ethical approval was granted from the UCL Royal Free Hospital BioBank Ethical Committee. Patients were identified from theatre lists and consented by author

on morning prior to surgery. Patients were given a written information sheet (Appendix 2)

2.3.2 Recording of Clinical and Demographic Data

Using history-taking, medical case notes and using the Royal Free Hospital electronic medical database records (EDMS) clinical and demographic data including age, reason for implant removal, duration of implantation, reason for original implant placement, smoking status, previous radiotherapy, chemotherapy and implant revisional surgery and complications were recorded.

2.3.3 Retrieval of Explanted Implants and Surrounding Capsule

Implant(s) and samples of the adjacent surrounding capsule tissue were collected from the theatre by the author. The implants were at first visually inspected to determine whether they were ruptured or intact prior to being cleaned with iso-propanolol and stored for further analysis. The sample of surrounding capsule tissue was placed in 10% neutral buffered formalin for at least 48 hours prior to further processing. All samples were labeled anonymously.

2.3.4 Non-implanted Breast Implant (Control)

Two non-implanted (Mentor Siltex™ Contour Profile™ Becker ™ 35 Expander, Cohesive II™, Lot 6811381, volumes 195cc and 295cc) were used the non-implanted, control implants for the purpose of our experiments.

2.4 Polymer and Nanocomposite Synthesis

2.4.1 Preparation of Polyhedral oligomeric silsesquioxane poly(carbonate-urea) urethane (POSS-PCU) nanocomposite

Dry polycarbonate polyol (2000 molecular weight) and trans-cyclohexanechloroydrinisobutyl-silsesquioxane were placed in a 250ml reaction flask with a mechanical stirrer and nitrogen inlet. The mixture was then heated to 135°C to dissolve the POSS cage into the polyol and then cooled to 70°C. Flake 4,4'-methylenebis(phenyl isocyanate), was added to the polyol blend and then reacted, under nitrogen, at 70°C-80°C for 90 minutes to form a pre-polymer. Dimethylacetamide (DMAC) was added slowly to the pre-polymer to form a solution and this was cooled to 40°C. Chain extension of the pre-polymer was carried out by the addition of a mixture of ethylenediamine in dimethylacetamide to form a solution

of POSS modified Polycarbonate urea-urethane in Dimethylacetamide. After the chain extension completion, 4g of 1-butanol in 80g of DMAC was added to the polymer solution to form the nano-composite.

2.4.2 Synthesis of Poly(carbonate-urea) urethane (PCU) polymer

Dry Polycarbonate polyol (2000mwt) was placed in a 250ml reaction flask equipped with mechanical stirrer and nitrogen inlet. The polyol was heated to 60°C and then flake MDI was added and reacted with the Polyol, under nitrogen, at 70°C - 80°C for 90 minutes to form a pre-polymer. Dry Dimethylacetamide was added slowly to the pre-polymer to form a solution; the solution was cooled to 40°C. Chain extension of the pre-polymer was carried out by the drop wise addition of a mixture of Ethylenediamine and Diethylamine in dry Dimethylacetamide. All reagents and chemicals were purchased from Sigma-Aldrich Ltd., Gillingham, UK.

2.4.3 Casting of POSS-PCU and PCU

The final polymer mixtures (15% mwt POSS-PCU and PCU) were separately casted onto 16 x 16cm stainless steel plates and then placed in an oven at 65°C overnight to allow the dimethylacetamide to evaporate. The casted polymer sheets were removed from the oven, allowed to cool to room temperature and carefully peeled off the plates before further analysis.

2.5 Mechanical Strength Properties

In the early 1990's, a moratorium on the use silicone breast implants was issued in response to reports of an association silicone gel leakage from the implants with autoimmune connective tissue. As a result, studies were performed upon explanted breast implants to determine the changes in the mechanical properties of the shell namely their tensile strength, elasticity and strain at break [63]. As part of the evaluation of retrieved implants in this study, these mechanical properties of implants were tested. The maximum tensile strength is the maximum force required to break the material apart. Elongation at break is the maximum stretch of the material tested as measured by percentage of its original size. Young's modulus or elastic modulus a measure of the stiffness of a given material and is calculated using the following equation

Young's modulus = (Force applied/Area) / (change in length/original length)

The higher the Young's modulus, the more resistant a material is to elastic deformation. Previous studies have shown that the longer the breast implant remains in vivo the mechanical properties of the implant shell fall [63].

2.5.1 Tensile Strength

Using the British Standards ISO 37:2005 tensile stress-strain properties of the polymeric sheets and breast implant shells were performed. A dumbbell-shaped, specimen type 3, shaft length 20mm with a width of 4mm (n=6) was cut using a die cutting press (Wallace instruments, UK). Three thickness measurements were performed using a digital electronic micrometer and the average thickness was inputed into the software. The sample was loaded onto the pneumatic grips of the Instron 5565 tensiometer equipped with a 500 N load (Instron, UK) and uniaxial testing at a rate of 100 mm/min was performed. The data was captured and analysed using Bluehill software. All experiments were performed on dry samples at room temperature.

2.5.2 Tear Testing

Tear testing was determined using the British Standard ISO 34-1:2004 standards and assessed using method 3. Crescent-shaped samples (n=3) were cut to shape with a single nick using a scalpel blade. The thickness of each specimen was measured thrice using a digital electronic micrometer and the average value used to input into the software. The ends of the crescent shaped specimens were loaded into the pneumatic grips of the Instron 5565 tensiometer and uniaxial tension was performed at a rate of 500 mm/min until the specimen was tore apart. Data was captured using Bluehill software. This was performed on dry samples at room temperature.

2.5.3 Statistical Analysis

Tensile testing was performed n=6 and tear testing was performed n=3. Mean and standard deviations were calculated using GraphPad Prism software Version 6.

2.6 Attenuated Total Reflectance-Fourier Transform Infra-Red Spectroscopy (ATR-FTIR)

To determine the surface chemical structure of any given polymer, and to determine compounds and functional groups, infra-red spectrosocopy is employed. Elemental analysis can be performed upon a material as discussed later in this chapter by

scanning electron microscopy however it cannot determine compounds [120]. Attenuated Total Reflectance-Fourier Transform Infra-Red (ATR-FTIR) spectroscopy is a newer method used to determine the molecular composition of a liquid, gas or solid substance without the need to prepare the sample. It detects concentrations of functional groups and molecules in any given sample by its ability to absorb infra-red (IR) light at a specified wavelength. Attenuated total reflectance allows only a limited path length of the IR beam to the sample and therefore avoids over-absorbance of the IR beam in samples that are highly absorptive. The sample's ability to absorb the IR beam at differing wavelengths is collected on an IR detector providing spectra with information on the molecular bonds present and composition of any given substance without causing damage or changes to the chemical structure [120].

2.6.1 ATR-FTIR

Fourier Transform Infrared Spectra (FTIR) recordings were obtained using a Jasco FT/IR 4200 Spectrometer with a diamond attenuated total reflectance accessory (Diamond Miracle ATR, Pike Technologies, US). From an average of 30 scans a spectra was produced over a range of 600cm^{-1} to 4000 cm^{-1} with a resolution of 4cm^{-1}. Each breast implant shell and casted polymer sheet was analysed at 5 different points. Each gel was analysed using 5 separate samples. A background scan was performed prior to every measurement. The data was collected, spectra and standard deviation were composed from the mean value of the 5 repeat measurements using Microsoft Excel worksheet (Microsoft Excel, 2011) and statistical analysis performed using GraphPad Prism software Version 6

2.7 Surface Properties of Retrieved Breast Implants and Polymers

To investigate the surface properties of the materials, contact angle measurements and scanning electron microscopy were performed. Contact angle measurement examines the wettability of a given material providing information on its free surface energy. Wettability describes the ease by which a fluid flows across a solid surface. Materials displaying contact angles of less than 90^{o} are generally considered hydrophilic and those materials greater than 90^{o} are hydrophobic. Protein adsorption and cell adhesion to a foreign material are influenced by its surface chemistry that naturally is key in developing a biomaterial [121,122]

Scanning electron microscopy is a method used to obtain high resolutions images of the surfaces of materials at high magnifications. This is achieved by scanning the material using a beam of electrons, rather than a light source as performed in light microscopy, which interacts with the atoms on the surface of the material generating forces which are analysed providing detailed images and information about the sample's surface topography. The samples are first coated with a conductive metal such as gold/palladium to avoid the sample absorbing electrons and providing a poor image or artifacts.

2.7.1 Surface Wettability/Contact Angle Measurements (θ)

Samples were cleaned using iso-propanolol and contact angle measurements were performed using DSA 100 Krűss Goniometer. Using the sessile drop technique, 5µl of deionized water was dropped onto the samples using an automated syringe with 10 seconds of dispensing and analysis was performed using the Drop Analysis software (EasyDrop DSA200, KrűSS) at room temperature. Four samples from each specimen were tested three times, n=12 and mean and standard deviation were calculated.

2.7.2 Scanning Electron Microscopy

Breast implant shell specimens were immersed in 1% Triton X100 and 1% sodium dodecyl sulfate for 16 hours, washed x2 with deionized water then washed with absolute ethanol followed by a further x2 washes with deionized water to remove any biological proteins. The samples were then dried in a 40 degrees oven for 1 hour. The samples were then mounted on aluminum stubs using carbon adhesive tabs and sputter coated with gold/palladium using a High Resolution Ion Beam Coater (Gatan Model 681). Images were taken using a scanning electron microscopy at magnifications ranging from x50 to x 1000 using a Field Emission Scanning Microscope (JEOL- JSM 7401F) and images saved as JPEG.

2.8 Determination of the Protein Content in Retrieved Breast Implant Shells (Bicinchoninic Acid Assay)

In keeping with previous literature examining the protein deposition found in silicone contact lenses [123] 0.25gm of breast implant shell were placed in 0.2% Trifluoroacetate and acetonitrile 50v/50v for 24 hours in a dark cupboard at room temperature. The solution was evaporated to dryness using a speedivac, the breast implant shell removed and the extracted proteins were re-suspended in 250µl of

water. Protein content of the resuspension was determined using the Pierce™ BCA Protein assay kit (Bicinchoninic acid assay), ThermoFisher Scientific, UK. This method relies on the Cu2+-protein reaction and measures Cu+ under alkaline conditions using the bicinchoninic acid which produces a vivid purple colour maximally detected at 592mm absorbance. A standard curve was performed using bovine serum albumin (BSA) as a control before the fluorescence of the samples at 592mm excitation wavelength were measured using a fluorescent plate reader (Fluoroskan Ascent FL™, ThermoScientific™, USA).

2.9 Histological Response to Retrieved implants

2.9.1 Preparation of Retrieved Surrounding Capsule Tissue, Processing and Staining

The retrieved capsular tissue was labeled anonymously and placed in 10% formalin for at 48-72 hours. The tissue was then cut into approximately 1 cm sections and placed in labeled tissue cassettes before being submitted to 15 hr automatic tissue processing using a Shandon Citadel 2000 Automatic Tissue Processor (Thermo Scientific™, UK). Processed samples were then removed from the cassettes and paraffin embedded using a Tissue Tek II™ tissue embedding centre.

Paraffin blocks were sent for sectioning and staining to Departments of Pathology at the Royal Free Hospital (Haematoxylin and Eosin, H&E stain) and at the UCL Institute for Neurology (Masson's Trichrome stain).

Sections were imaged and analysed using an EVOS XL Core Microscope and saved as TIFF images.

2.9.2 Scanning Electron Microscopy with Energy Dispersive X-Ray Spectroscopy (SEM EDX)

SEM EDX is a method similar to previously described in section 2.7 but incorporating an energy dispersive X-ray detector to conduct elemental analysis of a given sample. It relies on the unique atomic structure of individual elements and the impact of the electron beam on the sample produces characteristic x-rays of elements present that are absorbed and analysed by the detector. Combined with SEM, elemental mapping is achieved. The paraffin embedded tissue blocks were first sputter coated with a thin layer of carbon using a K975X Turbo Evaporator, Quorum

Technologies, UK. Images and analysis were performed using a scanning electron microscope Hitachi S-3400N with an EDS Oxford instrument. Analysis was performed using the INCA software package.

2.10 Cellular Response to Retrieved Implants and Materials

2.10.1 Culture and Seeding of Fibroblasts

HDFa fibroblasts were passaged in sterile T-75 flasks using Trypsin with Dulbecco's Modified Eagle's Medium, low glucose DMEM (Gibco, ThermoScientific™, UK) supplemented with 10% FBS (foetal bovine serum) and 1% PenStrep (penicillin-streptomycin, Gibco) or cryopreserved using DMSO until further required. HDFa's used in the experiment were between passage 7 and 11.

Discs of materials measuring 6mm (n=6 or otherwise stated in the text) were used for the cell seeding experiments as availability of material to test was limited.

A 6 mm heavy-duty office hole punch was used to cut 6mm discs from the breast implant shells and polymer. The specimens were then decellularised by being placed in 1% Triton X for 1 hour, washed twice in PBS followed by 70% ethanol followed by washing twice in PBS.

Specimens were then placed in a 96 well plate and covered with 100μl of warmed DMEM for approximately 2 hours prior to cell seeding. Each specimen was seeded with cells at density of 5 x 10^4 cells/cm^2 and incubated at 37^{o}C at 5% CO2 in air. Cells seeded onto tissue culture plastic served as a positive control and media only wells provided a negative control. Cell culture media was replenished on days 0, 1, 3 and 6 during the 7 day experiment.

2.10.2 Measurement of Cell Metabolism using Alamar Blue™ Assay

Cell metabolism was assessed using the Alamar Blue™ assay (Invitrogen, Paisley, UK). This commercially available assay kit contains the compound resazurin that in the presence of respiring cells, is reduced to resofurin which is pink and highly fluorescent. This oxidation reaction is a direct measure of the cells metabolic activity. The fluorescence is proportional to the number of respiring cells and can be measured objectively using a fluorescent plate reader.

At each timepoint, day 1, 3 and 7, media from the wells was removed and fresh media containing 10% Alamar Blue™ solution was added to each well. The plates were

immediately wrapped in aluminium foil and incubated for 4 hours. Then, 100µl of the media from each well was placed into a 96-well plate and analysed using a fluorescent plate reader (Fluoroskan Ascent FL™, Fluorescence Plate Reader, ThermoScientific, USA) at an excitation and emission wavelength of 530nm and 620nm. Values from the media only wells were subtracted from the other wells and recorded using Microsoft Excel and presented using the mean and standard deviation. Statistical analysis was performed using GraphPad Prism software Version 6.

2.10.3 DNA Quantification Assay – Cell proliferation

To assess cell proliferation, Hoechst 33258 DNA Quantification Kit™, Fluorescence Assay (Sigma-Aldrich, UK) was used. Following Alamar Blue™ analysis the specimens were washed with PBS and 100µl of molecular grade water was added to each well. The plates were then submitted to 6 freeze-thaw cycles to achieve cell lysis. Fluorescence was measured using a fluorescent plate reader (Fluoroskan Ascent FL™ Fluorescence Plate Reader, ThermoScientific, USA) at excitation and emission wavelengths of 360nm and 460nm (n=6). A standard curve was performed with known quantities of calf thymus DNA and the equation was used to calculate the DNA concentrations from the fluorescence of the specimens. Statistical analysis was performed using GraphPad Prism software Version 6.

2.10.4 Cell Morphology Imaging: Cytoplasmic Actin and Nuclear Fluorescent Staining

In order to assess cytoplasmic morphology, fluorescent green staining of actin (F-actin Phalloidin stain) and fluorescent blue staining of the cell nuclei (DAPI) was performed on day 7. Cells were fixed with 100 µl 10% neutral buffered formalin for 30mins then permeabilised using 1% Bovine Serum Albumin (BSA)/0.3% Tween in PBS for 1 hour. The cells were then washed thrice with PBS before being immersed in 1:500 solution of Alexa Fluor 488 Phalloidin (Molecular Probes, ThermoScientific™, UK) in PBS for 2 hours. Following a further wash with PBS, 1 droplet of Vectashield Antifade Mounting Medium with DAPI (Vector Labaoratories, USA) was added to each well prior to imaging to stain the nuclei. Images were captured using the EVOS fluorescent microscope (EVOS FL Imaging System, ThermoScientific™, UK) and saved as TIFF files.

Chapter 3: Determining the outcomes of post mastectomy radiation therapy delivered to the definitive implant in patients undergoing one and two stage implant based breast reconstruction: A systematic review

3.1 Introduction

Post mastectomy radiation therapy is increasing being offered to patients undergoing implant based breast reconstruction [97–99]. It has been shown in some studies that PMRT negatively impacts on the cosmetic outcome and increases the complication rate for patients undergoing implant based reconstruction however the results are conflicting [124]. Delayed reconstruction or autologous reconstruction can be offered to women who are likely to undergo radiotherapy [125,126] However, delayed reconstruction following treatment with radiotherapy can often be much more technically challenging thus resulting in a poorer cosmetic result and leaves the patient without a breast for a period of time [127]. There are various advantages of implant-based over autologous reconstruction including reduced operative time, avoidance of donor site morbidity, reduced cost and can be offered to those patients unsuitable for autologous reconstruction either due to co-morbidities or lack of available donor tissue [128].

In the two-stage setting of implant based breast reconstruction, radiotherapy can be given at one of three time-points, firstly to the un-expanded tissue expander, secondly to the fully expanded tissue expander prior to exchange to a permanent implant and lastly following implant exchange radiotherapy can be delivered to the permanent implant. In one stage implant reconstruction, radiation is delivered to the permanent implant.

Several studies have investigated the effect of PMRT on implant-based reconstruction including one and two stage reconstructions, however are limited to mostly single unit, retrospective cohort studies. Moreover, the studies are limited by the variability in the timing radiotherapy treatment regimes in relation to the reconstruction often dictated by local hospital protocols (radiation therapy delivered to tissue expander or permanent implant or delivered pre or post mastectomy) which incurs significant bias as operating on previously irradiated tissue is associated with a more complex procedure and an increased risk of post-operative complications. In addition, small patient sample size and lack of control population results in variable outcomes. A

meta-analysis is of use to determine the effect of PMRT onto the permanent implant and may identify significant results.

3.2 Aims of Chapter

The aim of this review was to systematically examine the effect of post mastectomy radiation therapy delivered to the permanent implant to determine the incidence of complications such as implant loss, capsular contracture and patient satisfaction to determine the impact of post mastectomy radiation therapy to permanent breast implants.

3.3 Methods

This systematic review and meta-analysis was performed according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement [119] (Fig. 3.1). The study protocol was registered with the University of York Centre for Reviews and Dissemination international prospective register of systematic review (2015:CRD42015026061). Ovid MEDLINE and Embase databases were searched up to and including the second week of October 2016 using the following search algorithm: ((breast reconstruction.mp. or Mammaplasty/) OR ((breast$ adj3 (reconstruction or implant)).mp.)) AND ((Radiotherapy, High-Energy/ or Radiotherapy, Intensity-Modulated/ or Radiotherapy/ or Radiotherapy, Computer-Assisted/ or Radiotherapy, Image-Guided/ or Radiotherapy, Adjuvant/ or Radiotherapy Dosage/) OR (radiotherapy.mp. or Radiotherapy/)). Medical literature was searched for studies comparing patients receiving radiotherapy post mastectomy directly to permanent implant with patients who did not receive PMRT. Patients undergoing delayed reconstruction post mastectomy, combined autologous reconstruction, radiation delivered to the tissue expander prior to implant exchange for the permanent implant and patients with a prior history of radiotherapy were excluded. Primary outcomes were implant loss, capsular contracture and revisional surgery. Secondary outcomes were cosmesis and patient satisfaction.

A manual search was also performed to search for relevant studies. Publications were excluded if not relevant to the topic, review articles, autologous breast reconstruction articles, letters, comments and conference abstracts.

All patients undergoing immediate one or two stage implant breast reconstruction were included in the study. Articles had to define that PMRT was delivered to the permanent implant or following tissue expander exchange to the definitive implant to be included. Patients who received PMRT to the tissue expander prior to exchange to PI and those patients who had combined implant autologous or autologous breast reconstruction were excluded. A time limit of the studies published in the last 20 years was chosen to reflect the improvements in breast implant technology and design as well as improvements in surgical and radiation techniques to limit bias.

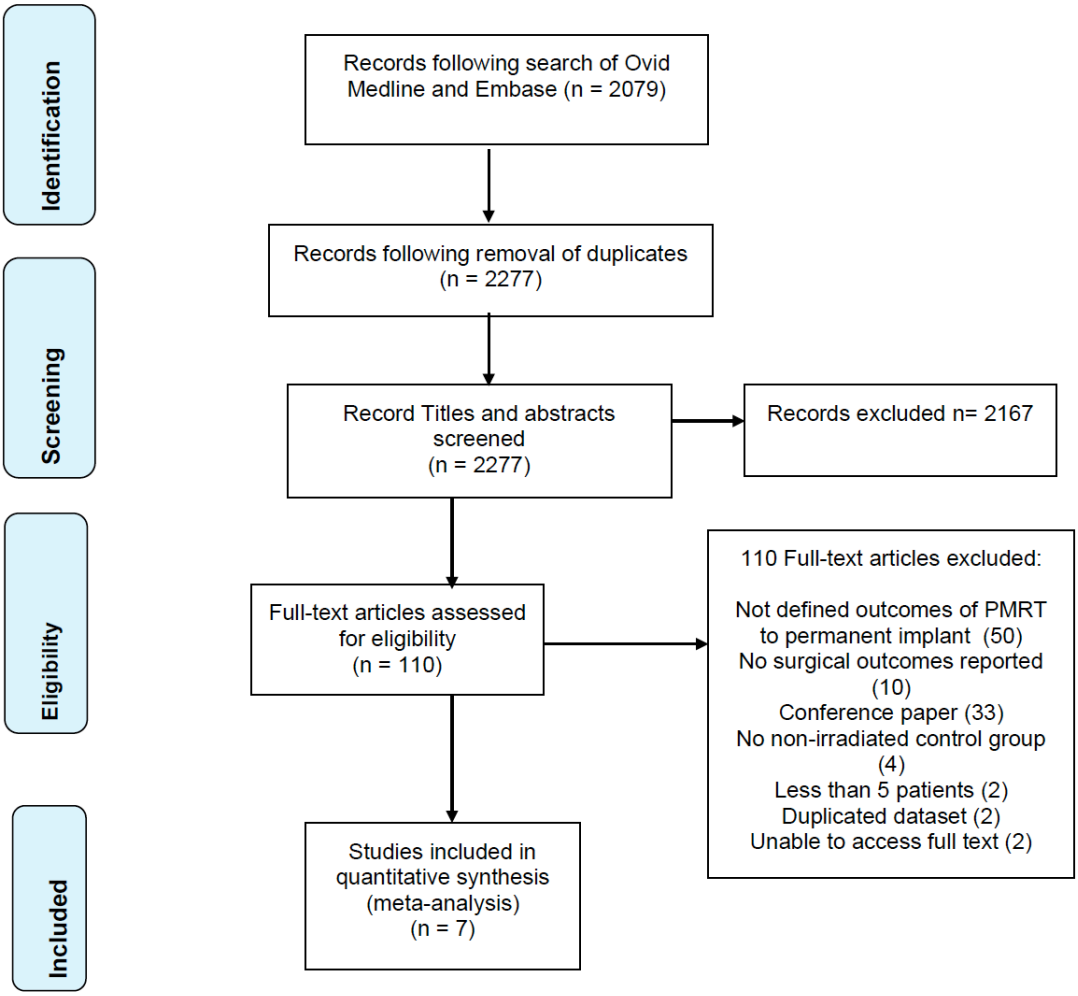
Primary outcomes were defined as capsular contracture (as defined as Baker Grade III or IV), revisional surgery and reconstructive failure (as defined as removal or replacement of the implant).

Secondary outcomes were defined as patient satisfaction and cosmetic outcomes. Patient satisfaction outcomes varied between studies and a good outcome was accepted as 'partially to fully satisfied', 'medium to good' and 'satisfied' for the purpose of our review. Cosmetic outcomes were similarly varied but defined by the operating surgeon.

3.3.1 Statistical Analysis

All primary and secondary endpoints were entered into and analysed using Revman 5® software (The Nordic Cochrane Centre, Copenhagen, Denmark) using a random effects DerSimonian-Laird model and results were reported with 95% confidence intervals. Heterogeneity was assessed using τ^2, χ^2, and I^2 measures and was deemed significant if $p<0.10$ or I^2 was greater than 30%.

Figure 3.1: PRISMA flow chart.



3.4 Results

A total of 2979 results were identified from combined Ovid Medline and Embase searches. Following electronic removal of duplicates, 2277 remained. Following review of the title and abstracts 1883 studies were considered irrelevant, 224 were reviews, case reports, letters and editorials, 48 were autologous breast reconstruction and radiation therapy and 12 were outside the defined time frame.

110 studies were selected for full text review, 50 described radiation therapy to tissue expander or combined outcomes of TE/PI and/or autologous reconstruction, 10 did not address surgical outcomes of radiation therapy, 33 were conference abstracts, 4 studies did not report outcomes for non-irradiated patients, 2 studies contained duplicate patient populations, 2 studies reported on less than 5 patients and 2 studies were not available in full text. Thus, seven studies [129–135] were selected for data extraction and inclusion in the final analysis containing 2921 patients (520 PMRT, 2401 control). A summary of the patient characteristics is outlined in Fig. 3.2.

	Benediktsson et. al 2005	**Cordeiro et. al 2004**	**Cordeiro et. al 2015**	**McCarthy et al 2005**	**Nava et. al 2011**	**Rella et. al 2015**	**Vandeweyer et. al 2000**
No. Of Patients radiotherapy group (PMRT)	98	81	1486	10	109	64	6
No. of Patients control group (Control)	107	542	210	10	98	80	118
Mean age (years)	54 (range 32-75)	48.5 (PMRT) 48.1 (control)	46.3 (PMRT) 47.8 (control)	47.9	49	46 (38-76)	45 (range 38-59)(PMRT) 47 (range 29-73)(control)
Mean follow-up (months)	56	33 (PMRT) 34 (control)	40.3 (PMRT) 45.6 (control)	23.5 (12-58.5)	50	10(PMRT) 9 (control)	64.5 (PMRT) 65 (control)
One vs Two stage reconstruction	One stage	Two stage	Two stage	Two stage	Two stage	One stage	One stage

Figure 3.2: Summary of Patient Characteristics

3.4.1 Primary End Points

All seven studies commented on capsular contracture [129–135] (2529 patients: 494 PMRT, 2035 control). There was significant increase in rate of capsular contracture in those patients receiving PMRT (OR 10.21, 95% C.I 3.74 to 27.89, p<0.00001). However, there was significant heterogeneity between the studies (I^2=88%, p<0.00001) indicative of retrospective cohort studies (Fig. 3.3)

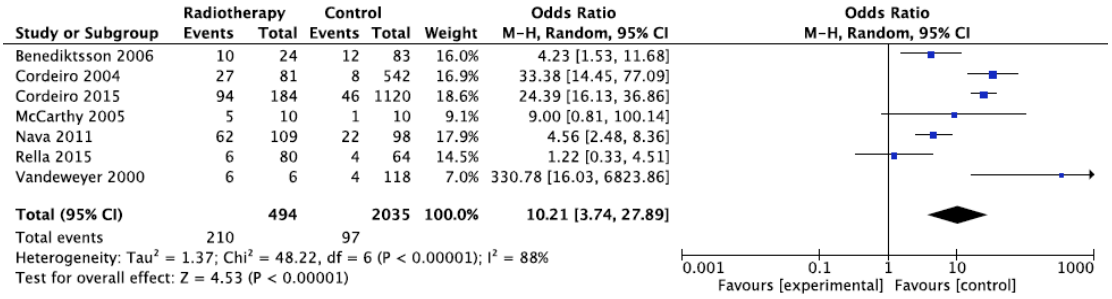


Figure 3.3: Forest plot demonstrating increased incidence of capsular contracture in patients undergoing PMRT

In addition, all studies reported patients undergoing revisional surgery including those with reconstructive failure [129–135] (7 studies, 2921 patients: 520 PMRT, 2401 control) (Fig. 3.4). There was no significant heterogeneity between the studies (I^2=30%, p=0.20). There was a significant increase in numbers of patients undergoing revisional surgery in the PMRT group (OR 2.18, 95% C.I 1.33 to 3.57, p=0.002).

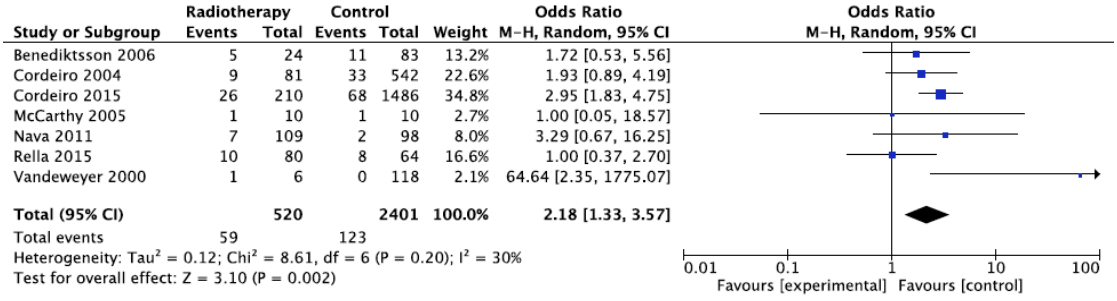


Figure 3.4: Forest plot demonstrating increased incidence of revisional surgery in patients undergoing PMRT

Six studies [130–135] (2814 patients: 496 PMRT, 2318 control) described reconstructive failure (as defined as implant removal or replacement) (Fig. 3.5). There was no significant heterogeneity between the studies (I^2=21%, p=0.28). PMRT was significantly associated with an increased number of patients with reconstructive failure (OR 2.52, 95% C.I 1.48 to 4.29, p=0.0007)

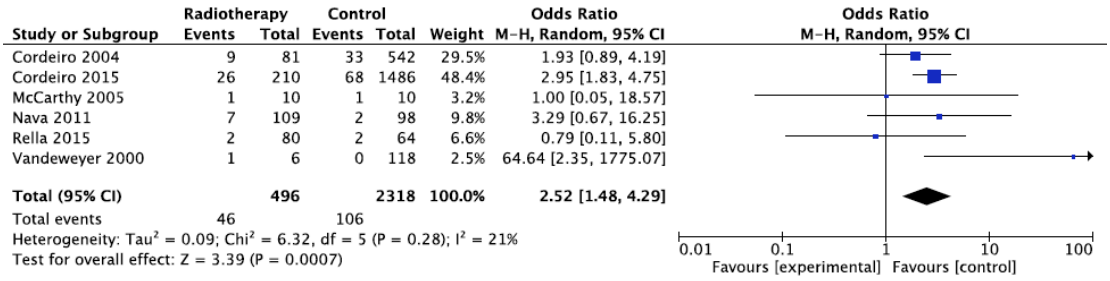


Figure 3.5: Forest plot demonstrating increased incidence of reconstructive failure in patients undergoing PMRT

3.4.2 Secondary End points

Four studies reported patient satisfaction outcomes [131,132,134,135] (468 patients: 174 PMRT, 294 control). There was no significant heterogeneity between the studies (I^2=0%, p=0.5). There was significant reduction in patient satisfaction rates in patients undergoing PMRT compared to the control group (OR 0.29 95% C.I 0.15 to 0.57, p=0.0003) (Fig. 3.6).

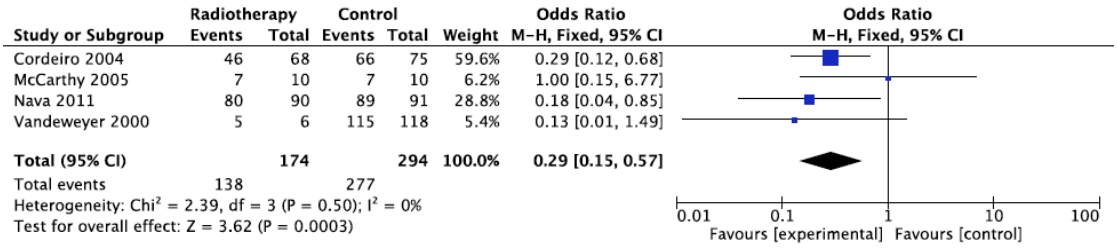


Figure 3.6: Forest plot demonstrating a reduction in patient satisfaction at reconstructive outcome following PMRT

Four studies reported cosmetic outcome [130,132,134,135] (1317 patients: 275 PMRT, 1042 control). There was significant heterogeneity in the studies (I^2=59%, p=0.09) with a significant reduction in acceptable cosmetic outcome in patients undergoing PMRT compared to the control group (OR 0.28 95% C.I 0.11 to 0.67, p=0.005) (Fig. 3.7).

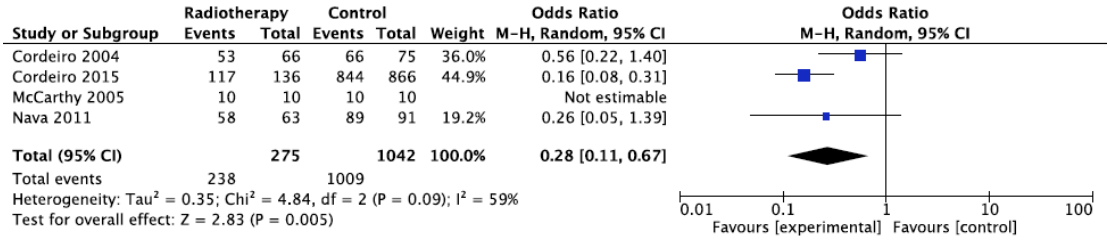


Figure 3.7: Forest plot demonstrating reduced cosmetic outcome as reported by the surgeons in patients undergoing PMRT

3.5 Discussion

Radiotherapy has now been shown to be increasingly efficacious in early stage breast cancer disease as well as those with established disease [99]. With an increasingly younger population of patients diagnosed with breast cancer, the numbers of patients undergoing implant based breast reconstruction and PMRT is set to increase. Implant based breast reconstruction is the most popular form of reconstruction and may represent increasing numbers of younger patients who lack the adipose reserves to perform autologous reconstruction or the patients desire to achieve a more aesthetically pleasing, non-ptotic breast. To date, studies on PMRT and permanent implants are limited due to their small patient sample size, retrospective nature, lack of randomization, variable reporting outcomes and often lack of control groups to compare their findings as well as variability in timing of radiation therapy in relation to the breast reconstruction – therefore a systematic review of this topic is important as it may demonstrate significant results from underpowered studies.

The results from this review demonstrate clearly that the deliverance of PMRT to a permanent implant is associated with significantly increased rate of capsular contracture. The incidence of capsular contracture increased from 5% in the control group to 43% in patients undergoing PMRT.

Furthermore these patient groups are more likely to suffer from a failure of their reconstructive surgery (9% vs. 6%, $p<0.001$) and to have to undergo further revisional surgery (11% vs. 5%, $p=0.002$).

Cosmetic outcome as reported by both patients and surgeons were significantly poorer in patients undergoing PMRT.

There are limitations to this review. There was significant heterogeneity in the method that each paper reported their outcomes. We included 'partially to fully satisfied' [131], 'medium to good' [132] and 'satisfied' [134,135] as acceptable patient satisfaction outcomes for the purpose of our review. In addition, this was echoed in the reported outcomes for cosmesis [130,132,134,135].

Radiotherapy was generally delivered 3-6 weeks following reconstructive procedure, however in a study published by Vandemeyer et al. two patients included in the study with permanent implants were irradiated for local recurrences months after reconstructive surgery [131]. In addition, in a study reported by Cordeiro et al. those

patients receiving PMRT to permanent implant had already undergone post mastectomy chemotherapy in comparison to those patients in the same study who did not require chemotherapy and had therefore PMRT delivered to the tissue expander [130]. This may therefore select a patient cohort with later stage disease requiring several adjuvant treatment modalities that may influence their overall quality of life and may impact on their psychological state and patient satisfaction scores. However, despite this, data published by Cordeiro et al. 2015 reported no difference in patient satisfaction scores between the PMRT to tissue expander and PMRT to permanent implant groups [130]

All studies stated that textured implants were employed, one study used only saline-filled implants [129] another study stated that eight of 12 patients underwent reconstruction with saline implants, the remaining patients having silicone breast implants and one study stated only 'textured' implants [131].

In addition, a study by Benediktsson et al. excluded 14 patients who had lost their implant before the two year follow up therefore this will have led to under-reporting the revisional surgery and reconstructive failure data [129] . Moreover, 6 patients in the study did not undergo revisional surgery due to personal choice or advanced disease which may have influenced the results [129].
In the study by Cordeiro 2015, not all patients in the study had capsular contracture outcomes recorded which might have led to bias in the results [130].
Interestingly, a study by McCarthy et al. reported outcomes for those patients undergoing bilateral reconstruction with unilateral radiotherapy using the non-irradiated breast as a control [135]. All patients described their cosmetic outcome as excellent/very good or good but only 70% of patients were satisfied with their reconstruction [135].
The average length of follow up in these studies was 31 months (range 9 – 65 months). There were a significant number of patients lost to follow up by five years in one study [129] therefore we used the data generated at 2 years follow up for the purpose of our review. No study followed patients up beyond five years and therefore the long-term outcome has not yet been reported.

3. 6 Conclusion

This meta-analysis has shown that there are significantly increased rates of capsular contracture, revisional surgery and reconstructive failure as well as reduced patient satisfaction scores and cosmetic outcome in those patients receiving PMRT to a permanent implant within the first five years of surgery. As this is the first meta-analysis to report patient outcomes for PMRT delivered to the permanent implant, it provides robust knowledge which can help guide informed decision making when deciding the most appropriate method and timing of breast reconstruction for the patient undergoing PMRT. Further long-term follow-up to determine the long-term complication rates of PMRT are required.

Chapter 4:Analysis of Retrieved Breast Implants from a Single Centre

4.1 Introduction

Breast implants have a role in aesthetic and reconstructive breast surgery however little is known of the mechanism of aging and in vivo response to breast implants. Complication rates or need for re-operation within the first two years following initial implant surgery have been reported up to 24.5% in primary augmentation patients and up to 42.9% in primary reconstruction patients [136]

Silicone gel breast implants are composed of an elastomer shell envelope containing a gel made from the polymer polydimethylsiloxane (PDMS $(CH_3)_2SiO$). The difference between the shell and gel composition is the degree of cross-linking between the polymer chains [56]. The mechanism of breast implants failure during implantation resulting in capsular contracture (a pathological fibrous encapsulation of the implant), gel bleed or leaking of silicone into the surrounding tissues and implant rupture is not yet fully elucidated.

Several studies have sought to establish the cause of breast implant failure. Research has shown that increasing implantation times negatively effect the mechanical strength properties in explanted breast implants [14,63,137]. Furthermore, mechanical weakening of the shells have been also postulated to be attributed to swelling of the breast implant shells with low molecular weight silicones diffusing from the gel into the shells during implantation [56,138]. Further chemical analysis through use of attenuated total reflectance–Fourier transform infra-red spectroscopy (ATR-FTIR) has been studied in breast implants highlighting potential functional groups with may interact with the surrounding host cellular environment [120] and surface chemical changes of the implants following radiation therapy [104] and implantation [43].

Prasad et al [139] demonstrated that increasing the surface roughness of silicone elastomers samples produced a decrease in fibroblast growth which may account for reduced incidence of capsular contracture using textured rather than smooth breast implants [140]. Valencia-Lazcano et al [86] showed that increased surface roughness of uninplanted breast implants resulted in greater fibroblast adhesion in an in-vitro model. However, no studies to our knowledge have investigated fibroblast behavior on retrieved breast implants.

Thus, the aim of this study was to characterize the effects of implantation and aging on the mechanical and surface chemical properties of the implants retrieved from

patients attending for elective removal or exchange of implants and their in vitro fibroblast response.

4.2 Aims

To assess the effect of increasing duration of implantation on the surface, chemical and mechanical properties of implants and to assess the fibroblast response to these implants.

4.3 Materials and Methods

4.3.1 Consent and Patient recruitment

Patients were consented by the author prior to surgery. Breast implants were collected from theatre, labeled anonymously, visually inspected and cleaned with iso-propanolol. The implants were cut using a scalpel into anterior shell and posterior shell (in contact with patient's chest wall) and the inner gel carefully inspected and removed. The anterior and posterior shells were then cleaned with iso-propanolol and left to air dry at room temperature prior to analysis (Fig. 4.1). For the purpose of control analysis in the Atomic Force Microscopy, an un-implanted textured silicone breast implant shell with the inner gel carefully removed (Mentor Siltex™ Contour Profile™ Becker ™ 35 Expander, Cohesive II™, Lot 6811381).

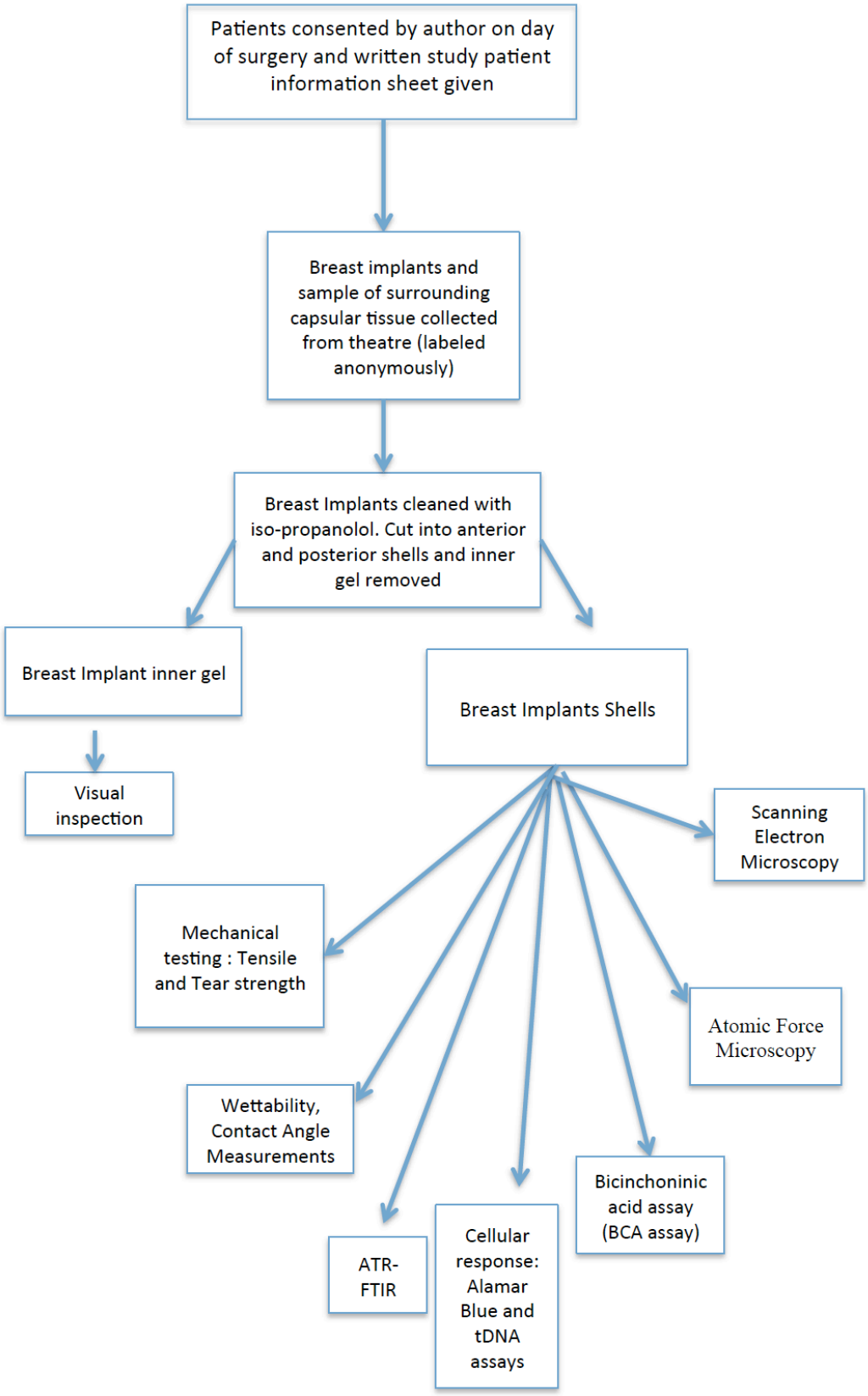


Figure 4.1: Timeline of sample collection, processing and analysis.

4.3.2 Tensile Testing and Tear Testing

From the implant shells, six dumbbell shaped (20mm x 4mm) specimens were cut using a Wallace cutting press and three crescent shaped specimens for tear testing from anterior and posterior parts of the implant shell in accordance with the ISO 37:2005 standards. Specimens were placed in the pneumatic grips of the tensiometer (Instron 5565 tensiometer equipped with a 500 N load, Instron, UK) and pulled apart at a rate of 100mm/min and 500mm/min for tensile and tear testing respectively. The data was captured using Bluehill software. Ultimate tensile strength, strain at break, Young's modulus and tear strength values were recorded.

4.3.3 Atomic Force Microscopy (AFM)

This was performed by selecting a control sample (un-implanted sample) and two of the retrieved breast implants from the same manufacturer (Allergan) of differing lengths of implantation. Using a Nanowizard 1 (JPK – Force Spectroscopy mode), AFM measurements of the selected shells were taken from the shells' cross section, outer and inner measurements, over 10 μm x 10 μm areas, using n= 256 per group with a sensitivity of 36.76 nm/V, k = 5.8311 (Bruker RFESPA), set point 64.304 nN and Z-length = 15 μm.

4.3.4 ATR-FTIR of Breast Implant Shells and Gels

To evaluate the chemical composition and quantity of molecules in any given substance, attenuated total reflectance-Fourier transform infrared spectra (ATR-FTIR) was used. This uses infrared radiation to identify chemicals and chemical bonds with breast implant shells tested and to determine if there were any chemical/molecular changes between them as result of implantation (n=5). Briefly, the midpoint of the anterior shell of each implant was tested (n=5). The breast implant shells were quantified using ATR-FTIR recordings using Jasco FT/IR 4200 Spectrometer with a diamond attenuated total reflectance accessory (Diamond Miracle ATR, Pike Technologies, US). A spectrum was produced (n=5) from an average of 30 scans a spectrum was produced over a range of 600cm^{-1} to 4000 cm^{-1} with a resolution of 4cm^{-1}. A background scan was performed prior to every measurement.

4.3.5 Surface Wettability/Contact Angle Measurements (θ)

Using a DSA 100 Krűss Goniometer, wettability analysis was performed on the breast implants outer shell surface. Using the sessile drop technique, 5ul of deionized water

was dropped onto the implants using an automated syringe with 10 seconds of dispensing and analysis was performed using the Drop Analysis software (n=12) (EasyDrop DSA200, KrűSS) at room temperature.

4.3.6 Scanning Electron Microscopy of Breast Implant Shells

Following the decellularisation protocol as outlined in 2.7.2, the breast implant samples were mounted on aluminum stubs using carbon adhesive tabs and sputter coated with gold/palladium using a High Resolution Ion Beam Coater (Gatan Model 681). Images were taken using a scanning electron microscopy at magnifications ranging from x50 to x 1000 using a Field Emission Scanning Microscope (JEOL-JSM 7401F) and images saved as JPEG.

4.3.7 Protein Quantification Assay (Bicinchoninic Acid (BCA) assay) of Breast Implant specimens.

The protein content of the breast implant shells specimens were analysed using the Bicinchoninic (BCA) assay. 0.25gram of implant shell and 0.25grams of gel were placed in 0.2% trifluoroacetate and acetonitrile 50v/50v for 24 hours in a dark cupboard at room temperature. The solution was evaporated to dryness using a speedivac, the implant shell removed and the extracted proteins were re-suspended in 250µl of water. Protein content of the resuspension was determined using the Pierce™ BCA Protein assay kit, (BCA assay) ThermoFisher Scientific, UK. The absorbance was measured at 592mm excitation using a fluorescent plate reader (Fluoroskan Ascent FL™, ThermoScientific™, USA)

4.3.8 In Vitro Cellular Response to Retrieved Breast Implants

4.3.8.1 Preparation of Breast Implant Shell specimens and Cell Culture

HDFa fibroblasts were passaged in sterile T-75 flasks using Trypsin with Dulbecco's Modified Eagle's Medium, low glucose (Gibco, ThermoScientific™, UK) supplemented with 10% FBS (foetal bovine serum) and 1% PenStrep (penicillin-streptomycin, Gibco) or cryopreserved using DMSO until further required. HDFa's used in the experiment were between passage 7 and 11.

Discs measuring 6mm (n=6) were cut using a heavy-duty office hole punch from the breast implant shells. Decellularisation of the specimens was achieved by placing them in 1% Triton X for 1 hour, washing twice in PBS followed by 70% ethanol followed by washing twice in PBS. Discs were then placed in a 96 well plate and

covered with 100µl of warmed DMEM for approximately 2 hours prior to cell seeding.

4.3.8.2 Alamar Blue™ Assay – Cell Metabolism

To assess cell metabolism, Alamar Blue™ assay (Invitrogen, Paisley, UK) was used. Implant disc specimens were seeded with HDFa cells at density of $5x10^4$ cells/cm^2. Cells seeded onto tissue culture plastic served as a positive control and media only wells provided a negative control. Cells were incubated at 37^oC at 5% CO2 in air and cell culture media was replenished on days 0, 1, 3 and 6. The Alamar Blue™ assay was conducted according to manufacturer's guidelines on days 1, 3 and 7. Media from the wells was removed and fresh media containing 10% Alamar Blue™ solution was added to each well. Following a 4 hr incubation wrapped in aluminium foil, 100µl of the media from each well was placed into a 96-well plate and analysed using a fluorescent plate reader (Fluoroskan Ascent FL™ Fluorescence Plate Reader, ThermoScientific, USA) at an excitation and emission wavelengths of 530nm and 620nm (n=6).

4.3.8.3 DNA Quantification – Cell proliferation

To assess cell proliferation, Hoechst 33258 DNA Quantification Kit, Fluorescence Assay (Sigma-Aldrich, UK) was used. Following Alamar Blue™ analysis the specimens were washed with PBS and 100µl of molecular grade water was added to each well. The plates were then submitted to 6 freeze-thaw cycles to achieve cell lysis. Fluorescence was measured using a fluorescent plate reader (Fluoroskan Ascent FL™ Fluorescence Plate Reader, ThermoScientific, USA) at excitation and emission wavelengths of 360nm and 460nm (n=6). A standard curve was performed with known quantities of calf thymus DNA and the equation was used to calculate the DNA concentrations from the fluorescence of the specimens.

4.3.12 Statistics

All statistics were performed using either linear correlation, non-parametric Spearman correlation, one-way ANOVA and 2 way ANOVA where significance was $p < 0.05$. All graphs were performed using GraphPad Prism software Version 6 apart from ATR-FTIR were presented using Microsoft Excel.

4.4 Results

A total of 15 patients were recruited to the study. All patients were female and mean age at time of implant removal or exchange was 42.3 years (SD 8.36 years). Eleven patients had undergone breast implant surgery for reconstructive purposes (9 unilateral procedures, 2 bilateral procedures) and four patients had undergone breast implant surgery for cosmetic augmentation (4 bilateral procedures). Three of fifteen patients were smokers. Twenty-one breast implants and 8 corresponding samples of surrounding capsular issue were retrieved. All implants retrieved featured a textured surface. The mean time from initial operation to removal or exchange of implant was 133.3 months (SD 90.1 months). Five implants (24%) were ruptured at time of retrieval. The reasons for removal were capsular contracture (suffering either Baker III or IV level of capsular contracture) (5 implants), exchange for permanent implant (5), suspected or confirmed rupture (4), pain/discomfort (3), complication with other breast (2), symmetrisation (1), presence of axillary silicone granuloma (1). A summary of patient and breast implant characteristics is provided in Table 4.1 and detailed in Appendix 1.

No. of Patients	**15 (21 implants)**
Unilateral procedure	**9 (60%) (9 implants)**
Bilateral procedure	**6 (40%) (12 implants)**
Age, Mean ± SD (years)	**42.3 ± 8.4**
Duration of Implantation, Mean ± SD (months)	**133.4 ± 90.1**
Smoker	**3 patients (18.6%)**
Indication for Implants	**No. of Patients**
Breast Reconstruction	**11 patients (9 unilateral, 2 bilateral)**
Cancer	*8 patients (8 unilateral)*
Risk Reducing Surgery	*2 patients (2 bilateral)*
Breast hypoplasia	*1 patients (1 unilateral)*
Breast Augmentation	**4 patients (4 bilateral)**
Indication for implant removal/exchange	**No. of Patients**
Capsular Contracture (Baker Grade III/IV)	**5(23.8%)**
Exchange for Permanent Implant	**5(23.8%)**
Suspected/Confirmed Implant Rupture	**4 (19.0%)**
Contralateral breast implant complication	**3 (14.2%)**
Pain/Breast distortion	**2 (9.6%)**
Symmetrisation	**1 (4.8%)**
Presence of Axillary Silicone Granuloma	**1 (4.8%)**
Implant integrity at time of retrieval	**No. of Implants**
Intact	**16 (76.2%)**
Ruptured	**5 (23.8%)**

Table 4.1. Summary of Patient and Breast Implant Characteristics

Retrieved gels varied in colour from clear, colourless to strong yellow. As shown in Fig. 4.2 ruptured implants contained strong yellow discoloured gels in comparison to intact implants.

Figure 4.2: Intact Implant (left) with ruptured implant with observed yellowing of inner gel (right)

4.4.1 Breast Implant Type and Manufacturer

Implants collected were found to be from a range of manufacturers as shown in Fig. 4.3 and included Allergan (5), McGhan (3), Mentor (2), Poly Implant Prothèsis (3) and Labaratoire Sebbin (2). Five of the 21 retrieved implants did not exhibit manufacturers details and were therefore labeled as 'unknown'. Furthermore, implants were determined as tissue expanders or permanent implants as shown in Table 4.2.

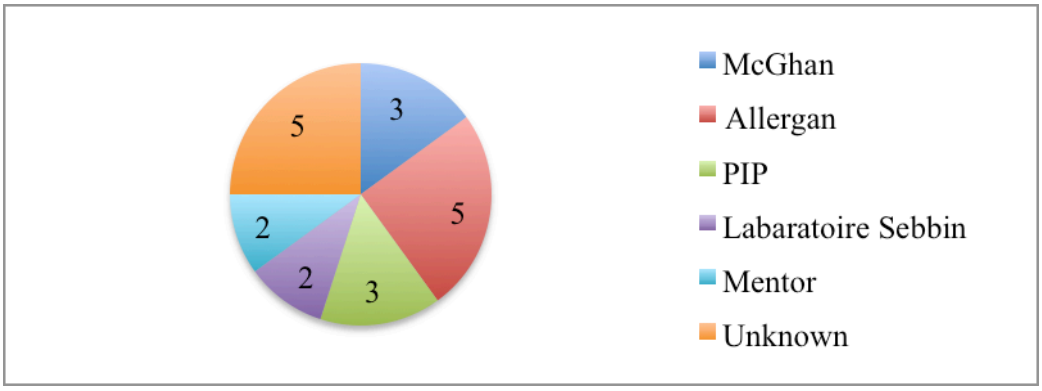


Figure 4.3: Analysis of Implant Type by Manufacturer. Five retrieved implants (23.8%) were manufactured by Allergan.

Implant Type	Implants By Manufacturer
Permanent Implant Total = 14	• Unknown (5) • Poly Implant Prothèse (3) • McGhan (3) • Laboratoires Sebbin (2) • Allergan (1) • Mentor (1)
Tissue Expander Total =7	• Allergan (4) • Mentor (2) • McGhan (1)

Table 4.2: Implant Type and Manufacturers

4.4.2 Tensile Mechanical Properties of Retrieved Breast Implants

A significant fall in ultimate tensile strength (UTS) is observed with increasing implantation times both for anterior shell (p=0.0003, r -0.708) and posterior shells specimens from the explanted implants (p=0.0312, r=-0.5085) as shown in Fig. 4.4A and B. In addition, Young's modulus for anterior shell specimens (p=0.0037, r=0.6049) was reduced however, posterior shell specimens showed no significant difference (p=0.2032, r=-0.3149) as shown in Fig. 4.5. Strain at break demonstrated a significant reduction in anterior shell specimens (p=0.0003, r=-0.7158) but no significant difference was observed in the posterior shell specimens (p=0.0654, r=-0.4433) as shown in Fig. 4.6.

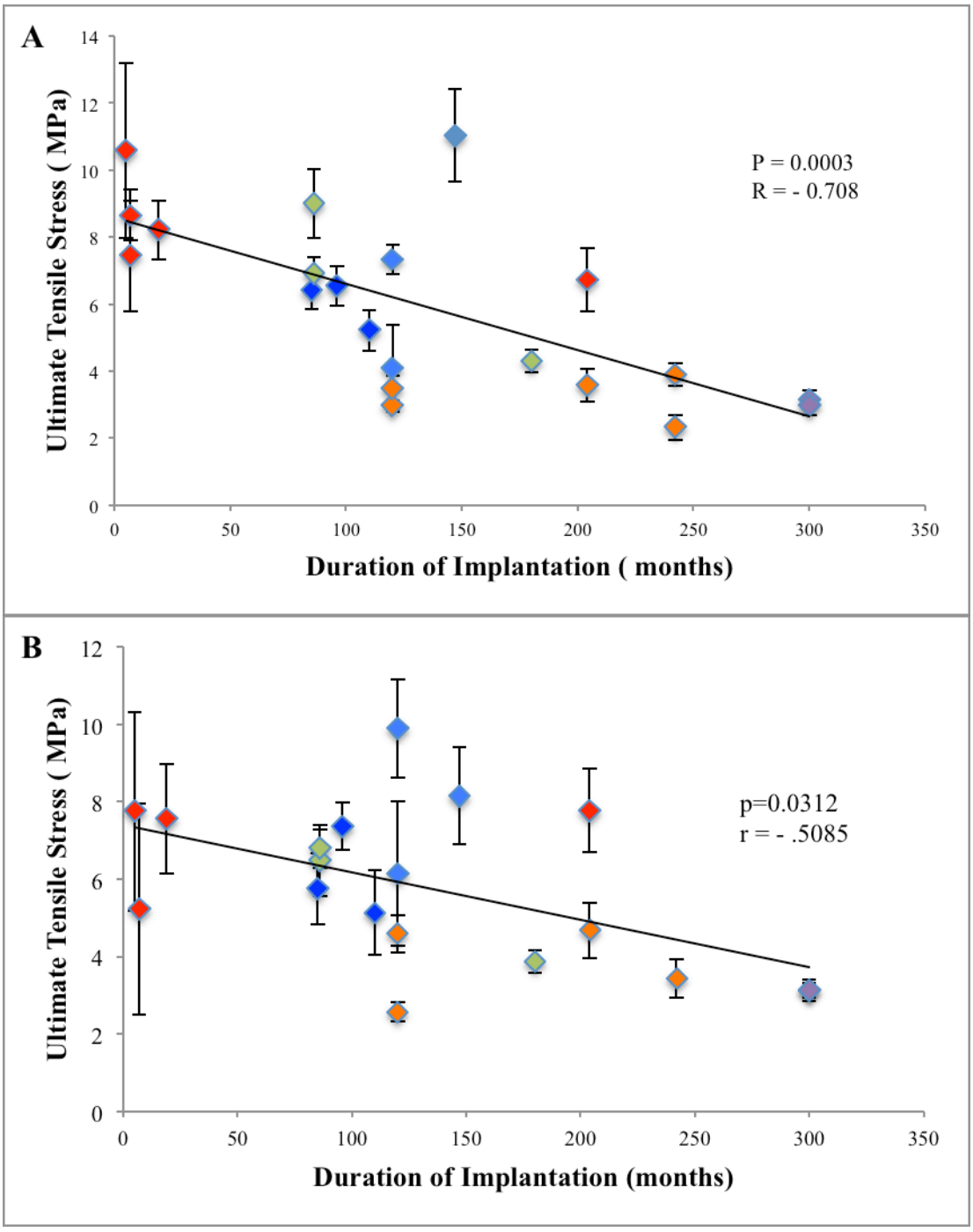


Figure 4.4: A fall in ultimate tensile stress (MPa) is significantly associated with increasing implantation times seen in **A** Anterior Shell Specimens (p=0.0003) and **B.** Posterior Shell Specimens (p=0.0312). Markers by manufacturer are displayed as follows: McGhan (dark blue), PIP (green), Labaratoire Sebbin (purple), Mentor (light blue), Allergan (red), Unknown (orange).

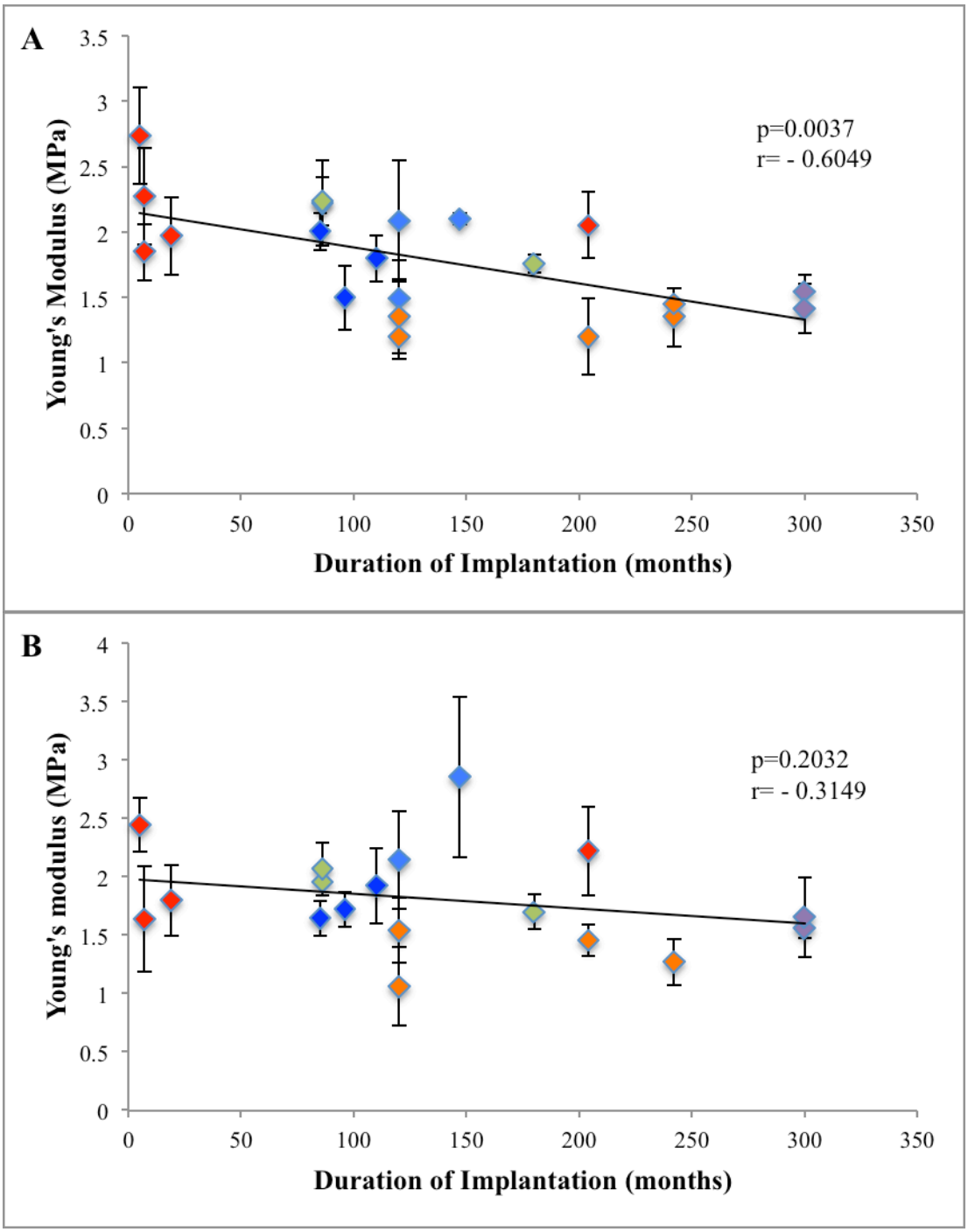


Figure 4.5: Young's Modulus of breast implants falls significantly with increasing duration of implantation in **(A)** Anterior Breast Implant Shells (p=0.0037) but no significant change is seen in **(B)** Posterior Breast Implant Shells (p=0.2032). Coloured markers by manufacturer as per Fig. 4.4.

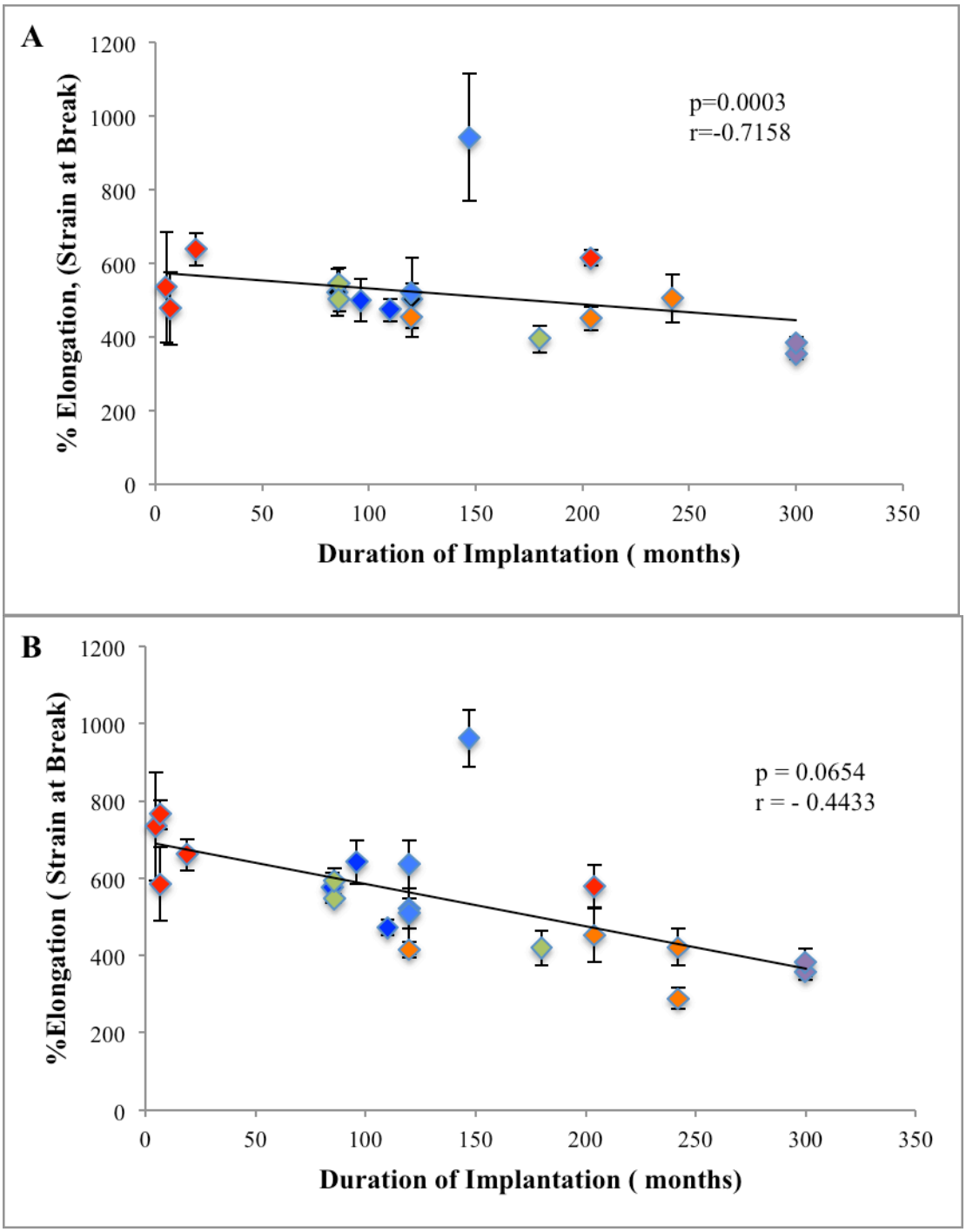


Figure 4.6: % Elongation, Strain at break of Anterior Breast Implant Shells **(A)** falls with increasing implantation times (p=0.0003) but no significant difference detected in **(B)** Posterior Shells (p= 0.0654).

4.4.3 Tear Strength of Retrieved Breast Implants

Ultimate tear strength demonstrated a significant reduction with increasing implantation times as shown in Fig. 4.7 A and B for anterior (p=0.0006, r=-0.6714) and posterior shell specimens (p=0.0254, r=-0.5108).

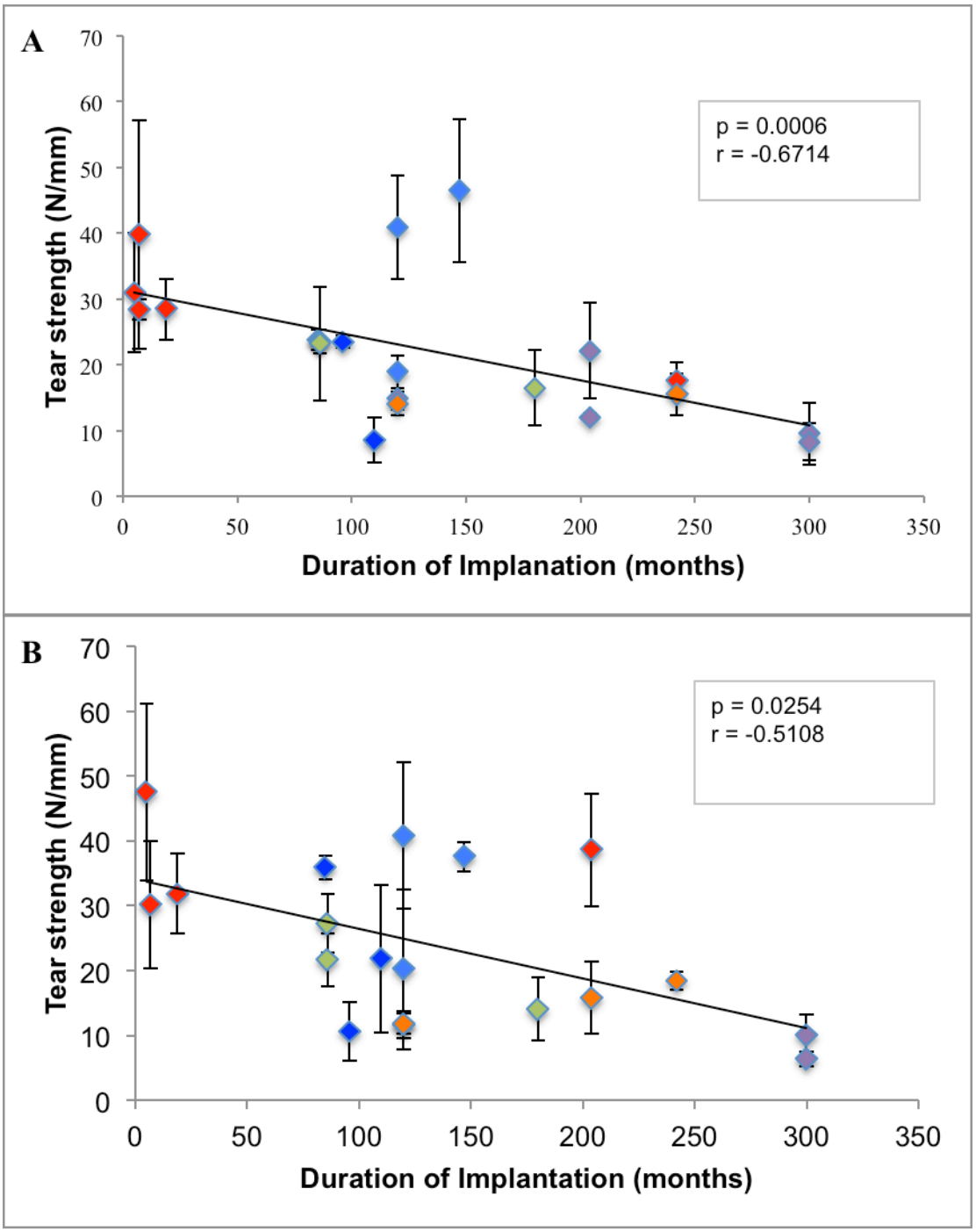


Figure 4.7: Tear Strength of Anterior **(A)** (p=0.0006) and Posterior **(B)** (p=0.0254) Breast Implant Shells fall significantly with increasing implantation times.

4.4.4 Atomic Force Microscopy of Retrieved Breast Implants

This demonstrated in the control specimen, no difference in cross section. In the 7 month implant, significant reduction in the young's modulus from inner to outer section of the shell. In the 204 month shell, there was no significant difference but the values were lower than the control specimen shown in Table 4.3.

Specimen Type	Young's Modulus (MPa) Inner	Young's Modulus (MPa) Outer
Control	6.53 (1.67)	7.70 (2.60)
7 months	12.14 (2.97)	0.37 (0.13)
204 months	4.35 (0.62)	4.32 (0.99)

Table 4.3: Atomic Force Microscopy (AFM) Young's Modulus values of control specimen, 7 month and 204 month retrieved implants.

4.4.5 ATR-FTIR of Retrieved Breast Implant Shells

Chemical analysis of the retrieved breast implant shells grouped into 5 year categories was determined by ATR-FTIR, outlined in Fig. 4.8. The average spectrum of implant shells (n=5) grouped into 5-year categories is shown in the overlaid spectra in Fig. 4.9 and Fig. 4.10. On review, there was statistically significant observed changes at the peak spectral height 784 cm^{-1} corresponding to $-CH_3$ rocking and –Si-C-stretching in $-Si-CH_3$ as shown in Fig, 4.11A (p=0.0224, one-way ANOVA, parametric data). However, there were no significant differences between the peak spectral heights at 1004 cm^{-1} corresponding to the asymmetric stretching of –Si-O-Si- (Fig. 4.11B p=0.2152, one-way ANOVA, parametric data) and at 1257 cm^{-1} corresponding to symmetric bending of $-CH_3$ in $-Si-CH_3$ (Fig. 4.11C p=0.1698, one-way ANOVA, parametric data). Furthermore, on statistical analysis of the peak spectral height between the intact group (n=17) versus ruptured shells (n=4) found no significant differences as shown in Fig. 4.12 and Fig. 4.13.

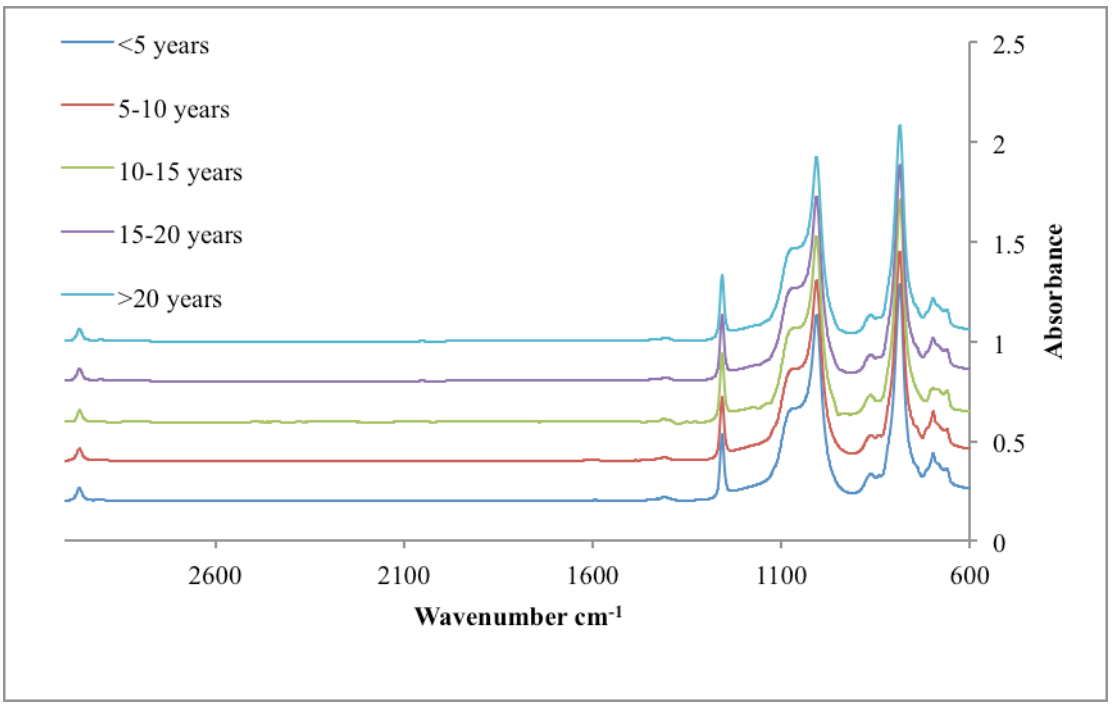


Figure 4.8: Overlaid ATR-FTIR spectra by 5-year categories offset by 0.2 absorbance

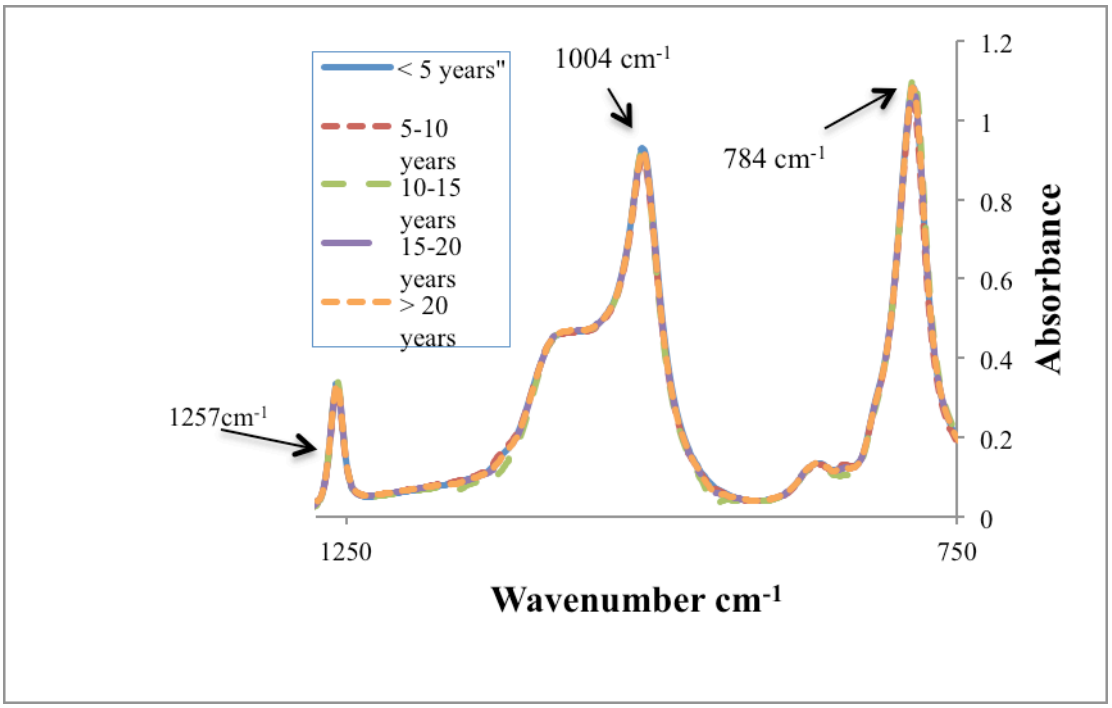


Figure 4.9: ATR-FTIR spectra from wavenumber 750–1300 cm^{-1} showing overlaid spectra from the 5-year categories. Significant differences in spectra height seen at peak 784 cm^{-1}, but not at peak height 1004 cm^{-1} and 1257 cm^{-1}.

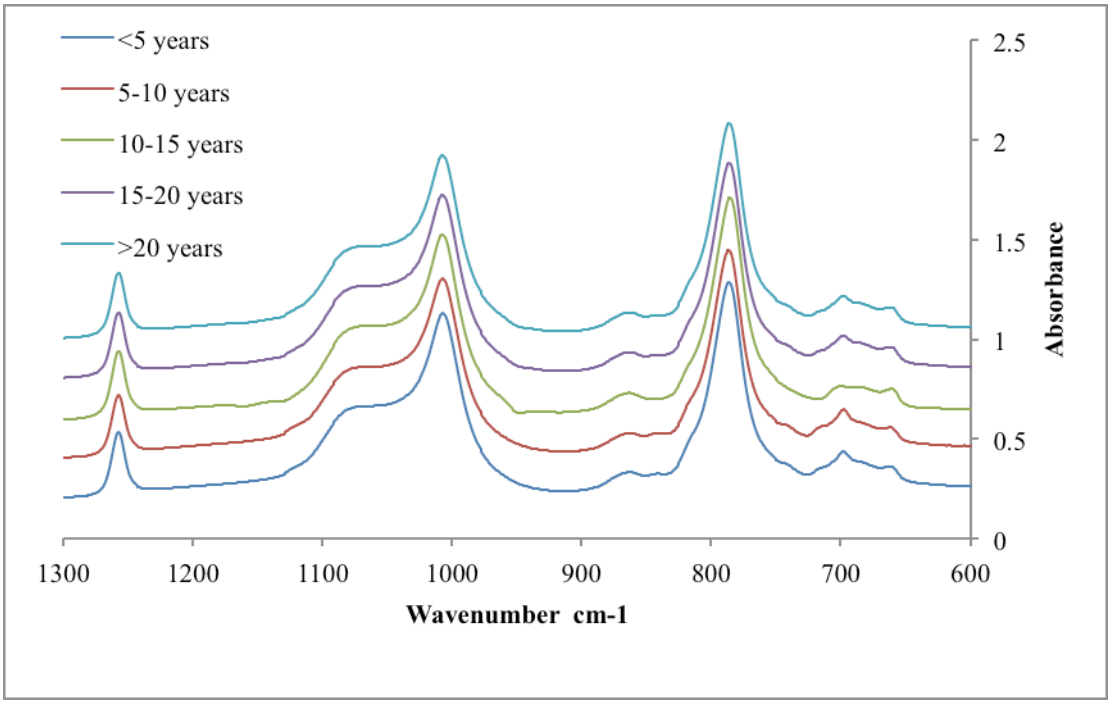
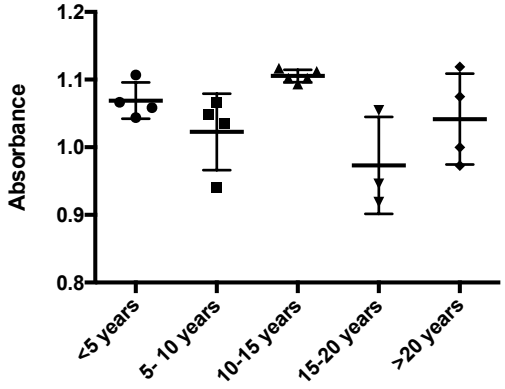
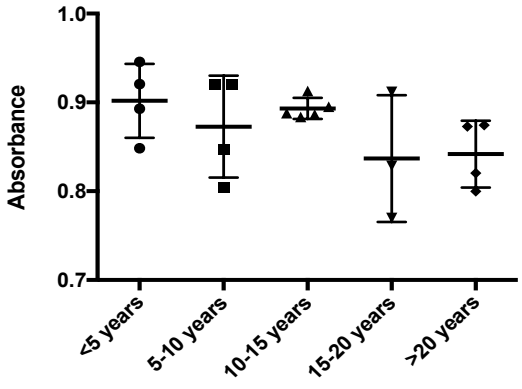


Figure 4.10: Overlaid ATR-FTIR spectra by 5-year categories offset by 0.2 absorbance from wavelength 600-1300cm^{-1}

A



B



C

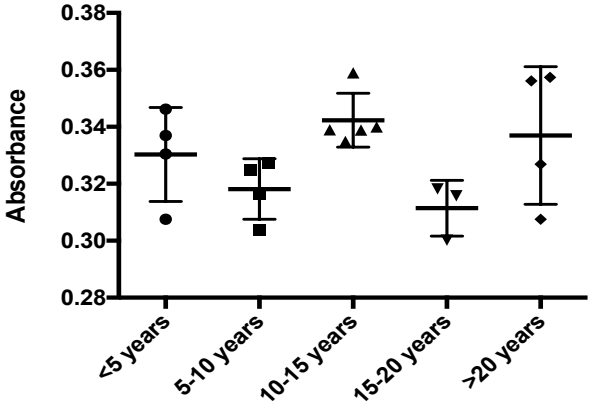


Figure 4.11: (A) Peak Spectral Height 784 cm^{-1} (p=0.0224, one way ANOVA, parametric data) **(B)** Peak Spectral Peak Spectral Height 1004 cm^{-1} (p=0.2152, one-way ANOVA, parametric data) **(C)** Height 1257 cm^{-1} (p=0.1698, one-way ANOVA, parametric data)

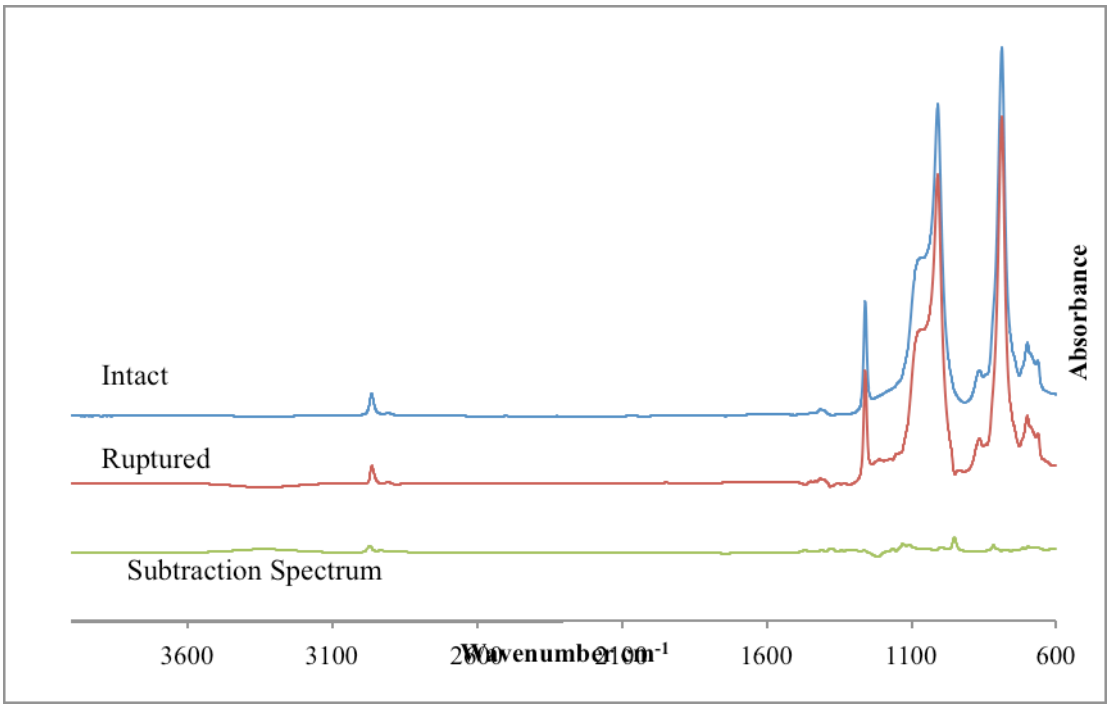


Figure 4.12: ATR-FTIR spectra of Intact and Ruptured shell specimens (staggered). A subtraction spectrum (Intact-Ruptured) is shown.

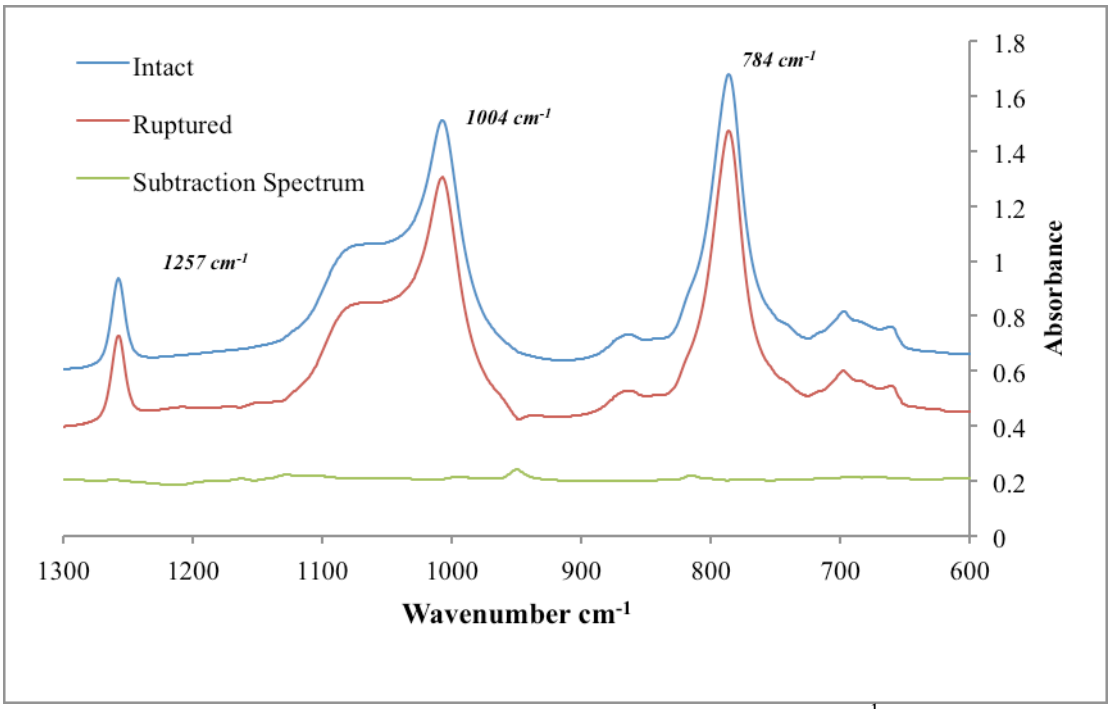


Figure 4.13: ATR-FTIR spectra from wavenumber 600 – 1300 cm^{-1} showing overlaid spectra from the intact breast implant shells (+0.6) and ruptured (+0.4) shells. No significant differences detected in the peak spectral values between each group: 784cm^{-1} p=0.8303, 1004cm^{-1} p=0.9569, 1257 cm^{-1} p=0.9342, two tailed, unpaired t-test. Subtraction spectrum is also shown.

4.4.6 Surface Wettability/Contact Angle Measurements (θ)

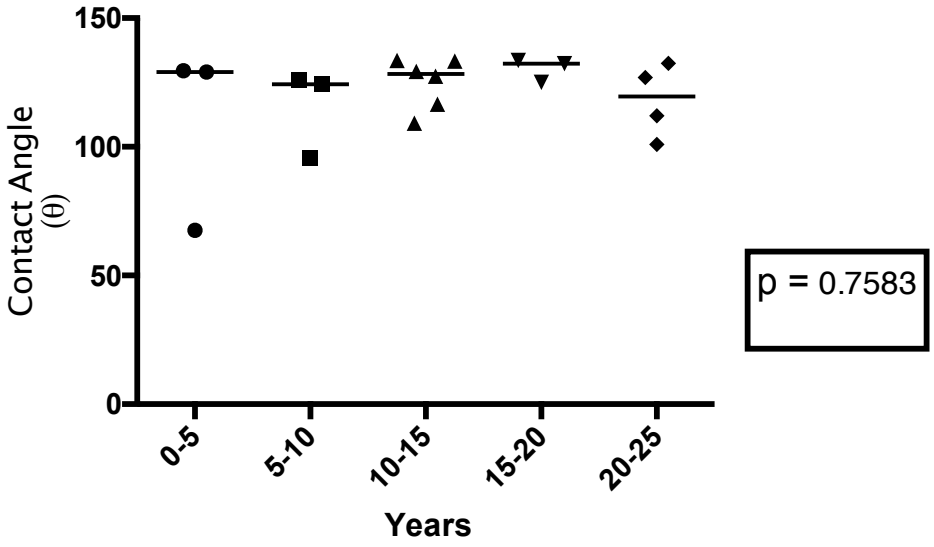


Figure 4.14: Contact angle measurements of retrieved breast implant shells grouped into 5-year categories.

There was no significant difference in contact angle/surface wettability in the implants grouped into 5-year duration of implantation categories as shown in Fig. 4.14 (p= 0.7583. one way ANOVA).

4.4.7 Scanning Electron Microscopy of Breast Implant Shells

The SEM images of retrieved implants from the same manufacturer of differing implantation times demonstrated within increasing implantation times the breast implant shell surface was roughened with pits, grooves and 'ragged' edges.

All retrieved implants irrespective of duration of implantation displayed evidence of surface wearing. The McGhan implants retrieved after 85months (Figure 15a) and 110 months (Figure 15b) showed increasing evidence of ragged edges of the manufactured pits with surface dents and grooves. In the Allergan implants retrieved after 7 months (Figure 16a) and 204 months (Figure 16b) surface degradation was evident with ragged edges, increasing irregularity of the pits outline more pronounced in the 204 months implant suggesting increasing surface degradation with time.

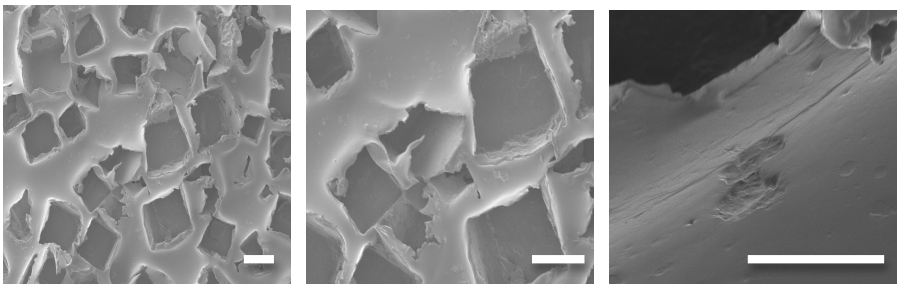


Fig. 4.15A: 85 months duration of implantation

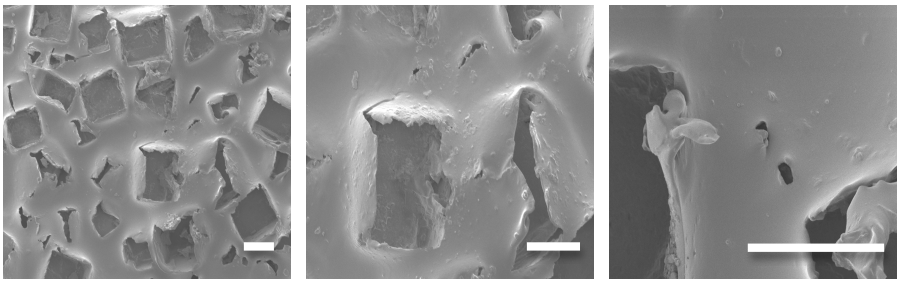


Fig. 4.15B: 110 months duration of implantation

Figure 4.15: SEM images of retrieved McGhan™ implant (scale bar represents 200 μms)

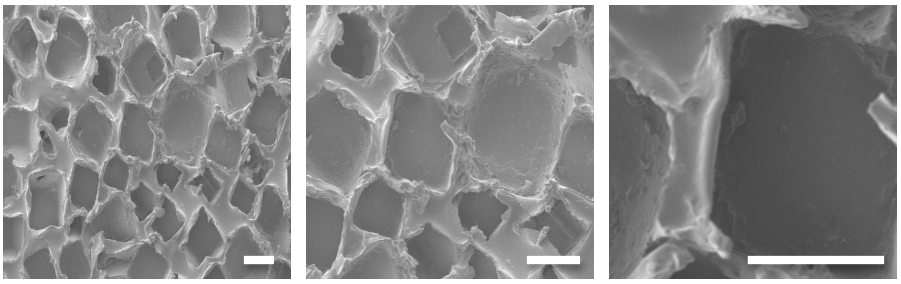


Fig. 4.16A: 7 months duration of implantation

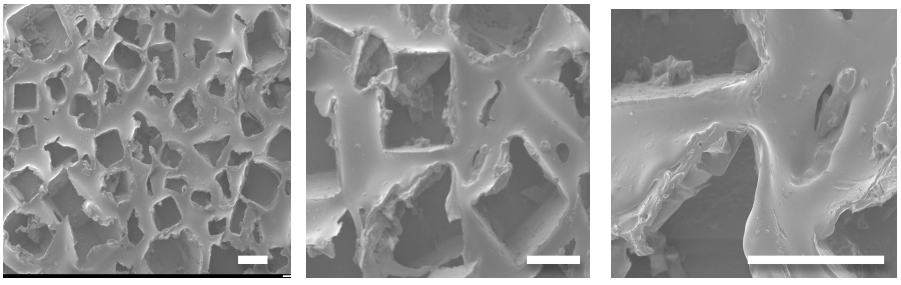


Fig. 4.16B: 204 months duration of implantation

Figure 4.16: SEM images of retrieved Allergan™ implants (scale bar represents 200 μms)

4.4.8 Protein Quantification of Breast Implant Shells – Bicinchioninic Acid (BCA) Assay

Volume of extracted protein from retrieved breast implant shells varied significantly as determined by Bicinchioninic Acid (BCA) assay (standard curve shown in Fig. 4.17). There was no statistically significant difference determined between protein content of the shells and duration of implantation (p = 0.91, two tailed correlation) as shown in Fig. 4.18.

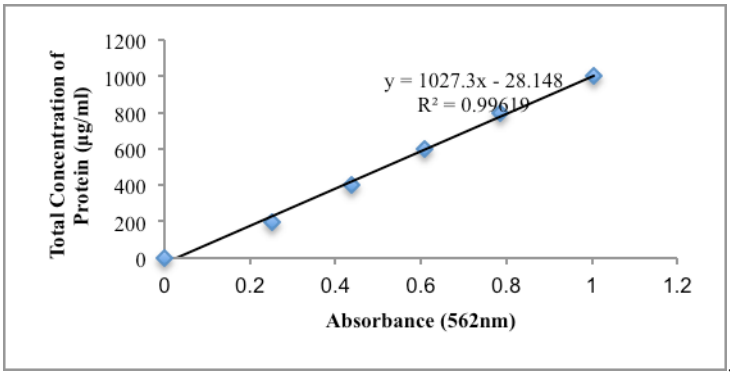


Figure 4.17: Bicinchoninic Acid Assay standard curve

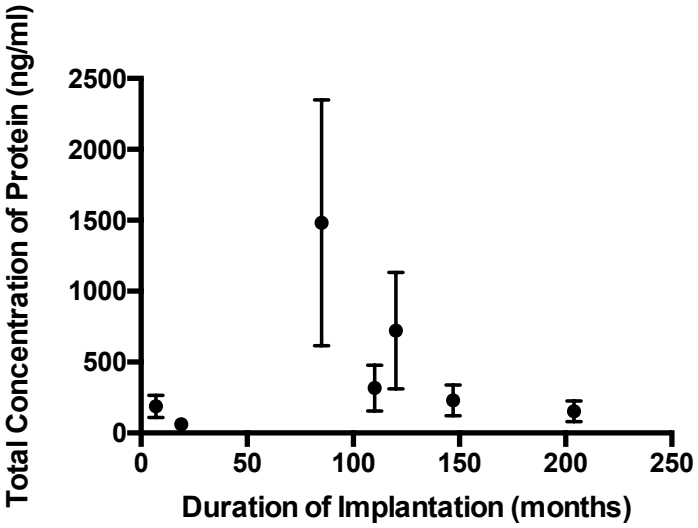


Figure 4.18: Protein content extracted from retrieved implants. No significant difference detected with duration of implantation (p=0.91)

4.4.9 Alamar Blue™Assay (Cell Metabolism)

HDFa cells were cultured for 7 days and alamar blue assays were performed at 24 hours, 72 hours and 7 days as shown in Figure 17. There were significant differences at Day 1 between TCP and Allergan 7 months and McGhan 85 months only. At Day 3 there were significant reduced cell metabolism on all implants apart from McGhan 85 months. At Day 7 there was significantly increased metabolism in all implants apart from McGhan 110 months. Analysis of the time points revealed significant differences between the timepoints ($p<0.0001$) and between the groups ($p = 0.003$, 2 way ANOVA, multiple comparisons)

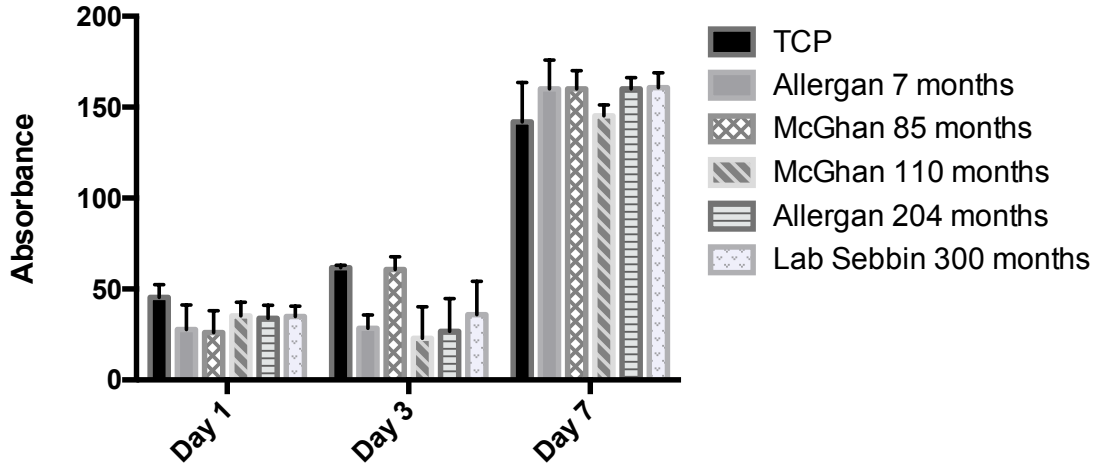


Figure 4.19: Alamar Blue Assay cell metabolism on tissue culture plastic (TCP), cells grown on retrieved implant shells over 7 days as assessed by Alamar™ Blue assay. Significant differences between the timepoints ($p<0.0001$) and between the groups ($p = 0.0030$, 2 way ANOVA, multiple comparisons)

4.4.10 DNA Quantification (Cell Proliferation)

Hoechst 33258 DNA Quantification Kit™, Fluorescence Assay showed significant increased cell proliferation in comparision to TCP Day 1 apart from implant Lab Sebbin 300months. There was no significant difference at Day 3 apart from McGhan 85 months. At Day 7 there was no significant difference in cell proliferation detected between the materials tested and TCP. On 2 way ANOVA, multiple comparisons, there was significant differences in cell proliferation between the timepoints ($p<0.0001$) and the implants tested ($p = 0.0001$, 2 way ANOVA, multiple comparisons).

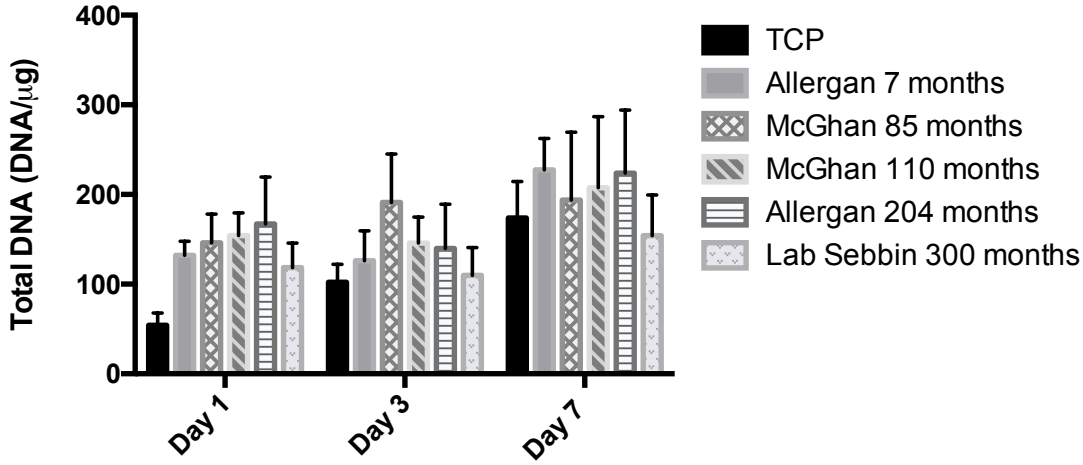


Figure 4.20: Hoechst 33258 DNA Quantification Kit™, Fluorescence Assay, cell quantification as measured on cells grown on tissue culture plastic and retrieved implant shells over 7 days as assessed by total DNA assay over 7 days.

4.5 Discussion

At the time of inception in 1962, the breast implant manufactured by Dow Corning Corporation featured a thick silicon envelope and viscous inner gel that ultimately led to high rupture and silicone gel bleed rates. After five generations of manufacturing adjustments the current fifth generation breast implants are comprised of a textured surfaced silicone elastomer envelope containing a cohesive silicone gel to minimize capsular contracture, gel leakage and implant rupture rates. All patients who were approached participated in the study agreed to take part. Increasing implantation times demonstrated a significant reduction in the shells ability to withstand stretch both for the anterior and posterior components of the implant. In addition, tear strength was significantly reduced with increasing implantation times. This is in-keeping with previous work by Greenwald et al. [63] of 25 retrieved implants who showed that increasing implantation times resulted in reduced shell strength and elasticity [14,57,60]. In addition, Brandon et al. [141] showed, in un-implanted control breast implants, evidence of significant lot to lot variability as well as between different models of implant by same manufacturer suggesting variability in the manufacturing process of implants.

However, to our knowledge this is the first study to examine the micro-mechanical properties in relation to the breast implant shells through atomic force microscopy. This showed significant changes in young's modulus across the cross-sectional layer in 2 of 3 specimens indicated that either the surrounding host environment or the inner silicone gel is exerting an influence on the material characteristics of the implant. Swelling of the breast implant shells caused by diffusion of low molecular weight silicones from the gel have been implicated in weakening the mechanical properties [56,138]. Lipid infiltration to the shells has also been implicated in causing degradation of breast implant shells in vivo [62]. In addition, our study showed that contact angle measurements/wettability was not statistically changed with increasing duration of implantation. Previous work by Wei et al. [142] demonstrated that increasing hydrophilic surfaces promote cell adhesion in a mouse fibroblast model and hydrophobic surfaces promote cell spreading. Valencia-Lazcano et al. [86] examined new un-implanted implants from a range of manufacturers and reported contact angles for textured implant surfaces between 130° and 142° in-keeping with

our work. Furthermore, protein deposition levels in this study showed no significant changes in relation to the implantation times.

ATR-FTIR analysis showed significant changes only at the peak spectral height 784 cm^{-1} corresponding to CH_3 rocking and –Si-C-stretching in –Si-CH_3. This may represent degradation of the surface of the implant however, there was no significant differences seen in the other peak spectral heights nor was there any statistically significant differences demonstrated in all peak spectral heights comparing the ruptured and intact shells. The spectra and peak intensities produced are in keeping with previous work by Yildrimer et al [43]. However, they found there was a statistically significant difference in peak intensity at 1007.6cm^{-1} corresponding to stretching of the Si-O-Si polymer when comparing explanted PIP implants containing industrial grade silicone and explanted implants produced by Allergan and Mentor manufacturers which may have influenced their results.

Gel colour changes identified on naked eye inspection from clear, colourless gels in intact, short duration of implantation implants to deeply yellow gels and in one case cloudy gels in older and ruptured implants is in-keeping with previous work [60]. The cause of this is yet unknown but has been postulated to be an unknown biological component which diffuses from the host through the silicone elastomer to interact with the gel. However, this does not uniformly occur in all explanted implants [60]. Yildirimer et al. [43] demonstrated protein-like peaks intensities on FTIR analysis of gels from ruptured implants suggesting a bacterial contaminant. Further work analyzing the gels of explanted breast implants revealed evidence of lipid infiltration [62] but no evidence of protein or peptides in the gels from intact implants sampled by proteonomic analysis [143]. A further area of study would be to analyse the colour intensity of the retrieved gels and correlate this with the implant shell mechanical properties.

Scanning electron microscopy imaging revealed visual changes in the surface of the implants with roughening of the pits and ragged edges. Comparing retrieved breast implant shells from same manufacturer, through scanning electron microscopy there was evidence of increasing surface degradation with increasing implantation times. However, further research is required to quantify this further using retrieved implants from a single manufacturer and type and a range of implantations times to further detect the true effect of in vivo aging. In addition, a possible explanation may be trauma to the implant at time of retrieval surgery and separation from the surrounding

encapsulated implant could account for the surface changes encountered as shown in cadaver studies [54].

The cellular response results show no significant differences in cell metabolism between the implants tested but increased with each timepoint. Cell quantification measurements however, did not show statistically significant changes between the timepoints and the implants tested. For the purpose of this study we used human dermal fibroblasts to establish the cellular response, however, future research is required examining the behavior of other cell types as well as measurement of inflammatory cytokines would be of value.

The limitations of our study included retrieval of breast implants from a range of different manufacturers and small sample size. In addition, the implants were retrieved from patients who had originally received breast implants both for augmentation and for reconstructive purposes which may have also influenced the results. Two of sixteen patients had undergone pre-operative radiation therapy prior to implant placement and this may have contributed to increased risk of capsular contracture and implant failure as previously been reported [144].

Chapter 5: Analysis of the Cellular Response to the Retrieved Implants and of the Surrounding Capsular Tissue

5.1 Introduction

The host inflammatory response is known to play a significant role in the development of capsular contracture (a pathological fibrous encapsulation of the breast implant causing firmness, pain and distortion of the breast) and has been reported to occur in up to 9.8%-18.9% of primary augmentations and in 14.5%-24.6% of primary reconstructions at 10 years follow up [2,53] in implant manufacturers pre-market approval studies. It has also been shown to be the commonest cause of re-operation in long-term studies [145]. The pathogenesis is as yet unknown but thought to be multi-factorial in origin and factors such as haematoma, biofilm formation, radiation therapy, implant surface, implant rupture and gel leakage have been implicated [146–148].

The host response to a foreign body is to provide protection for the body. It is characterized by injury, blood-interaction, acute inflammation followed by chronic inflammation and fibrous encapsulation [149]. A degree of fibrous encapsulation around the implant is necessary to prevent unwanted movement of the implant within the breast. However, in the development of capsular contracture, the foreign body response to the implant is chronically perpetuated by an unknown trigger(s) with inflammation and recruitment of fibroblasts, which produce collagen fibres and myofibroblasts that exert contractile forces upon the collagen fibres. This continuing chronic inflammatory process allows the development of more collagen fibres and recruitment of myofibroblasts to exert tight forces upon them resulting in a firm capsule surrounding the implant [77]. This often leads to a painful condition with distortion and firmness of the breast often necessitating further corrective surgery.

In order to reduce the incidence of capsular contracture, several approaches have been used including pocket antibiotic washes [150], minimal surgical handling of the implant, using textured surface implants (to disrupt the parallel alignment of collagen fibres) [151], delayed reconstruction following radiation therapy [152], use of acellular dermal matrix [90,153] and using submuscular implant placement compared to subglandular [78].

To date, histological analysis of capsules have shown evidence of synovial metaplasia at implant surface, inflammation, and calcification as well as evidence of silicone bleeding. Thickness of the capsule has been previously shown to be positively related to duration of implantation and severity of capsular contracture [154], whilst Dolores et al. [155] showed that fibroblasts and macrophages constituting the majority of cell type present within retrieved capsules. Hwang et al. [156] showed in 31 retrieved surrounding breast implant capsules, the tensile strength of capsular tissue was related to the degree of capsular contracture. However, to our knowledge, no studies have compared the mechanical and chemical properties of the implants to the characteristics of the corresponding surrounding capsular tissue.

5.2 Aims

To assess the histological host response to retrieved implants and correlate this with the characterization of the breast implants mechanical properties.

5.3 Materials and Methods

5.3.1 Consent and Patient recruitment

Patients were consented by the author on day of surgery. Only in cases where capsulotomy was clinically indicated in addition to removal or exchange of implants, consent was obtained to collect the collagenous capsules. Ethical approval was obtained as outlined in Chapter 2.

5.3.2 Histological Analysis of the Retrieved Capsule tissue

Retrieved capsule tissue were collected from theatre by author at time of surgery, labeled anonymously and placed in 10% formalin for 48-72 hours. The tissue was then cut into approximately 1 cm sections, placed in labeled tissue cassettes and underwent a 15 hour automatic tissue processing using a Shandon Citadel 2000 Automatic Tissue Processor (Thermo Scientific™, UK). Once complete, the processed samples were removed from the cassettes and paraffin embedded using a Tissue Tek II™ tissue embedding centre and labeled anonymously (Fig. 5.1).
Paraffin blocks were sent for sectioning and staining to Departments of Pathology at the Royal Free Hospital (Haematoxylin and Eosin stain) and at the UCL Institute for Neurology (Masson's Trichrome stain).
Sections were imaged and analysed using an EVOS XL Core Microscope and saved as TIFF images.

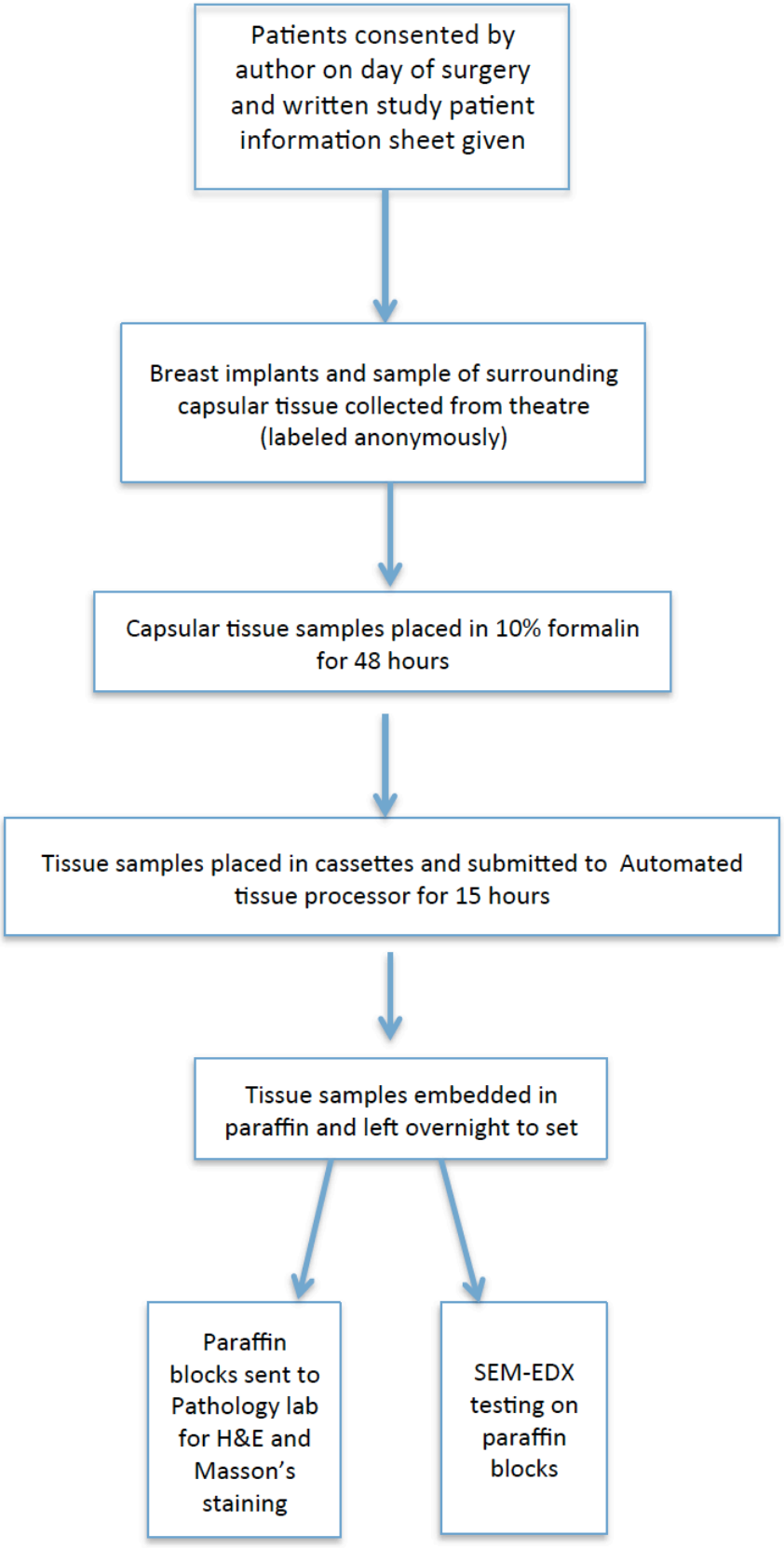


Figure 5.1: Flowchart of sample collection, processing and analysis.

5.3.3 Scanning Electron Microscopy with Energy Dispersive X-Ray Spectroscopy (SEM-EDX)

The paraffin embedded tissue blocks were sputter coated with a thin layer of carbon using a K975X Turbo Evaporator, Quorum Technologies, UK. Images and analysis were performed using a scanning electron microscope Hitachi S-3400N with an EDS Oxford instrument. Elemental analysis was performed using the INCA software package.

5.3.4 Tensile Strength of Corresponding Retrieved Breast Implant Shells

Using the methods outlined in Section 2.5.1, briefly the retrieved implant shells were cut into dumbbell shapes 20mm x 4mm, specimen type 3 using a cutting press (Wallace instruments, UK).). Three thickness measurements were performed using a digital electronic micrometer and the average thickness was inputed into the software. The sample was loaded onto the pneumatic grips of the Instron 5565 tensiometer equipped with a 500 N load (Instron, UK) and uniaxial testing at a rate of 100 mm/min was performed. The data was captured and analysed using Bluehill software. All experiments were performed on dry samples at room temperature.

5.3.5 Statistics

All statistics were performed using non-parametric Spearman correlation where significance was $p < 0.05$. All graphs were performed using GraphPad Prism software Version 6.

5.4 Results

5.4.1 Histological Analysis of Retrieved Capsule

Histological examination of the retrieved surrounding capsule revealed overall reduced cellularity and presence of thick, dense layers of aligned collagen fibres (Fig. 5.2) in keeping with previous work [77]. At the surface in direct contact with the implant, there was evidence of synovial metaplasia and engulfment of silicone particles which appeared birefringent under the microscope in keeping with silicone 'bleeding' as evidenced in capsules from ruptured implants (Figure 1 and 3) as well as intact implants (Figure 2). Only in the capsule at 85 months from an explanted intact breast implant there was evidence of loose areolar tissue (Figure 8). There was no evidence of acute inflammatory response or infection as predicted. A summary of patient characteristics are documented in Table 5.1.

No. of breast implant capsule specimens retrieved	**9**
Mean age (years)	42.8 (SD. 9.2)
Mean duration of implant (months)	122.1 (SD. 59.2)
Reason for implant placement	
Augmentation	4
Reconstruction	5
Reason for exchange or removal of implant	
Capsular Contracture (Baker Grade III/IV) and implant rupture	4
Capsular Contracture (Baker Grade III/IV) and implant rupture	2
Implant rupture	1
Contralateral removal/exchange of implant	1
'Rippling' of the implant	1
Radiotherapy	
Pre-operative radiotherapy	2

Table 5.1. Summary of Patient Characteristics

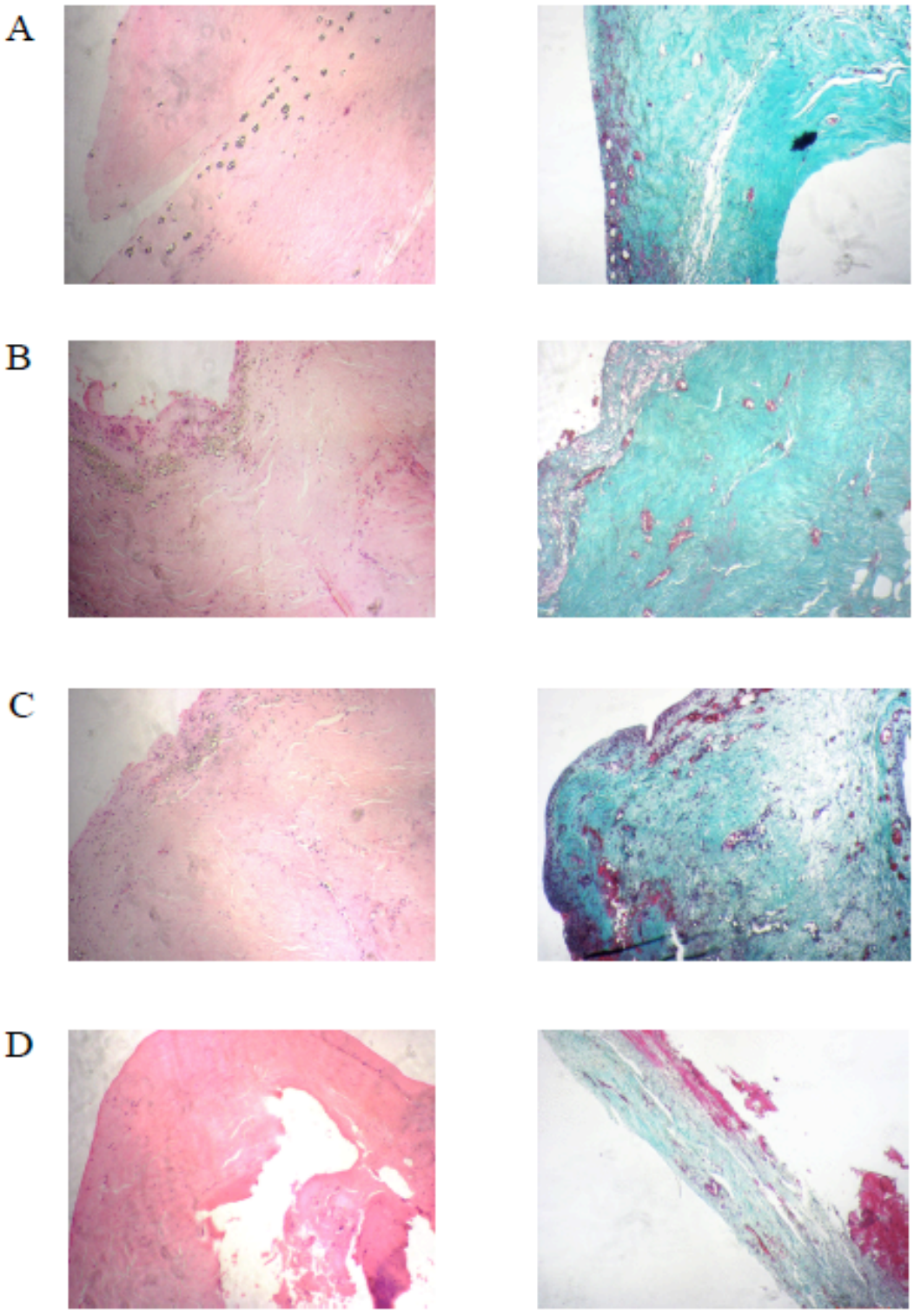


Figure 5.2: Representative Haematoxylin & eosin (left) and Masson's trichrome (right) staining of retrieved capsules from **(A)** 96 month ruptured implant, CC, **(B)** 120 month implant removed due to contralateral rupture **(C)** 120 month ruptured implant **(D)** 120 month intact implant CC. x 20 magnification Arrows point to engulfed silicone particles.

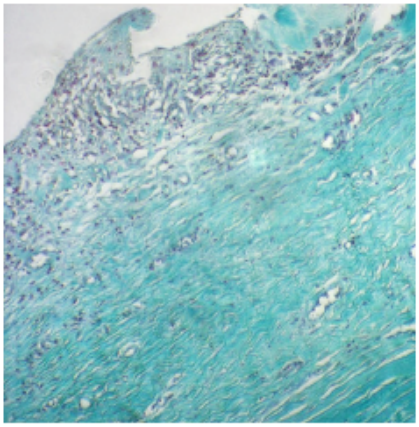


Figure 5.3: Retrieved capsule from 19 month implant with densely aligned collagen fibres and thickened relatively acellular area.

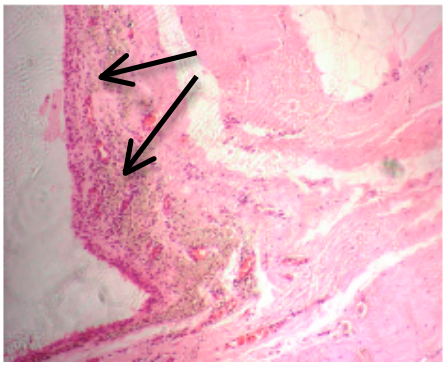


Figure 5.4: Retrieved capsule from 215 month intact implant, Grade III CC with dense highly aligned collagen fibres with synovial metaplasia at the implant surface edge (arrows).

5.4.2 Correlation of Fibrotic Capsule Thickness and Duration of Implantation/Mechanical Properties of Corresponding Breast Implant Shell

In order to assess the relationship between the formed surrounding implant capsule tissue and the implant, the thickness of the fibrotic capsule was measured from surface edge to limit of aligned collagen fibres as shown in Fig. 5.5. There was no significant correlation between fibrotic capsular thickness and duration of implantation ($p = 0.8503$, $r = 0.07833$, Spearman two-tailed correlation). In addition, there was no significant correlation between fibrotic capsular thickness and ultimate tensile strength properties of the corresponding retrieved breast implant shell ($p = 0.1206$, $r = 0.5667$, Spearman two-tailed correlation) as shown in Fig. 5.6. Measurements are outlined in Table 5.2.

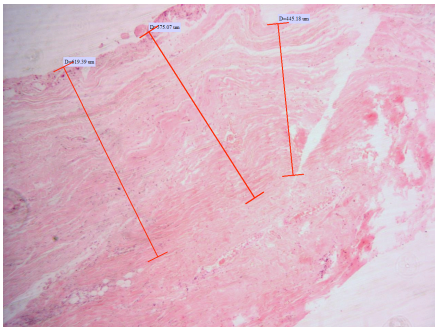


Figure 5.5: Fibrotic capsule stained with H&E at x4 magnification demonstrating measurements of aligned collagen fibres from patient 15, retrieved implant and capsule at 19 months.

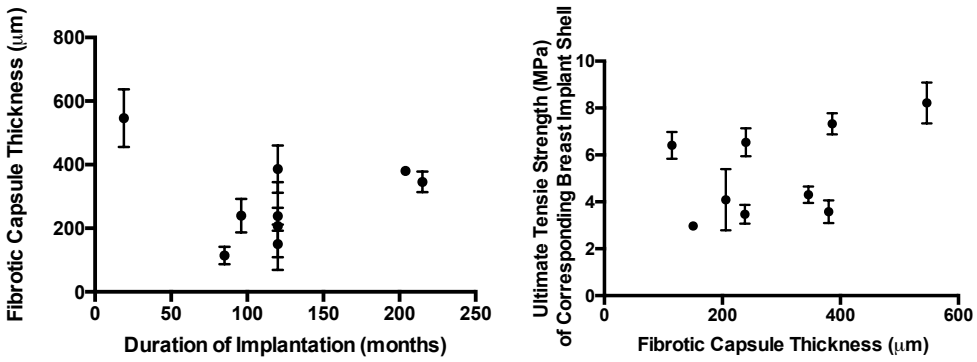


Figure 5.6: Graphs demonstrating no significant relationship between fibrotic capsule thickness and duration of implantation, $p = 0.8503$, $r = 0.07833$, Spearman two tailed correlation, (left). No significant relationship between fibrotic capsule thickness and UTS of corresponding breast implant shell, $p = 0.1206$, $r = 0.5667$, Spearman two tailed correlation, (right).

Implant	Duration of Implantation (months)	Capsular Thickness Measurements (µm)	Mean Thickness (µm)	Ultimate Tensile Strength + SD (MPa)
1	96	263.32 276.59 179.60	239.84	6.54 + 0.595
2	120	361.81 95.72 163.38	205.97	4.09 + 1.305
3	120	447.15 408.02 303.45	386.21	7.33 + 0.450
7	120	149.37 109.73 192.72	150.61	2.97 + 0.147
8	120	249.10 257.39 208.02	238.17	3.47 + 0.399
9	204	370.38 387.32 382.78	380.16	3.58 + 0.481
11	215	312.62 377.02 347.96	345.87	4.30 + 0.350
12	85	140.12 85.70 117.48	114.43	6.41 + 0.571
15	19	619.39 575.07 445.18	546.55	8.22 + 0.871

Table 5.2: Measurements of capsule thickness, duration of implantation and ultimate tensile strength measurements.

5.4.3 Scanning Electron Microscopy with Energy Dispersive X-Ray Spectroscopy (SEM EDX) of Surrounding Retrieved Capsule

Six specimens were selected for SEM EDX. In all ruptured implants (n=3) and in 1 intact implants (n=3) there was evidence of silicone particles engulfed into foreign body giant cells a shown in Fig. 5.7.

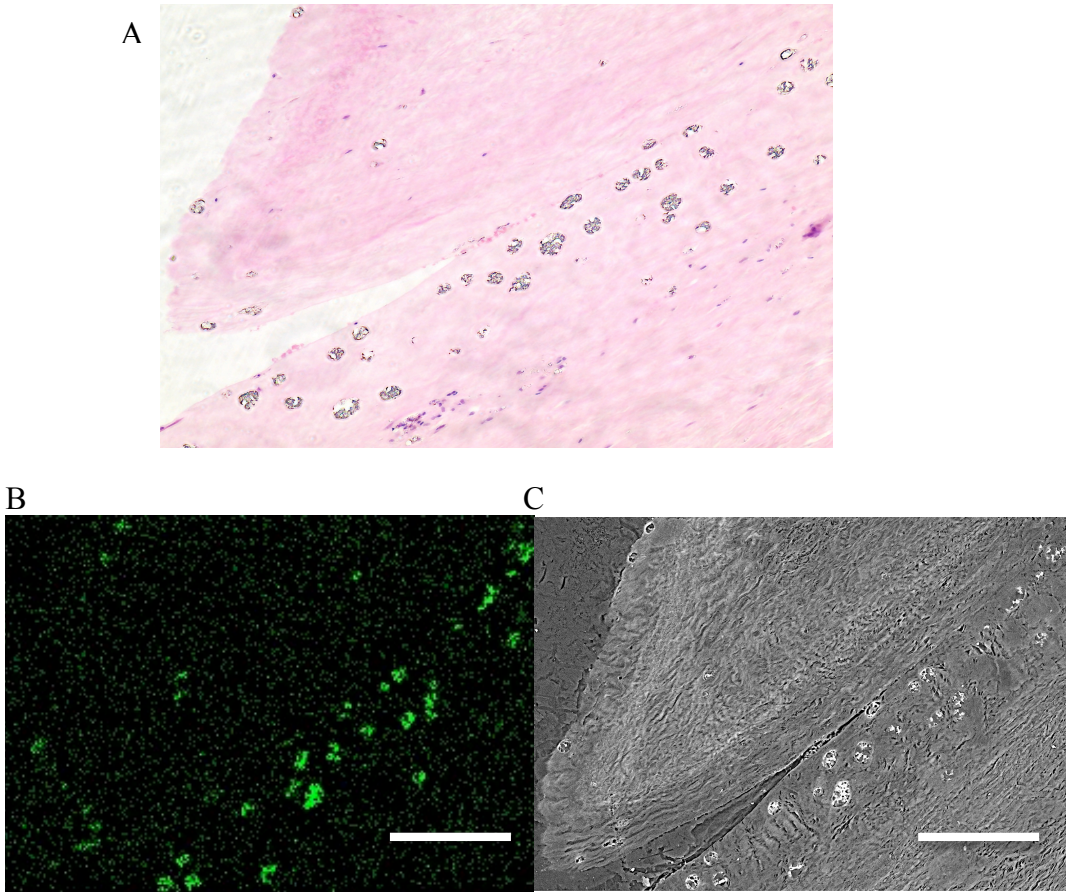


Figure 5.7: Ruptured Implant 96 months duration **(A)** H&E staining at x10 magnification **(B)** SEM-EDX imaging displaying Silicon element **(C)** SEM image (scale bar =200µm).

5.5 Discussion

On histological analysis, the capsules were overall relatively acellular with an abundance of thick, highly aligned collagen fibres in keeping with previous research [77]. Interestingly, in capsules sampled from patients with both ruptured and intact breast implants there was evidence of engulfment of silicone particles by foreign body giant cells demonstrating silicone bleeding in-keeping with previous work by Siggelkow et al. [154]. As shown by Ko et al. [157], synovial metaplasia was seen at the host/implant interface in our specimens and this may serve as a lubricating mechanism in response to mechanical stress between the surrounding cells and the implant. In contrast to Bui et al. [76] the thickness of the capsules was not significantly correlated with length of implant duration. However, in this study the implants were not all derived from the same implant manufacturer and were implanted for either cosmetic or reconstructive purposes that may have influenced the results. In addition, two patients had undergone radiation therapy to the breast prior to implant insertion that could have contributed to increased risk of capsular contracture seen in these patients. To our knowledge, the thickness of the surrounding capsule has not yet been correlated mechanical properties of the implant shells in the literature. In this study, it was demonstrated that capsular thickness is not significantly associated with tensile strength of the corresponding implant. Overall, this suggests that the fibrotic encapsulation occurring is not simply the result of changes alone in the mechanical properties of the corresponding breast implant relating to changes in substrate stiffness as shown in previous in vitro studies [81,83]and indeed is multifactorial.

Chapter 6: Impact of Post Mastectomy Radiotherapy on the Silicone Breast Implant

6.1 Introduction

Breast reconstruction following mastectomy can be performed using either an implant-based technique or autologous tissue reconstruction or using a combination of both. Rates of mastectomy and breast reconstruction are increasing, even in patients who are deemed suitable for breast conserving surgery and those with early stage disease [158,159]. This may be attributed to improved aesthetic outcome associated with reconstruction, anxiety associated with possible breast cancer recurrence and increased in rates of prophylactic mastectomies [158,160]. Breast reconstruction offers improved psychological, body-image, emotional and sexual well-being for patients undergoing mastectomy [161] and therefore is offered to those patients deemed suitable. Following the publication of the Danish and British Columbia trials there is increasing evidence of the benefits of post mastectomy radiotherapy (PMRT) in reducing loco-regional recurrence in certain cohorts of patients [96,97]. The recent guidelines published by the American Society of Surgical Oncology advise all patients with T1-2 tumours and 1-3 positive lymph nodes should be considered for PMRT as well as patients with T1-2 tumours with one positive node on sentinel node biopsy who do no undergo further axillary clearance should be considered [100]. According to the UK National Audit of Mastectomy and Breast Reconstruction, over half of breast reconstructions performed in the UK were implant based. The advantages of implant-based breast reconstruction (IBBR) over autologous tissue reconstruction include shorter operative time and inpatient stay, reduced cost, avoidance of donor site morbidity and can be offered to patients whose co-morbidities would prevent them from undergoing autologous reconstruction [162]

However, IBBR is associated with long term complications including most commonly capsular contracture, causing breast distortion, pain and firmness as well as implant rupture and gel leakage. Capsular contracture has been reported to occur in 24.6% of patients at ten years in patients undergoing implant based reconstruction [53]. The exact mechanism of the cause of breast implant failure is unknown and several factors have been postulated in both augmentation and reconstructive procedures including surgical handling of implant, biofilm formation, peri-operative haematoma, exposure to silicone and peri-operative radiotherapy [163,164] As demonstrated in the systematic review of Chapter 3, post mastectomy radiotherapy delivered to the

permanent implant increases the rate of capsular contracture, implant failure and revisional surgery [103,165]. Moreover, it is associated with poor cosmetic outcome and patient satisfaction [131,132]. However, the role of radiation on the physicochemical material properties of the implant or the material biological interface remains unclear. Here we investigate if radiation (at similar doses to that delivered to the patient) changes the material properties of the implant.

Breast implants are composed of polydimethylsiloxane (PDMS, $(CH_3)_2SiO$). The outer envelope of the implant is composed of highly crossed-linked PDMS chains buttressed by silica whereas the inner gel is composed of the same linear PDMS polymer, minimally cross-linked PDMS chains by vinyl-hydrogen bonding [1]. The difference in the outer elastomer shell and the inner gel although composed of the same polymer is the degree of crosslinking between the chains. Silicone is recognised for its relatively low toxicity and is widely used in several medical applications [139] including aesthetic implants, cardiovascular grafts, hydrocephalic shunts, soft joint replacements and in bariatric gastric bands [110]. It is well known that material surface properties, including chemistry and topography play important roles in determining protein and cellular interactions, that can influence short and long-term host responses [139]. Surface texturisation of silicone breast implants has been shown to reduce the incidence of capsular contracture in comparison to smooth surface implants [78].

The influence of radiation therapy on the material chemical properties is unclear, with previous studies showing that radiation had no significant effect upon the surface and bulk properties of PDMS based materials [105,106], but others demonstrating change in the surface chemical changes following treatment dose radiation (50 Gy in 25 fractions) of commercially available silicone breast implants [104]. The aim of this study was to examine the effect of treatment dose radiation therapy upon the mechanical and surface chemical properties as well as the cellular response of silicone breast implants pre and post radiation exposure to gain a deeper understanding of the role of radiation therapy on breast implant failure.

6.2 Aims of Chapter

Aim 1 – To examine the effect of treatment dose radiation therapy on the mechanical and surface chemistry properties of silicone breast implants by performing tensile and tear strength, attenuated total reflectance-fourier transform infra-red spectroscopy (ATR-FTIR) and wettability measurements.

Aim 2 - To assess the fibroblast response to treatment dose radiation therapy on silicone breast implants.

6.3 Methods

6.3.1 Preparation and Irradiation of Silicone Breast Implant Shells

Un-implanted textured silicone breast implants (Mentor Siltex™ Contour Profile™ Becker ™ 35 Expander, Cohesive II™, Lot 6811381) were used. The implant inner gel was removed and the outer shells were subjected to radiation. The radiation delivered to the implants was based upon the recommended chest wall dosing schedule for patients with invasive breast cancer after mastectomy (40.05Gy in 15 fractions) [166]. The breast implant shells were then categorized into three groups according to full treatment dose radiotherapy (40.05 Gy), one daily fraction dose radiotherapy (2.67 Gy) and a non-irradiated shell was used as the control.

The implant shells were surrounded by blocks and adjuncts to simulate surrounding soft tissue and radiated at a rate of 6 Gy/min courtesy of the Department of Radiotherapy, Royal Free Hospital, London.

6.3.2 Mechanical Testing of Breast Implant Shells

All samples were measured using the Instron 5565 tensiometer equipped with a 500 N load (Instron, UK). From the implant shells, for each condition, six 20mm x 4mm dumbbell shaped specimens were cut from the implant shells using a Wallace cutting press for tensile testing and 3 crescent shaped specimens were cut for tear testing in accordance with the ISO 37:2005 standards. Specimens were placed in the pneumatic grips of the tensiometer. The specimen was pulled apart at a rate of 100mm/min and 500mm/min for tensile and tear testing respectively. The data was captured using Bluehill software. Ultimate tensile strength, strain at break, Young's modulus and tear strength values were recorded.

6.3.3 ATR-FTIR of Breast Implant Shells

Fourier Transform Infrared Spectra (FTIR) recordings were obtained to determine the surface chemical fingerprint of the implants using a Jasco FT/IR 4200 Spectrometer with a diamond attenuated total reflectance accessory (Diamond Miracle ATR, Pike

Technologies, US). A total of 5 silicone shell samples from each of the radiated groups and the control group were analysed. From an average of 30 scans a spectra was produced over a range of 600cm^{-1} to 4000 cm^{-1} with a resolution of 4cm^{-1} (n=5) for each group. The resulting spectra and the peak spectral intensities were identified and one-way ANOVA was performed using Graph Pad Prism software Version 6 to detect changes between the groups.

6.3.4 Surface Wettability/Contact Angle Measurements (θ)

Using a DSA 100 Krűss Goniometer, wettability analysis was performed on the implant samples from each of the 3 groups. Using the sessile drop technique, 5µl of deionized water was dropped onto the samples using an automated syringe with 10 seconds of dispensing and analysis was performed using the Drop Analysis software (EasyDrop DSA200, KrűSS) at room temperature. Four samples from specimens from each group were tested three times (n=12) and statistical analysis performed using Graph Pad Prism software Version 6.

6.3.5 Cell Metabolism, Growth and Morphology

6.3.5.1 Alamar Blue™ and Total DNA Assay

In order to assess cell metabolic activity and number, 6mm round disc cut samples (n=8) from each of the three groups: silicone shell radiated at 40.05Gy, silicone shell radiated at 2.67Gy and non-irradiated (control) silicone shell were sterilized by being placed in 1% Triton X for 1 hour, washed twice in PBS followed by 70% ethanol followed by washing twice in PBS. The discs (n=8) were placed in a 96 well plate, covered with 100µl of warmed DMEM for approximately 2 hours then seeded with HDFa cells at a density of 5 x 10^4 cells/cm^2. HDFa cells used were between passage 7 and 11. Cells seeded onto tissue culture plastic served as a positive control and media only wells provided a negative control. Cells were incubated at 37^{o}C at 5% CO2 in air. Cell culture media was replenished on days 0, 1, 3 and 6 during the 7 day experiment. Cell metabolism was assessed using Alamar Blue™ assay (Invitrogen, Paisley, UK) and conducted in accordance with protocol guidelines on days 1, 3 and 7. Fluorescent analysis was performed using a Fluoroskan Ascent™ Fluorescence Plate Reader (ThermoScientific, USA) at excitation of 530nm and 620nm. The

Hoechst 33258 DNA Quantification Kit™, Fluorescence Assay (Sigma-Aldrich, UK) was used to quantify number of cells and was performed in accordance with protocol guidelines on days 1, 3, and 7. Analysis was performed using the Fluoroskan Ascent™ Fluorescence Plate Reader (ThermoScientific, USA) at an excitation of 360nm and 460nm.

6.3.5.1 Cell Morphology

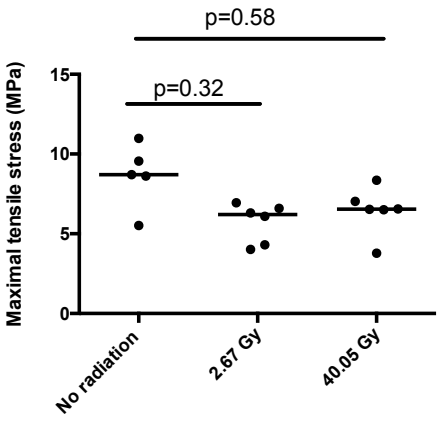
Using commercially available fluorescent green cytoplasmic actin (Alexa Fluor 488 Phalloidin, Molecular Probes, ThermoScientific™, UK) and fluorescent blue nuclei staining kit (Vectashield Antifade Mounting Medium with DAPI, Vector Laboratories, USA), cell morphology was examined at day 7 of seeded HDFa cells on tissue culture plastic and on non-irradiated silicone implant shells. This was performed in accordance to the manufacturers protocols. Images were captured using an EVOS Fluorescent Microscope (EVOS FL Imaging System, ThermoScientific™, UK).

6.4 Results

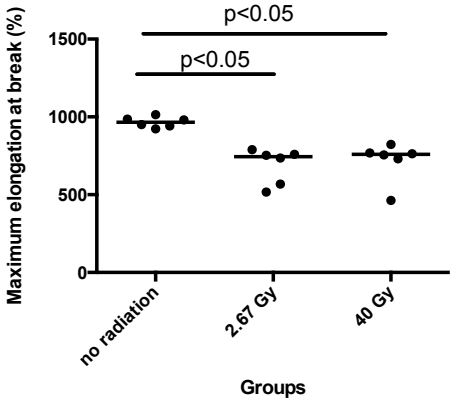
6.4.1 Material Mechanical Properties

There were no significant differences in maximal tensile strength (Fig. 6.1A) and tear strength (Fig. 6.1C) between each of the groups. There was no significant difference in Young's modulus between the control and full treatment dose group (40.05G) but there was a significant decrease in the 2.67 Gy group (Fig. 6.1.D). A significant reduction in the maximum elongation strain at break was, however, evident (Fig. 6.1B, $p<0.05$) suggesting the samples are less flexible under strain following irradiation.

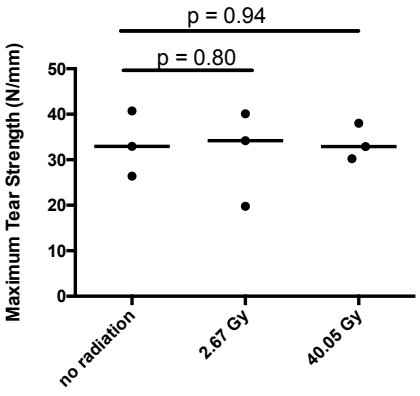
A Maximal tensile strength (MPa**)**



B Maximum elongation at break (%)



C Maximum Tear Strength (N/mm)



D Young's modulus (MPa)

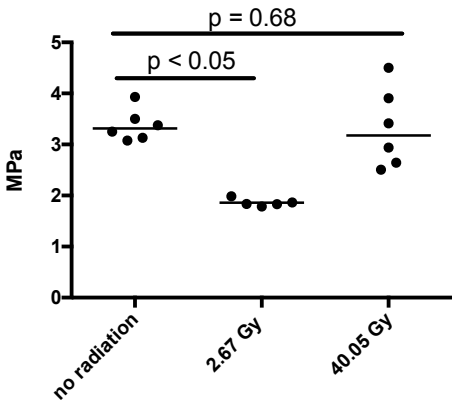


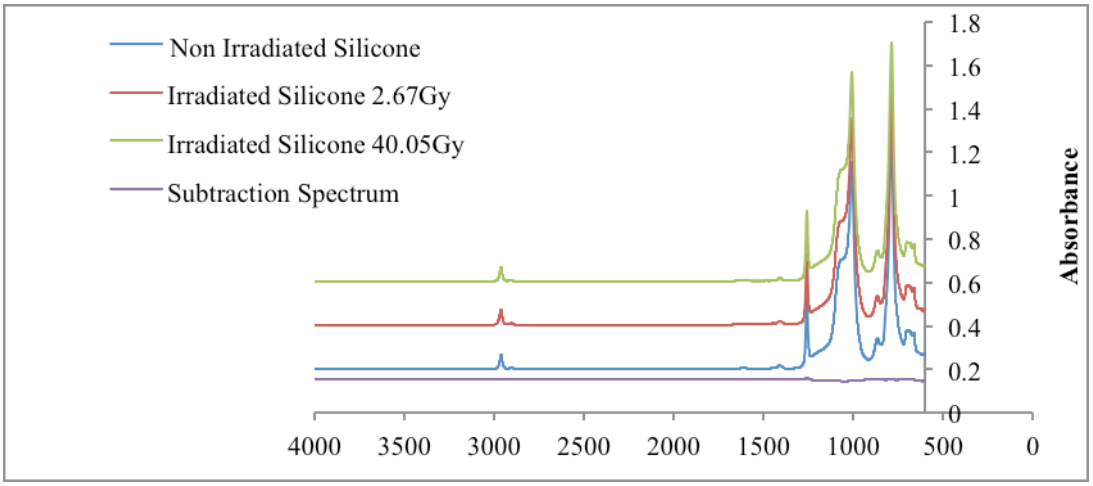
Figure 6.1: Mechanical Characterisation of Samples

No significant difference detected in **A.** maximal tensile strength and **C.** maximal tear strength. Significant differences were seen in **C.** maximum elongation at break. No significant difference in Young's modulus detected between control and full dose radiation groups **D.**

6.4.2 Material Surface Chemistry

6.4.2.1 ATR-FTIR of Breast Implant Shells

A



B

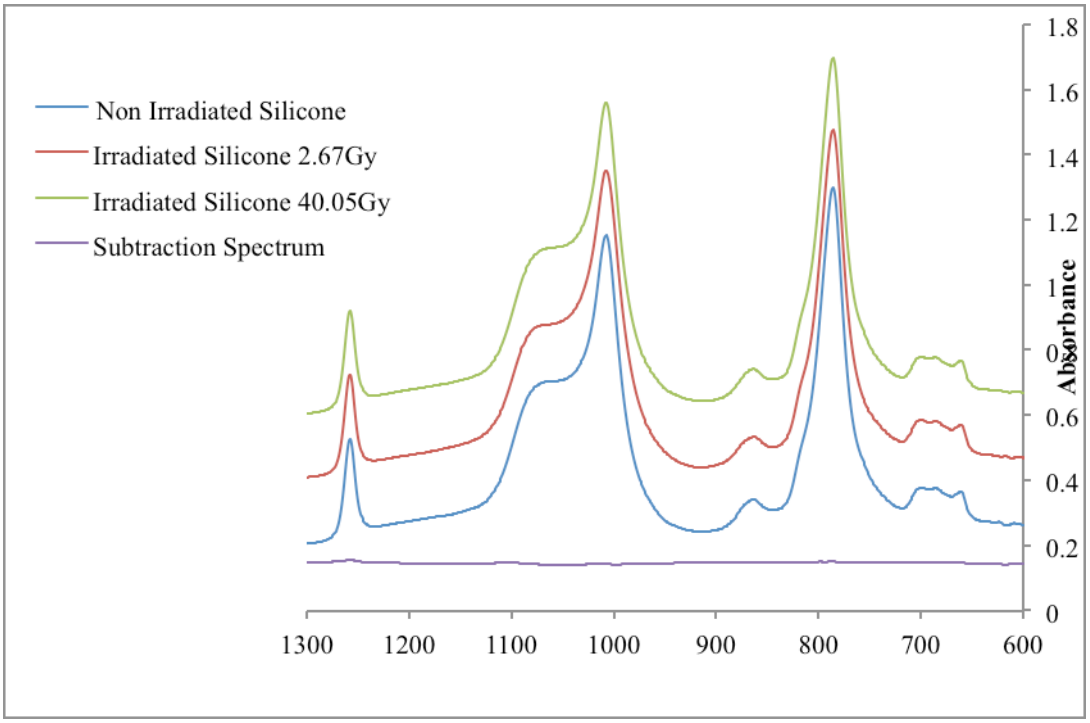


Figure 6.2: A. Overlaid ATR-FTIR spectra of non-irradiated (control) and irradiated silicone breast implant shells at 2.67 Gy and at 40.05 Gy offset by +0.2 with subtraction spectrum (Non Irradiated Silicone – Irradiated Silicone 40.05 Gy). **B.** Spectra between 600 and 1300 cm^{-1} wavelength showing subtraction spectrum

Analysis of the material surface properties was performed using ATR-FTIR. Despite some spectral difference observed upon spectral subtraction (Fig, 6.2A) of the ATR-FTIR spectra, there was no significance difference between the peak heights at 784cm^{-1} corresponding to –CH_3 rocking and –Si-C-stretching in –Si-CH_3 (p=0.33) one way ANOVA, parametric data), at 1004 cm^{-1} corresponding to the asymmetric stretching of –Si-O-Si- (p =0.87), one –way ANOVA, parametric data) and at 1257cm^{-1} corresponding to symmetric bending of –CH_3 in –Si-CH_3 (p = 0.67, one way ANOVA parametric data) as shown in Fig. 6.3

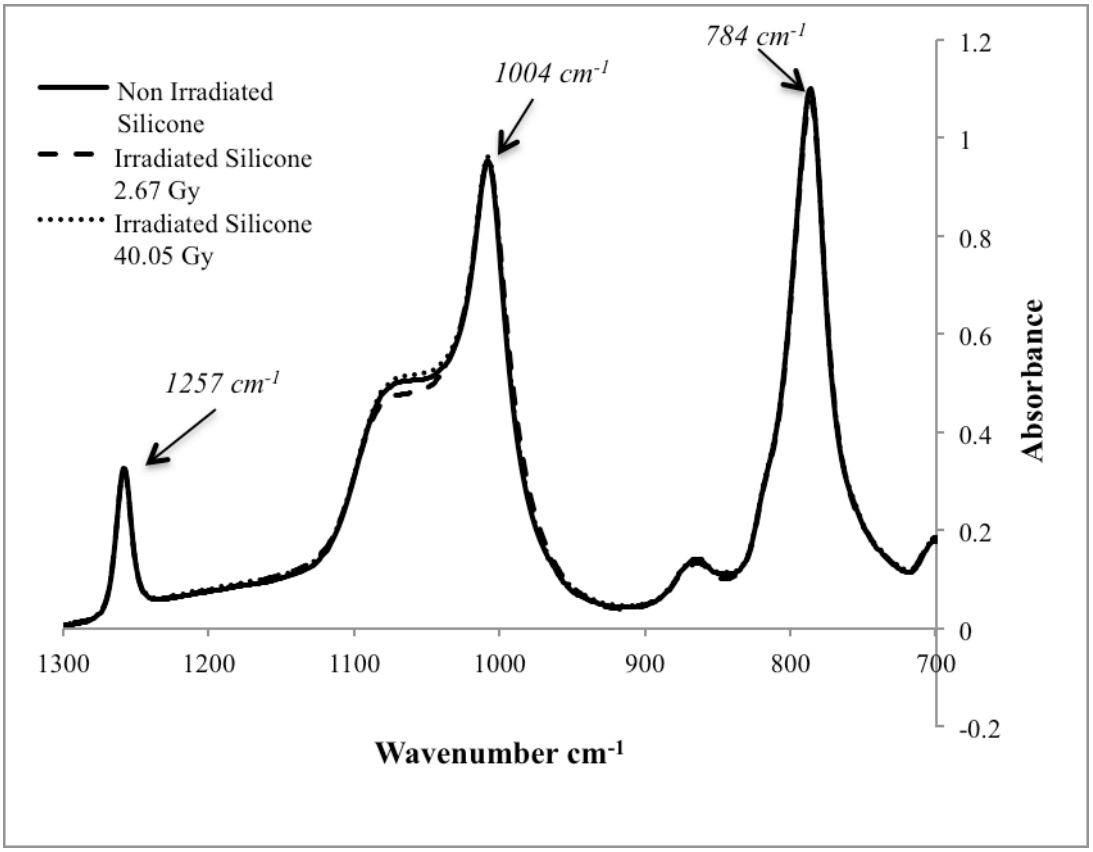


Figure 6.3: ATR-FTIR spectra from wavenumber 700 – 1300 cm^{-1} showing overlaid spectra from non irradiated and irradiated specimens. No significant differences in spectra height seen at peak 784 cm^{-1}, 1004 cm^{-1} and 1257 cm^{-1}

6.4.2.2 Surface Wettability/Contact Angle Measurements (θ)

On analysis of the contact angle measurements there was no significant differences detected between each of the groups (p=0.23, Kruskal Wallis test) as shown in Fig. 6.4.

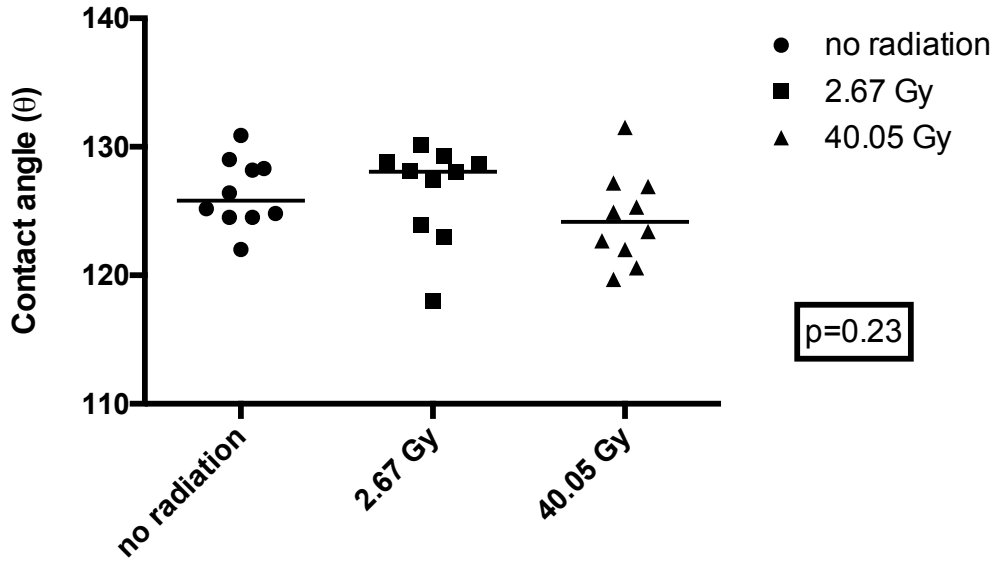


Figure 6.4: Contact Measurements showed no significant differences in each of the 3 groups (p= 0.23), Kruskal-Wallis test)

6.4.3 Cell Metabolism, Growth and Morphology

6.4.3.1 Cell metabolism

Cell metabolic activity increased with time (Day 1 and Day 7) as cells proliferated on all samples, but no significant difference was detected between irradiated (40.05Gy) and non-irradiated silicone ($p=0.79$, 2-way ANOVA) (Fig. 6.5). Significant difference was detected in cell activity between the days ($p < 0.0001$).

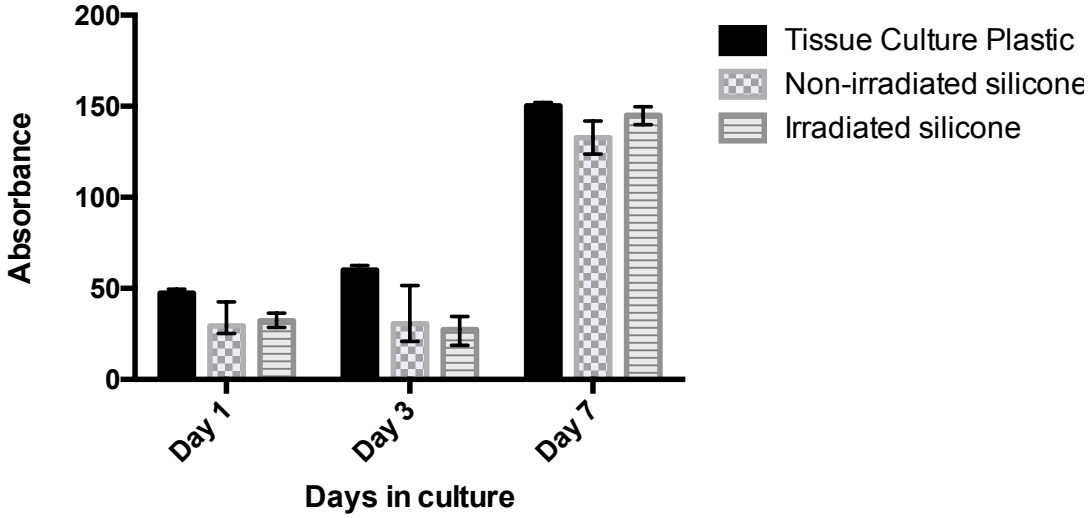


Figure 6.5: Cell metabolism on tissue culture plastic (TCP), cells grown on non-irradiated silicone breast implant shell and silicone breast implant shell subjected to full treatment dose radiation (40.05 Gy) (Irradiated Silicone) as assessed by Alamar Blue™ assay over 7 days. No significant differences were detected between the tested material groups ($p=0.79$, using 2-way ANOVA test).

6.4.3.2 Cell proliferation

Total DNA assay showed no significant differences in cell numbers between silicone and non-irradiated silicone when compared to the TCP (p=0.61, 2 way ANOVA) as shown in Fig. 6.6.

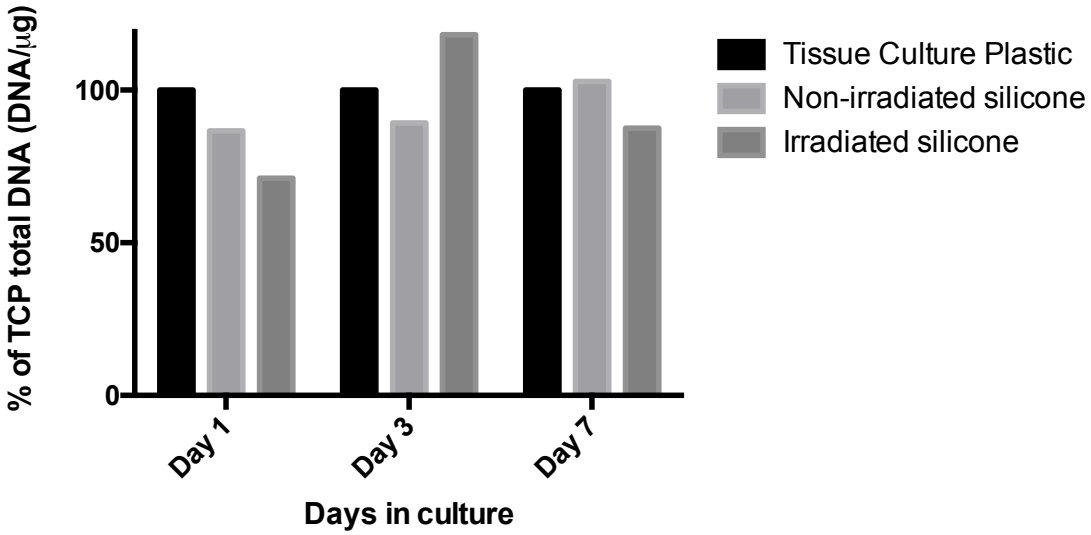


Figure 6.6: Total DNA assay, cell quantification as measured on cells grown on tissue culture plastic, non-irradiated silicone breast implant shells and silicone breast implant shells subjected to full treatment dose radiation (40.05Gy) (Irradiated silicone) as assessed by total DNA assay over 7 days. No significant differences detected between the groups were detected in comparison to TCP (p=0.61, 2 way ANOVA).

6.4.3.3 Cell Morphology (Immunofluorescence staining)

The cell morphology of cells grown on tissue culture plastic (TCP) demonstrated highly aligned, thin, long, spindle like projections in parallel with an abundance of nuclei. In contrast, the cells cultured upon non-irradiated silicone breast implant shells showed thickened projections with random orientation with fewer nuclei evident

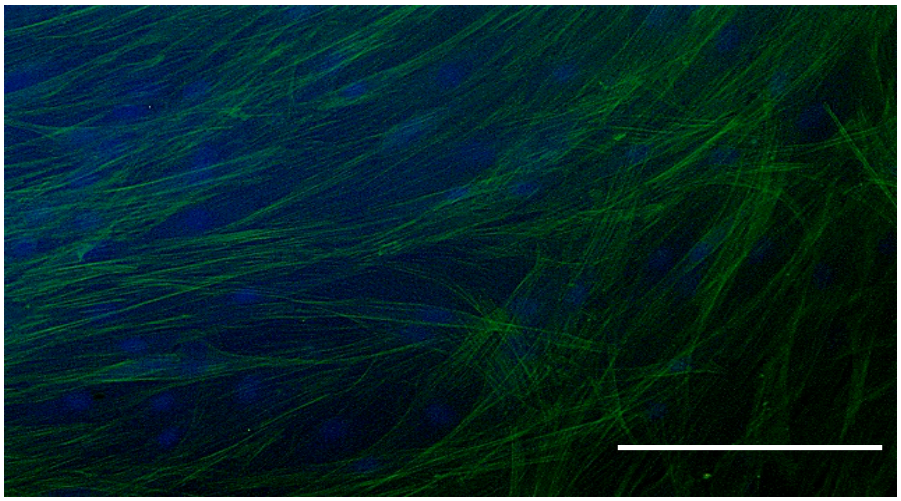


Fig. 6.7A. Cells seeded on TCP at Day 7

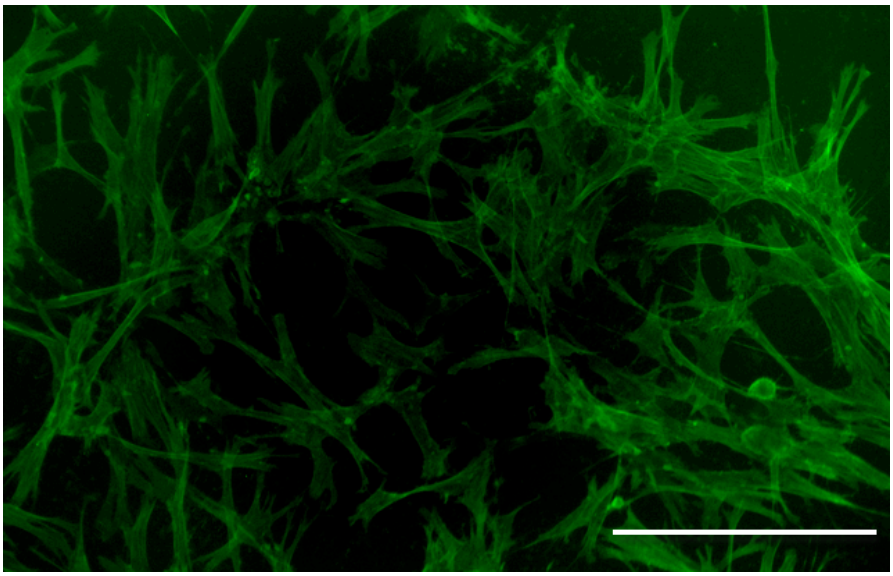


Fig. 6.7B. Cells seeded on non-irradiated Silicone Breast implant shells at Day 7

Figure 6.7: Fluorescent images capturing HDFa cells cultured at Day 7 stained with fluorescent F-actin (green) and DAPI nuclei (blue) staining on **A**. TCP versus cells seeded on **B**. non-irradiated Silicone Breast implant shells. The white line represents 200µm.

6.5 Discussion

As the indications for PMRT are expanding [100] and as patients undergoing implant based breast reconstruction who receive PMRT have been shown in our meta-analysis to have an increased rate of surgical complications such as capsular contracture and revisional surgery (Fig. 3.3, Fig. 3.4, Chapter 3), there is a clinical need to understand the mechanism of failure, which could lead to the creation of new materials or different treatment approaches. This is further supported by a recent systematic review by Lam et al. who reported patients undergoing a two stage implant based breast reconstruction demonstrated a higher rate of complications (capsular contracture, reconstructive failure and poor cosmetic outcome) of 18.6% in those receiving PMRT versus 3.1% ($p < 0.00001$) in those without [167]. This study sought to establish if the physicochemical properties and consequently cellular response to silicone breast implants change following radiation treatment.

In examining the mechanical properties of the implant shells, there were no significant changes in tensile strength between the groups in keeping with previous literature [104] and tear strength (Fig. 6.1). Young's modulus was not significant changed following full dose radiation (40.05Gy) but showed a decrease following single treatment fraction dose (2.67 Gy). In addition, elongation at break was significantly reduced after both single treatment fraction radiation dose (2.67 Gy) and after full dose radiation (40.05 Gy) suggesting that samples are less flexible after radiation treatment. Limitations to these results include testing implants of one type and from one manufacturer (Mentor Siltex™ Contour Profile™ Becker ™ 35 Expander implants). These implants were available and commonly used for breast reconstruction in the unit but limited samples were available for analysis and this may have influenced the results. Furthermore, our methods of material characterization assessed bulk mechanical properties in dry conditions at room temperature that may not be consistent with *in-vivo* conditions. There may have been micro-mechanical changes that were therefore not detected by the analyses performed. Further modalities of investigations including atomic force microscopy are warranted.

Contact angle measurements, performed to assess the hydrophobicity of the tested material that directly influences protein and cell attachment, were not significantly different between the irradiated and non-irradiated groups. ATR-FTIR analysis showed no significant differences detected between the spectral peaks amongst the three groups. This is not in keeping with Ribuffo et al. who detected changes in ATR-FTIR following treatment dose radiation therapy with the observance of smaller fragments suggesting scission of the polymers chains [104]. Although sample sizes were similar, this may possibly be explained by the higher radiation dose delivered in the study (50 Gy versus 40.05 Gy) delivered over a total of 25 fractions over 5 weeks in comparison to this study which delivered the full treatment radiation dose in one sitting.

Cellular response to the breast implant as demonstrated by fibroblast activity revealed no significant differences in terms of the cell metabolism and cell proliferation between the tested groups but a significant reduction in comparison to the control group (TCP) as demonstrated by Alamar Blue™ and Total DNA assays. Immunofluorescence staining revealed the effect on cell orientation and morphology between the 'smooth' TCP surface and the textured surface of the silicone implant. The cells grown on the TCP showed highly aligned, stretched out fibroblasts with increased nuclei in comparison to the cells grown upon the textured non-irradiated silicone implant which showed random orientation of shortened cells and reduced numbers of cells. This is in-keeping with clinical studies describing increased rates of capsular contracture in patients with smooth implants in comparison to textured implants [78].

Several theories as to the mechanism of capsular contracture exists including biofilm formation leading to chronic inflammation [69], surgical handling of the implant, peri-operative complications including haematoma and seroma, implant filler, radiation therapy and submuscular implant placement [164] but the actual mechanism is not fully understood and thought to be multi-factorial. It is well documented that radiation causes damage to normal cells through damage to DNA and cellular components leading to alterations in the cells signaling pathways could explain the increased rate of implant failure in those patients receiving PMRT [168]. Another possible factor could be the tumour microenvironment, which could be contributing to

implant failure as those patients receiving PMRT may in general have more advanced disease than patients who do not require PMRT.

This study shows that treatment dose radiation therapy administered to the silicone breast implant does not have an overall effect on the mechanical, surface chemistry and fibroblast cellular response and this is in keeping with previous literature examining the effect of radiation on PDMS based materials [105,106]. However, of note these studies used significantly greater doses of radiation for the purpose of examining the effect of material sterilisation than the doses required in the clinical setting and thus used in our experiment. Further in-vitro research is required to examine the effect of radiation therapy to the cells and the silicone breast implants in parallel to elucidate the mechanisms of development of capsular contracture and breast implant failure.

Chapter 7: A Comparison of the Effect of Treatment Dose Radiation on POSS-PCU, PCU and Silicone: Implications for future breast implant design

7.1 Introduction

Current day silicone breast implants are associated with complications such as capsular contracture, implant rupture and silicone gel bleeding/leakage often necessitating further corrective surgery. As shown in Chapter 4 and 5 their mechanical and chemical properties change over time and a fibrotic encapsulation can occur as well as leakage of silicone particles into the surrounding tissues.

Several attempts to produced the ideal breast implant using a variety of materials such as polyurethane [30] as well as fillers including soybean oil [36], hydrogels [1] and polyvinyl-pyrrolidone (PVP)-hydrogels [39] been withdrawn due to increased complications and carcinogenic potential. Current day breast implants are composed of the man made repeating unit polymer silicone, polydimethylsiloxane (PDMS, $(CH_3)_2SiO$). Silicone is well recognised for its inert qualities, low toxicity and is used in several medical applications [139].

The quest remains to produce a desirable breast implant with improved mechanical properties, biocompatibility and impermeability to leakage as well as the ability to mimic the natural breast mound.

Attempts to create the ideal implant are ongoing. Lim et al. [111] have described the potential application of a new polymer linear triblock poly(styrene-*b*-isobutylene-*b*-styrene) (SIBS) in comparison to silicone as a breast implant and shown promising results in a two week in vivo rabbit model. Furthermore, coating of silicone breast implants with extracellular proteins, namely collagen I and fibronectin has shown in an in vitro model to improve fibroblast adhesion which in turn may reduce micro-movement and shearing forces at the host-implant interface thereby reducing fibrous encapsulation [87].

Over recent years, the field of nanotechnology has grown and shown promise in drug delivery, medical diagnostics and tissue engineering. Previous research has demonstrated the effectiveness of a new nanocomposite polymer, composed of a poly (carbonate-urea) urethane (PCU) backbone integrated with the silica nanoparticle

polyhedral oligomeric silsesquioxane (POSS). The incorporation of the silica nanoparticle (POSS) cage into the polymer significantly enhances the mechanical properties and biocompatibility of the polymer [169]. Following research it has now been used in formation of lacrimal duct [170], human tracheal replacement [118] and ear and nose reconstruction [117] and in vascular bypass grafts [171].

The potential application of POSS-PCU as a possible alternative breast implant material thus is the focus of this chapter. The purpose of this study was to evaluate the mechanical, surface chemical properties and cellular response of POSS-PCU compared to PCU controls and current day silicone implants and also determine if these properties are altered by treatment dose radiation.

7.2 Aims of Chapter

Aim 1 – To evaluate the mechanical and surface chemistry properties of silicone breast implants versus POSS-PCU and PCU polymers and the effect of radiation upon these materials

Aim 2 - To assess the fibroblast response to treatment dose radiation therapy on silicone breast implants, POSS PCU and PCU.

7.3 Methods

7.3.1 Preparation and Irradiation of Materials

7.3.1.1 Silicone Breast Implant Shells

As described in Chapter 6.3.1.Un-implanted textured silicone breast implants (Mentor Siltex™ Contour Profile™ Becker ™ 35 Expander, Cohesive II™, Lot 6811381) were used. The implant inner gel was carefully removed and the outer shells were cut longitudinally into halves.

7.3.1.2 Preparation of POSS PCU nanocomposite

The synthesis of POSS-PCU has previously been described [172]. Polycarbonate polyol (2000 molecular weight) and trans-cyclohexanechloroydrinisobutyl-silsesquioxane were placed in a 250ml reaction flask possessing a mechanical stirrer and nitrogen inlet. The mixture was then heated t0 135°C to dissolve the POSS cage into the polyol and then cooled to 70°C. Flake 4,4'-methylenebis(phenyl isocyanate), was added to the mixture and then reacted, under nitrogen, at 75°C - 85°C for 90 minutes to form a pre-polymer. Dimethylacetamide was added slowly to the pre-polymer to form a solution; the solution was cooled to 40°C. Chain extension of the pre-polymer was carried out by the drop wise addition of ethylenediamine in dimethylacetamide to form a solution of POSS modified Polycarbonate urea-urethane in Dimethylacetamide. All reagents and chemicals were purchased from Sigma-Aldrich Ltd., Gillgham, UK.

7.3.1.3 Synthesis of polymer PCU

Dry Polycarbonate polyol (2000mwt) was placed in a 250ml reaction flask equipped with mechanical stirrer and nitrogen inlet. The polyol was heated to 60°C and then flake MDI was added and reacted with the Polyol, under nitrogen, at 70°C - 80°C for 90 minutes to form a pre-polymer. Dry Dimethylacetamide was added slowly to the pre-polymer to form a solution; the solution was cooled to 40°C. Chain extension of the pre-polymer was carried out by the drop wise addition of a mixture of Ethylenediamine and Diethylamine in dry Dimethylacetamide. All reagents and chemicals were purchased from Sigma-Aldrich Ltd., Gillgham, UK.

7.3.1.4. Casting of polymer sheets (POSS-PCU and PCU)

The final polymer mixtures were separately casted onto 16 x 16cm stainless steel plates and then placed in an oven at 65°C overnight to allow the dimethylacetamide to evaporate. The casted polymer sheets were then carefully peeled off the plates for further testing.

7.3.1.4 Irradiation of Materials

The breast implant shells and casted polymer sheets were categorized into three groups according to full dose radiotherapy (equivalent of 15 fractions delivered over 3 weeks) 40.05 Gy, one treatment dose radiotherapy (equivalent of one daily fraction) 2.67 Gy and non-irradiated samples (control). Implant shells and casted sheets of polymer were surrounded by blocks and adjuncts to simulate surrounding soft tissue and radiated at a rate of 6 Gy/min courtesy of the Department of Radiotherapy, Royal Free Hospital, London.

7.3.2 Mechanical Testing of Materials (Implant Shells and Casted Polymer Sheets)

All samples were measured using the Instron 5565 tensiometer equipped with a 500 N load (Instron, UK). From the implant shells and the casted sheets of POSS-PCU and PCU, for each condition, six dumbbell shaped specimens were cut using a Wallace cutting press for tensile testing and 3 crescent shaped specimens were cut for tear testing in accordance with the ISO 37:2005 standards. Specimens were placed in the pneumatic grips of the tensiometer and pulled apart at a rate of 100mm/min and 500mm/min for tensile and tear testing respectively. The data was captured using Bluehill software. Ultimate tensile strength, strain at break, Young's modulus and tear strength values were recorded.

7.3.3 ATR-FTIR of Samples

Samples of silicone implant, casted sheets of POSS-PCU and PCU subjected to each condition radiation condition were collected (n=5) and Fourier Transform Infrared

Spectra (FTIR) recordings were obtained using a Jasco FT/IR 4200 Spectrometer with a diamond attenuated total reflectance accessory (Diamond Miracle ATR, Pike Technologies, US). Spectra were produced from an average of 30 scans at a $4cm^{-1}$ resolution over a range of $600cm^{-1}$ to 4000 cm^{-1}. A background scan was performed prior to every measurement. The spectra were composed from the mean value of the 5 repeat measurements using Microsoft Excel worksheet (Microsoft Excel, 2011).

7.3.4 Surface Wettability/Contact Angle Measurements (θ) of Samples

Using a DSA 100 Krűss Goniometer, wettability analysis was performed on the implant samples, casted POSS-PCU samples and PCU samples from each of the 3 groups. Using the sessile drop technique, 5µl of deionized water was dropped onto the samples using an automated syringe with 10 seconds of dispensing and analysis was performed using the Drop Analysis software (EasyDrop DSA200, KrűSS) at room temperature. Four samples from specimens from each group were tested three times (n=12).

7.3.5 Cell Metabolism, Proliferation and Morphology

7.3.5.1 Cell Culture of HDFa

As previously described, HDFa fibroblasts were passaged in sterile T-75 flasks using Trypsin with Dulbecco's Modified Eagle's Medium, low glucose (Gibco, ThermoScientific™, UK) supplemented with 10% FBS (foetal bovine serum) and 1% PenStrep (penicillin-streptomycin, Gibco) or cryopreserved using DMSO until further required. HDFa's used in the experiment were between passage 7 and 11.
Discs measuring 0.9cm (n=8) were cut using a heavy-duty office hole punch from the breast implant shells and casted polymer sheets. All specimen discs were placed in 1% Triton X for 1 hour, washing twice in PBS followed by 70% ethanol followed by washing twice in PBS. Discs were then placed in a 96 well plate and covered with 100µl of warmed DMEM for approximately 2 hours prior to cell seeding.

7.3.5.2 Alamar Blue™ Assay – Cell metabolism

To assess cell metabolism, Alamar Blue™ assay (Invitrogen, Paisley, UK) was used. Disc specimens were seeded with HDFa cells at density of $5x10^4$ cells/cm^2. Cells seeded onto tissue culture plastic served as a positive control and media only wells provided a negative control. Cells were incubated at 37^oC at 5% CO2 in air and cell culture media was replenished on days 0, 1, 3 and 6. Alamar Blue™ assay was conducted according to manufacturer's guidelines on days 1, 3 and 7. Media from the wells was removed and fresh media containing 10% Alamar Blue™ solution was added to each well. Following 4 hr incubation, 100μl of the media from each well was placed into a 96-well plate and analysed using a fluorescent plate reader (Fluoroskan Ascent FL™ Fluorescence Plate Reader, ThermoScientific, USA) at an excitation and emission wavelengths of 530nm and 620nm (n=8).

7.3.5.3 DNA quantification – Cell proliferation

To assess cell proliferation, Hoechst 33258 DNA Quantification Kit, Fluorescence Assay (Sigma-Aldrich, UK) was used. Following Alamar Blue™ analysis the specimens were washed with PBS and 100μl of molecular grade water was added to each well. The plates were then submitted to 6 freeze-thaw cycles to achieve cell lysis. Fluorescence was measured using a fluorescent plate reader (Fluoroskan Ascent FL™ Fluorescence Plate Reader, ThermoScientific, USA) at excitation and emission wavelengths of 360nm and 460nm (n=8). A standard curve was performed with known quantities of calf thymus DNA and the equation was used to calculate the DNA concentrations from the fluorescence of the specimens.

7.3.5.4 Cell Morphology

Using commercially available fluorescent green cytoplasmic actin (Alexa Fluor 488 Phalloidin, Molecular Probes, ThermoScientific™, UK) and fluorescent blue nuclei staining kit (Vectashield Antifade Mounting Medium with DAPI, Vector Laboratories, USA), cell morphology was examined at day 7 of seeded HDFa cells on tissue culture plastic and on non-irradiated silicone implant shells. This was performed in accordance to the manufacturers protocols. Images were captured using an EVOS Fluorescent Microscope (EVOS FL Imaging System, ThermoScientific™, UK).

7.3.6 Statistical Analysis

Statistical analysis was performed using Graph Pad Prism software Version 6 with $p<0.05$ as significant.

7.4 Results

7.4.1 Material Mechanical Properties

Stress strain curves of silicone breast implant shells, POSS-PCU and PCU are shown in Fig. 7.1 and mechanical characteristics outlined in Table 7.1. POSS-PCU and PCU had significantly higher tensile strength than silicone breast implant shells both for non-irradiated and full dose irradiated specimens as shown in Figure 7.2. No significant difference in maximum tensile strength was detected between non-irradiated and radiated silicone breast implant shells and POSS-PCU (Fig. 7.3). However, there was a significant difference in maximal tensile strength of non-irradiated and irradiated PCU (Fig. 7.3). There were no significant differences in maximal tear strength in all samples detected following irradiation as outlined in Fig. 7.4.

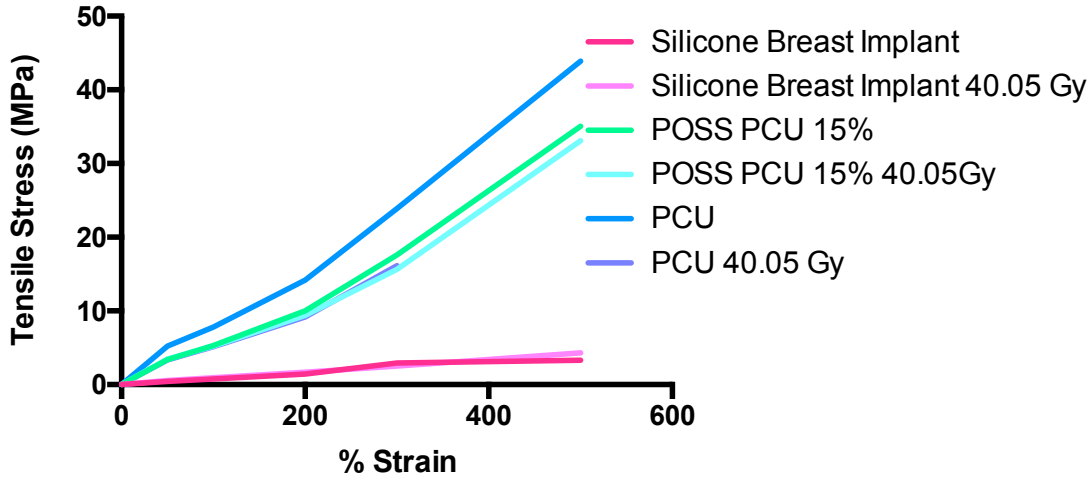


Figure 7.1: Overlaid Stress Strain Curves of Irradiated and Non-irradiated POSS-PCU, PCU and Silicone Breast Implant Shells. POSS-PCU and PCU demonstrate greater stress-strain profiles than Silicone breast implant shells.

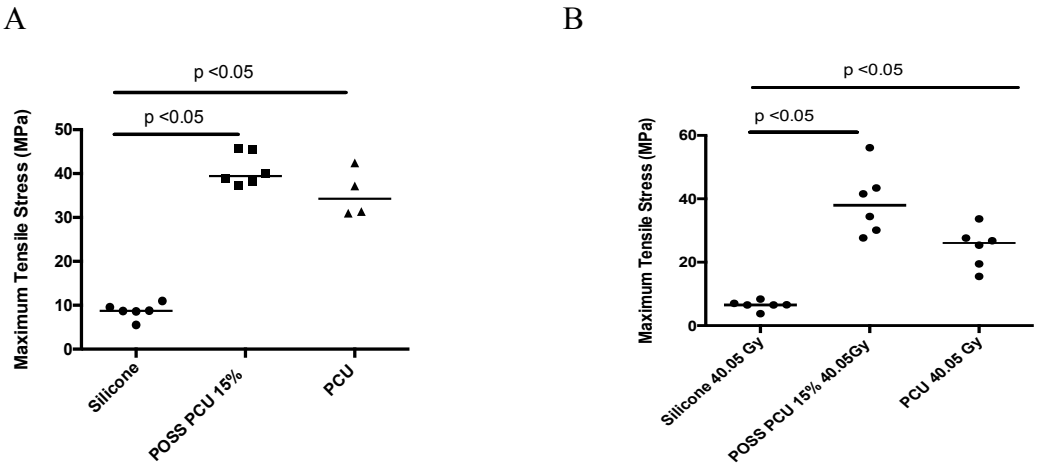


Figure 7.2: Maximal tensile strength (MPa) of **A**. Non-Irradiated specimens and **B.** Maximum Tensile Strength of Irradiated Specimens

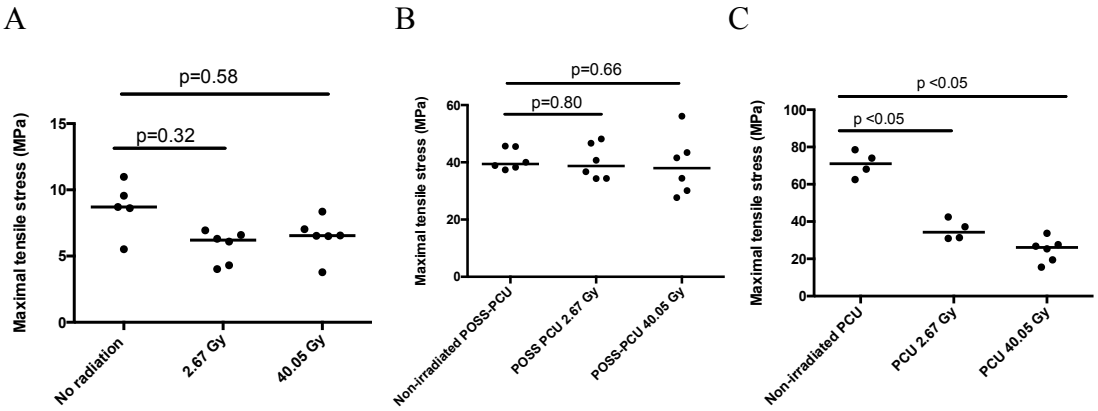


Figure 7.3. Maximum Tensile Strength of **A** irradiated and non-irradiated Silicone samples. No significant differences detected between the samples **B** irradiated and non-irradiated POSS-PCU samples. No significant differences detected between the samples **C.** Maximum Tensile Strength of irradiated and non-irradiated PCU samples. Significant differences detected between the non-irradiated and irradiated samples

Sample	*Max. Tensile Strength (MPa) + SD*	*Young's Modulus 5-10mm (MPa)+ SD*	*Strain at Break (%)+ SD*
Silicone Breast Implant Shell	8.68 ± 1.79	0.91 ± 0.09	1181.51 ± 194.65
Silicone Breast Implant Shell – 40.05Gy	6.46 ± 1.49	1.12 ± 0.11	717.37 ± 128.42
POSS- PCU	40.95 ± 3.70	5.46 ± 0.41	526.45 ± 43.08
POSS-PCU - 40.05Gy	35.13 ± 11.28	4.99 ± 1.22	560.95 ± 70.19
PCU	70.82 ± 6.99	8.03 ± 0.64	765.53 ± 61.77
PCU - 40.05Gy	24.74 ± 6.42	5.30 ± 0.98	412.83 ± 51.99

Table 7.1. Mechanical characteristics of irradiated and non-irradiated materials.

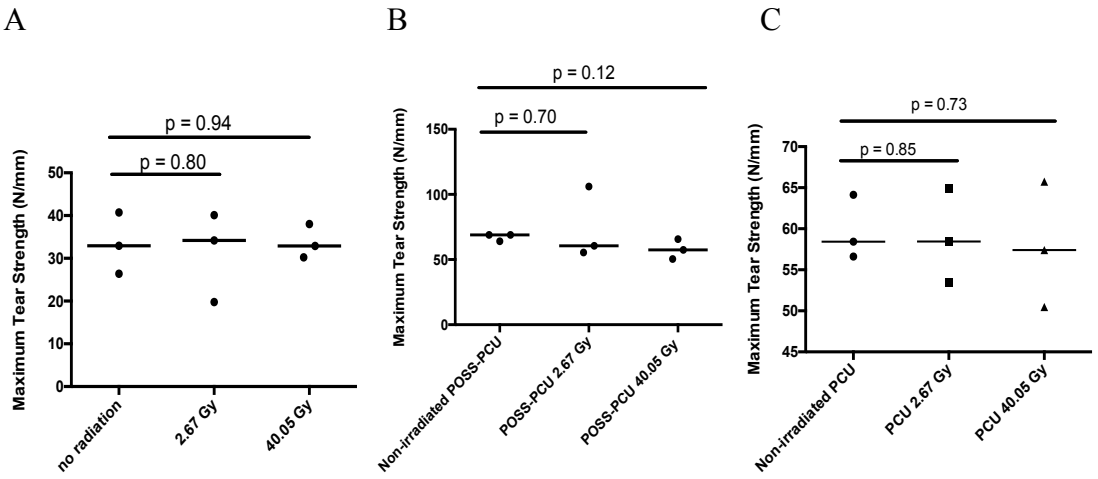


Figure 7.4: Maximum Tear Strength of irradiated and non-irradiated materials. **A.** Silicone breast implant shells showed no significant differences (p=0.94). **B.** No significant differences detected between irradiated and non irradiated POSS-PCU (p=0.12). **C** No significant differences detected between irradiated and non-irradiated PCU (p=0.73).

7.4.2 Material Surface Chemistry

7.4.2.1 ATR-FTIR

Surface chemical properties of the tested specimens were performed using ATR-FTIR. The overlaid spectra for non-irradiated and irradiated silicone breast implant shells, shown in Chapter 6 appeared the same. As described in the previous chapter, there were no significant differences detected in the peak spectral intensities in the irradiated and non-irradiated silicone breast implant shells. No observed differences between the peak spectral heights at 784 cm^{-1} corresponding to $–CH_3$ rocking and –Si-C-stretching in $–Si-CH_3$ ($p=0.3260$, one way ANOVA, parametric data), at 1004cm^{-1} corresponding to the asymmetric stretching of –Si-O-Si- ($p =0.8746$, one – way ANOVA, parametric data) and at 1257 cm^{-1} corresponding to symmetric bending of $–CH_3$ in $–Si-CH_3$ ($p = 0.6676$, one way ANOVA parametric data)

The overlaid spectra for the POSS-PCU samples, shown in Figure 5, appear the same. On statistical analysis, there was no significant difference in the peak spectral intensities at (carbonate C=O stretching from carbonate) ($p=0.0686$, one way ANOVA), at 1111.7cm^{-1} (carbonate C-O-C stretching) ($p= 0.2094$, one way ANOVA) and at (POSS Si-O-Si stretching) ($p =0.1183$, one way ANOVA) between the non-irradiated and irradiated POSS-PCU specimens.

The overlaid spectra for the non-irradiated and irradiated PCU specimens are shown in Figure 6 and again appear similar. There were no significant statistical differences detected at the peak spectral intensities at 1737cm^{-1} ($p=0.2903$, one way ANOVA) and 1241cm^{-1} ($p=0.4318$, one way ANOVA) between the non-irradiated and irradiated specimens.

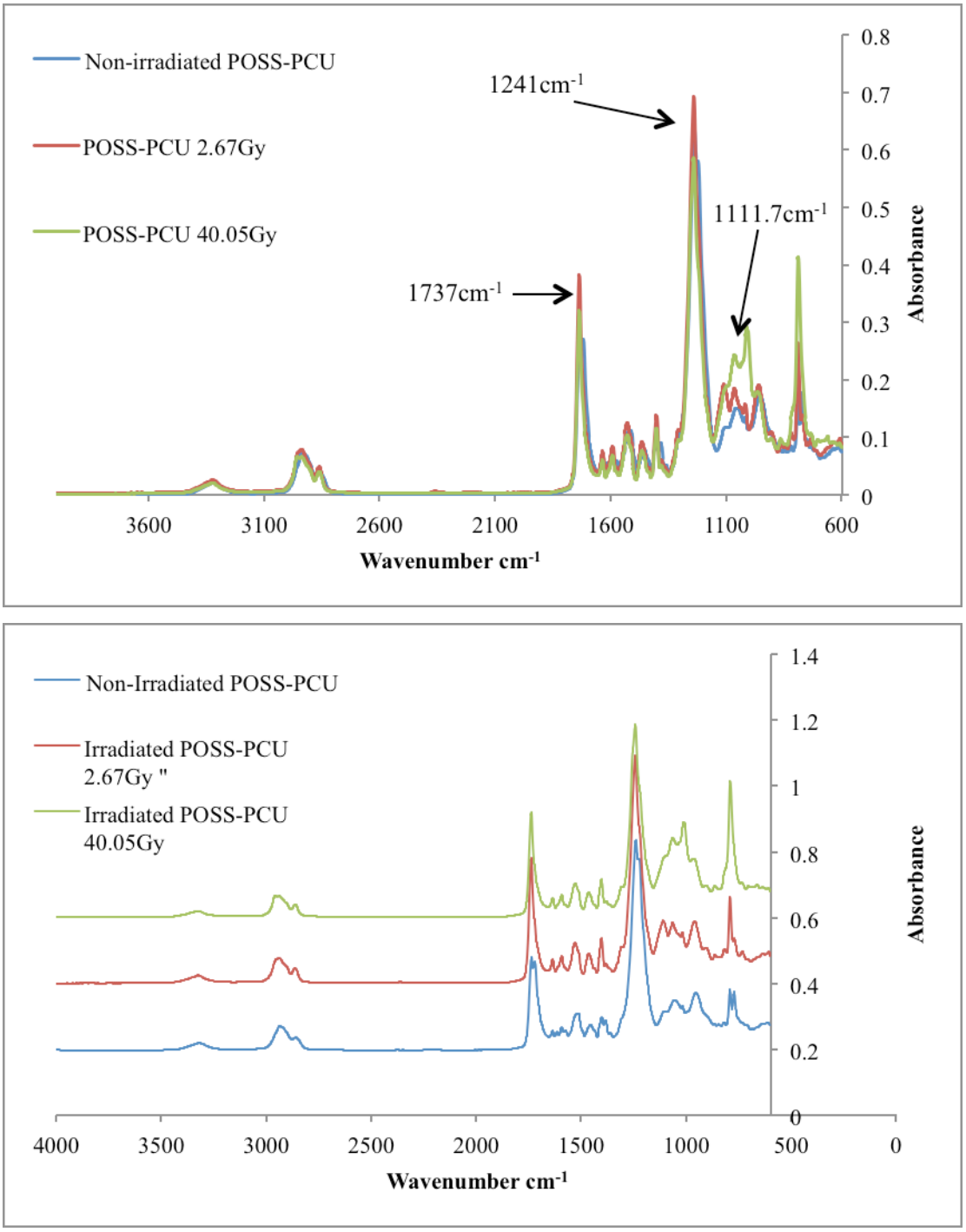


Figure 7.5: A. Overlaid ATR-FTIR spectra of tested POSS-PCU samples **B**. ATR-FTIR spectra of non-irradiated (+0.2) and irradiated POSS-PCU at 2.67 Gy (+0.4) and at 40.05 Gy (+0.6). No significant differences were seen in peak spectral differences.

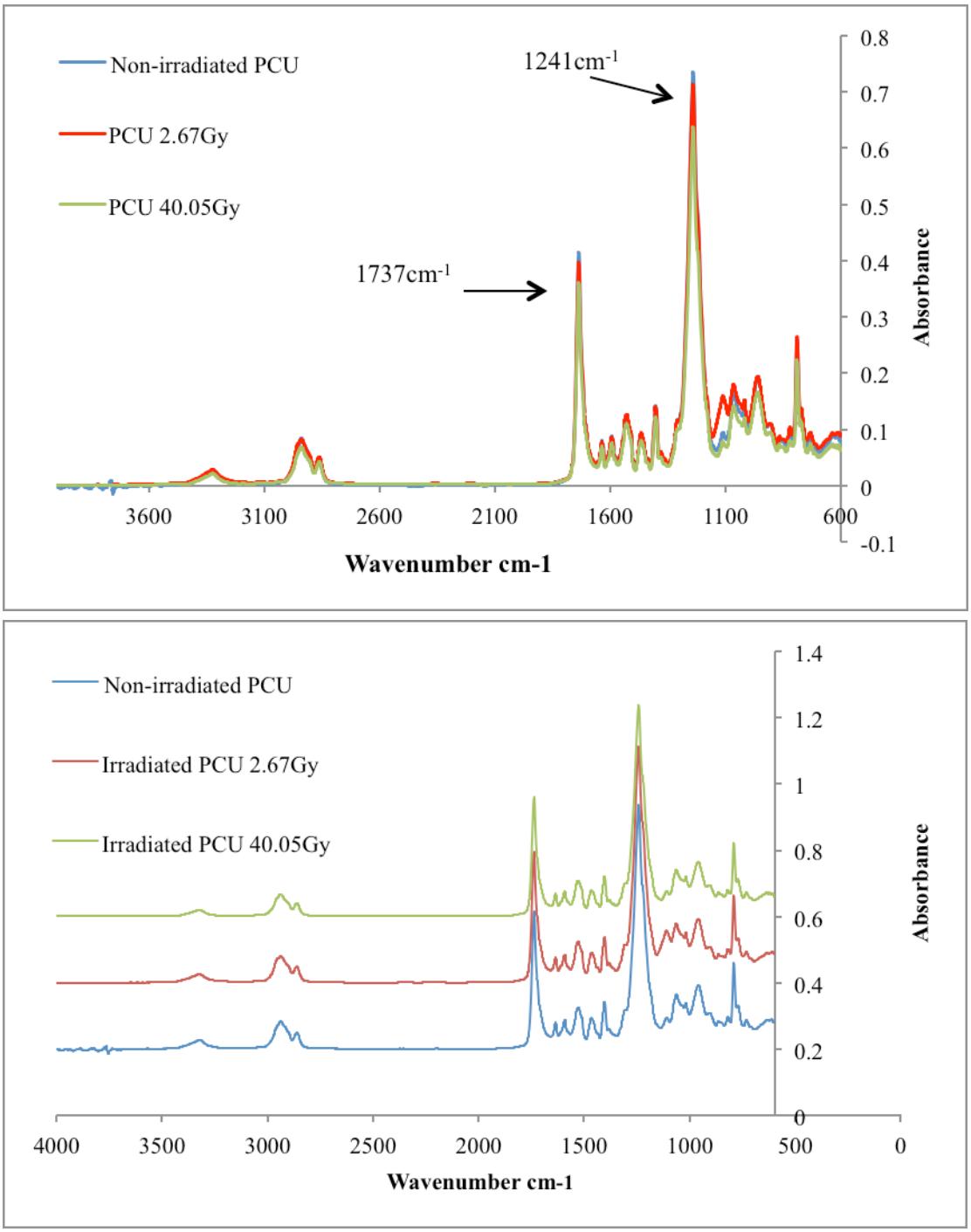
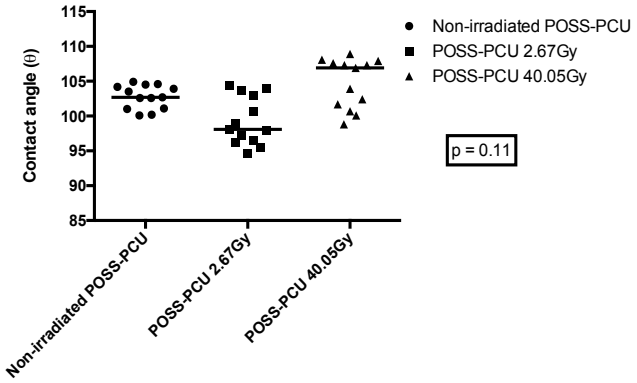


Figure 7.6: A. Overlaid ATR-FTIR spectra of non-irradiated and irradiated PCU samples. **B**. ATR-FTIR spectra of non-irradiated (+0.2) and irradiated PCU at 2.67 Gy (+0.4) and at 40.05 Gy (+0.6). No significant differences were seen in peak spectral differences.

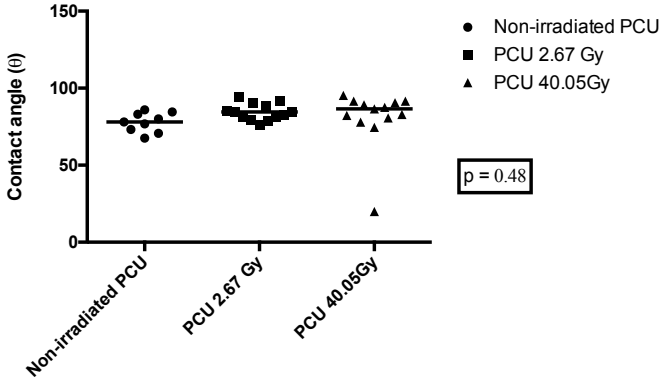
7.4.2.2 Surface Wettability/Contact Angle Measurements (θ)

There were significant differences in contact angle measurements detected between each of the POSS-PCU groups (p=0.0002, one way ANOVA) as shown in Fig. 7.710. On further analysis, there were statistically significant differences between non-irradiated POSS-PCU and 2.67 Gy POSS PCU (p = 0.0115, Mann-Whitney test) but no significant difference was detected between non-irradiated POSS PCU and 40.05Gy POSS-PCU (p=0.1987). There were no significant differences shown between the PCU non-irradiated and irradiated specimens, Fig. 7.7B (p = 0.4760, one way ANOVA). As shown in Chapter 6, there was no significant difference following irradiation in silicone breast implant shells, Fig. 7.7C.

A



B



C

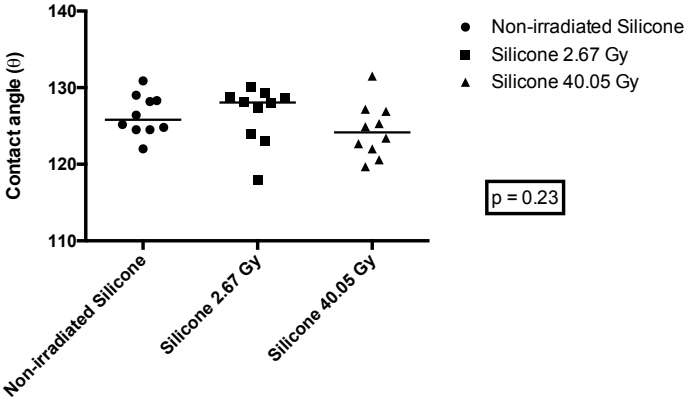


Figure 7.7: Contact Measurements showed no significant differences in contact angle measurements **A.** No significant differences between non-irradiated POSS-PCU and POSS-PCU 40.05 Gy ($p = 0.11$, Mann Whitney test) **B.** No significant differences between the PCU groups ($p = 0.48$, Kruskal-Wallis test) **C.** No significant differences between the silicone breast implant shell groups ($p= 0.23$, Kruskal-Wallis test)

7.4.3 Cell Metabolism, Growth and Morphology

7.4.3.1 Alamar Blue™ Assay (Cell metabolism)

There were similar cell metabolism seen when seeded upon tissue culture plastic (TCP) and the irradiated and non-irradiated samples as shown in Figure 8. At day 1 only HDFa cells seeded upon irradiated POSS-PCU 40.05Gy showed statistically significant reduction in cell metabolism ($p<0.05$). On Day 3, a significant reduction in cells seeded on irradiated silicone and non-irradiated POSS-PCU was observed ($p<0.05$). On day 7, cells seeded on upon non-irradiated POSS-PCU, non-irradiated PCU and irradiated PCU 40.05Gy showed a significant reduction in cell metabolism ($p<0.05$, 2 –way ANOVA).

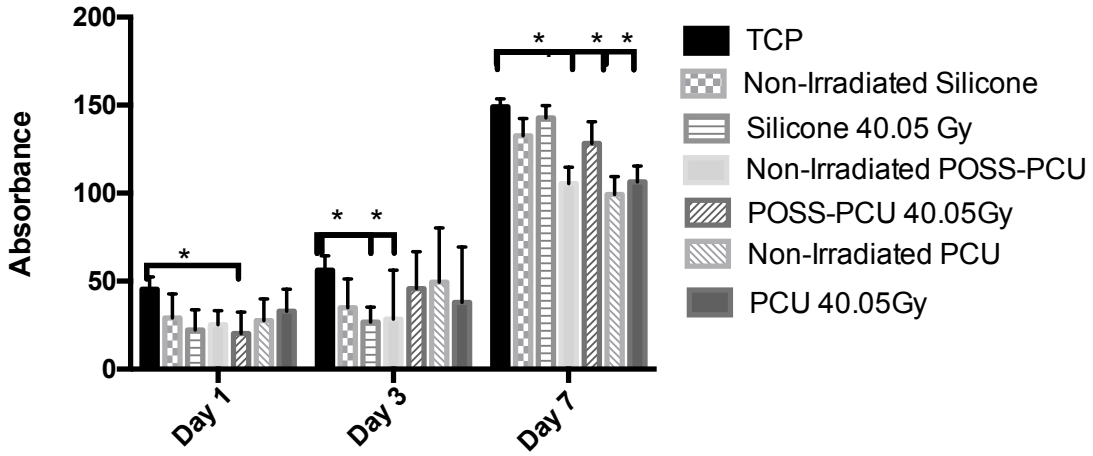


Figure 7.8: Cell viability on tissue culture plastic (TCP), cells grown on non-irradiated and irradiated silicone breast implant shells, POSS-PCU and PCU as assessed by Alamar Blue™ assay over 7 days. In comparison to tissue culture plastic (TCP) after 7 days there was significant reduction cell metabolism of HDFa cells seeded upon non-irradiated POSS-PCU, non-irradiated PCU and irradiated PCU 40.05Gy (* = $p<0.05$, 2 –way ANOVA).

7.4.3.2 Total DNA assay (cell proliferation)

Hoechst 33258 DNA Quantification Kit™, Fluorescence Assay showed no significant differences in cell growth on cells seeded on the irradiated or non-irradiated samples in comparison to tissue culture plastic as shown in Fig. 7.9.

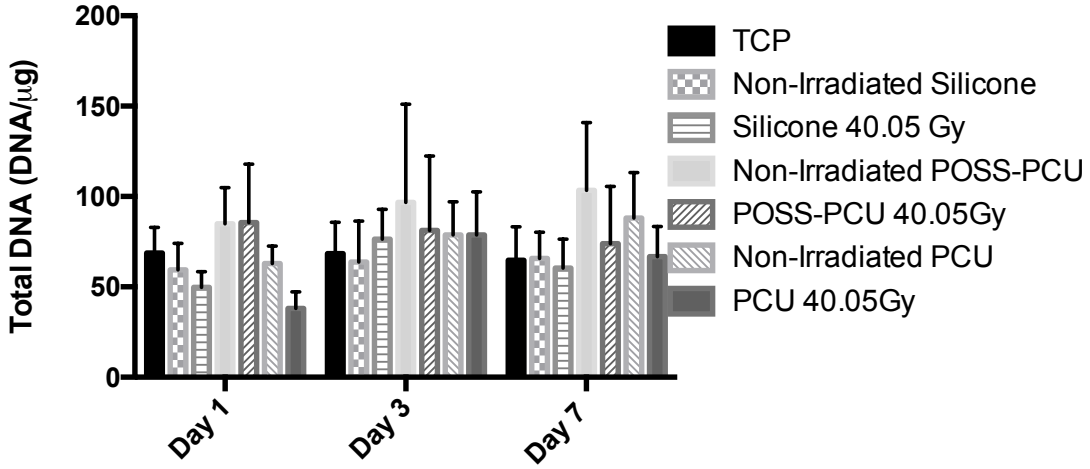


Figure 7.9: Hoechst 33258 DNA Quantification Kit™, Fluorescence Assay over 7 days performed on irradiated and non-irradiated silicone breast implant shells, POSS-PCU and PCU. No significant differences in cell growth detected between the samples.

7.4.3.3 Cell Morphology at Day 7 (Immunofluorescence staining)

Cells grown on tissue culture plastic (TCP) demonstrated highly aligned, thin, long, spindle like projections in parallel with an abundance of nuclei (Fig. 7.10A). In contrast, the cells cultured upon non-irradiated silicone breast implant shells showed thickened projections with random orientation with fewer nuclei evident (Fig. 7.10B). Cells grown on casted PCU (Fig. 7.10C) showed orientated, thicker, long spindle projections of the fibroblasts and cells grown on POSS-PCU showed fewer nuclei, with thicker projections of fibroblasts in comparison to TCP (Fig. 7.10D).

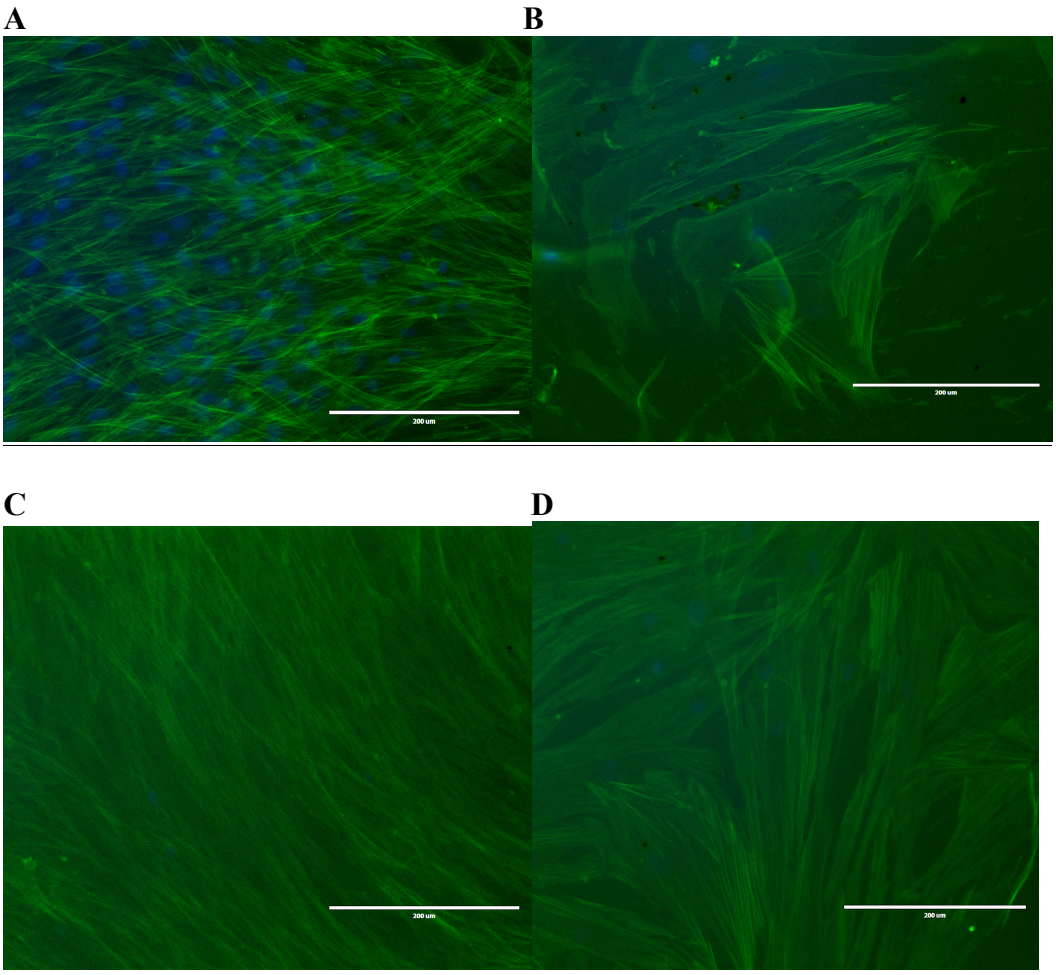


Figure 7.10. Fluorescent images capturing HDFa cells cultured at Day 7 stained with fluorescent F-actin (green) and DAPI nuclei (blue) **A.** Tissue culture plastic TCP **B**. Non-irradiated silicone breast implant shells **C.** casted PCU **D**. casted POSS-PCU

7.5 Discussion

The development of the ideal breast implant remains a challenge. It must possess improved mechanical properties, be impermeable to leakage and promote a minimal inflammatory host response in order to reduce or eliminate the known complications of implant rupture, leakage and capsular contracture. In addition, it must also be soft to palpate mimic the natural breast tissue to make this an acceptable implant for patients.

This study sought to compare the mechanical and surface chemical properties and cellular response of nano-composite POSS-PCU, PCU polymer and silicone breast implants shells to assess its potential material for future breast implant manufacture. In terms of mechanical properties, POSS-PCU showed greater tensile strength than current day silicone breast implant shells and in addition, showed that these properties were unchanged in response to treatment dose radiation therapy. This is a key feature in creating an improved alternative breast implant to improve implant rupture and leakage rates as it is documented from previous research that increasing duration of implantation of silicone breast implants leads to shell weakening and reduced mechanical properties [60,63]. In addition, POSS-PCU demonstrated no detectable changes in mechanical properties response to treatment dose radiation therapy further supporting its potential application in breast implant prosthesis manufacture.

In addition, there was no statistically significant difference in the peak spectral intensities between the irradiated and non-irradiated specimens as demonstrated by ATR-FTIR analysis, thereby indicating no significant changes in the surface chemistry of the materials in response to irradiation. The hydrophobic surfaces of the textured silicone breast implant shells and POSS-PCU in comparison to PCU may explain the reduction in cellular proliferation and viability seen in the study. In addition, the cellular response was reduced in comparison to TCP but was unaffected by radiation therapy. Hydrophobic surfaces reduce protein adsorption at the material surface. As demonstrated with cell morphology, the ‘smooth’ POSS-PCU featured less cell nuclei and thickened projections of the fibroblasts in comparison to TCP and

PCU. In assessing the cellular response, there was increased cell metabolism between the timepoints but no overall significant increase in cell proliferation.
The surface chemistry of any given material directs the cellular response, cell adhesion and migration. The addition of functional groups can alter the biocompatibility of the material. Studies by Barr et al. have already shown providing specific coatings to silicone breast implants can alter the cellular fibroblast response [87] In addition, preliminary studies have shown promise in functionalizing the surface of POSS-PCU to promote endothelisation in vascular bypass grafts [173]. A potential further area of research could assess the impact of altering topographical surface of POSS-PCU to create a textured surface in keeping with the current day textured silicone implants. Textured silicone breasts implants in comparison to smooth have been reported to produce reduced rates of capsular contracture [146]. Barr et al[174] demonstrated that the textured surface of the silicone breast implant disorientates the planar alignment of collagen fibres produced by fibroblasts which may explain the observed reduced capsular contracture rates. Therefore, it would be pertinent to conduct further research comparing smooth and textured POSS-PCU surfaces with current day textured silicone breast implants.

Preliminary studies have already compared the in vivo response to POSS-PCU to siloxane in a sheep model at 36 months [175] and reported a reduction in capsule formation around the POSS-PCU implants. However, further in vivo work, comparing POSS-PCU to current day silicone implants and the response to treatment dose radiation therapy is required to further evaluate POSS-PCU as a new potential material for breast implant manufacture, particularly for use in those patients undergoing implant based breast reconstruction.

Chapter 8: Discussion

Current day silicone breast implants have been subjected to five generation of manufacturing changes in response to complications. Despite this however, silicone breast implants are still associated with complications such as capsular contracture, gel leakage and implant rupture.

The aim of this thesis was to further understand and investigate the mechanism of breast implant failure with particular emphasis on the role of radiation therapy in its pathogenesis.

There is a clinical need to further understand this mechanism of injury with radiation therapy. The indications for radiation therapy post mastectomy are expanding and often in patients who have undergone implant based breast reconstruction. Thus the timing and method of breast reconstruction and radiotherapy has become a controversial topic in managing patients who require post mastectomy radiation therapy. One of the aims of this thesis was to determine the long-term clinical outcomes of radiation therapy delivered to patients with a permanent implant post mastectomy. As such, it was found that patients receiving PMRT directly to the permanent implant have increased risk of capsular contracture, implant rupture and failure of their reconstruction. In addition, these patients were also found to have poorer cosmetic outcome and satisfaction levels. This study is particularly of value to breast surgeons and oncologists when planning breast cancer treatment taking into account the timing of PMRT and the potential risks of implant based breast reconstruction in order to allow patients to make informed choices regarding their treatment.

With the knowledge that post mastectomy radiation therapy delivered to patients with breast implants increases the likelihood of breast implant failure, this thesis sought to further investigate the failure mechanisms of breast implants. Firstly this was done by retrieval of explanted breast implants from patients and their surrounding capsule from a single centre. A fall in mechanical properties of the breast implant shells was witnessed with increasing duration of implantation as described in previous studies. The limitations to the study were that the implants collected were derived from a range of manufacturers and consisted of both tissue expanders and permanent implants. Moreover, the indication for implants was for both cosmetic and

reconstructive purposes which may have influenced the results as patients who underwent reconstructive procedure may be followed up routinely by physicians and therefore may present earlier to hospital services with implant related complications. Further study to compare breast implants from a single manufacturer is warranted. In some of the breast implant shells, due to shell rupture and the retrieval process, there was in some cases not enough sample material to test the mechanical properties that may have influenced the results. Atomic force microscopy showed interesting initial results with a change in mechanical properties across the cross section of the implant shell suggesting the inner gel or the surrounding host environment is exerting changes upon the shell. However, due to limited samples, further study examining implants from a range of manufacturers and of varying ages of implants is required.

In assessing the cellular response, HDFa cells were employed as these are a reliable, easily accessible cell line. However, future study to mimic the true conditions a breast implant is subjected to in vivo would be to use breast derived cells including fibroblasts as well as other inflammatory cell types (macrophages and monocytes) as well as conducting studies to assess cytokine release.

Another limitation of our study was the histological analysis of the surrounding capsule. The capsules were retrieved by different surgeons and the area the sample was take from in relation to the position of the breast implant within the breast was variable which may have influenced the results which may have influenced the results. In addition, only six of the samples were submitted from SEM-EDX testing due to time and financial constraints and further study assessing all retrieved samples would be warranted.

In an attempt to explain the increased adverse events reported in patients receiving radiation therapy directly to the breast implant, a study of the effect of treatment dose radiation therapy in isolation on the silicone breast implant shells alone was performed. This showed no significant changes upon the bulk mechanical properties and surface chemical properties and the cellular response. Therefore, it has been shown in this thesis it is not the radiation therapy causing a fall in mechanical properties of the implants but the host response to the material. A further area of research would be to measure the cellular response to fibroblasts seeded upon the

silicone breast implants which are then both subjected to radiation therapy and assess the cellular behaviour upon the breast implant shells following the treatment.
The development of the ideal breast implant remains a challenge. It must possess improved mechanical properties, be impermeable to leakage and promote a minimal inflammatory host response in order to reduce or eliminate the known complications of implant rupture, leakage and capsular contracture. In addition, it must also be soft to palpate mimic the natural breast tissue to make this an acceptable implant for patients.

In this thesis radiation therapy has been shown in increase the complication rate for patients with breast implants however it has been shown that radiation therapy in isolation does not produce changes in the bulk mechanical and surface chemical properties of silicone implants. Therefore, it is hypothesised that the radiation therapy is causing changes in the cellular behaviour towards the breast implants which is in turn responsible for the increased complications seen. Future work therefore should be directed at understanding the cellular response in combination with the breast implants and the effect of radiation therapy delivered to both. POSS-PCU, a nanocomposite polymer has been shown to have different bulk mechanical properties and surface chemical properties to that of silicone breast implant shells and in this thesis have been shown to be unaffected by radiation therapy therefore could be a potential new material for future development of breast implants.

There will be a continuing need for breast implant both in the cosmetic and reconstructive setting and further research into understanding the cellular behaviour on the implant is necessary to allow researchers to design an improved tailor made polymer material that reduces the inflammatory response whilst retaining its mechanical and surface chemical properties.

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Appendix 1: Summary of Patient and Breast Implant Characteristics.

Implant	*Age*	*Duration of Implant (months)*	*Reconstruction Vs. Augmentation*	*Reason for Explantation*	*Adjuvant Therapy*	*Intact vs. Ruptured*
1	53	96	Reconstruction	CC Suspected Rupture	Pre-op Radiotherapy	Ruptured
2	29	120	Augmentation	Contralateral revision	Nil	Intact
3	29	120	Augmentation	Suspected Rupture	Nil	Ruptured
4	48	300	Augmentation	MRI confirmed intra-capsular rupture	Nil	Ruptured
5	48	300	Augmentation	Contralateral revision	Nil	Intact
6	36	5	Reconstruction	Exchange for PI	Nil	Intact
7	48	120	Augmentation	CC Grade 3	Nil	Ruptured
8	48	120	Augmentation	CC Grade 3	Nil	Intact
9	37	204	Reconstruction	CC Grade 4	Nil	Intact
10	44	84	Reconstruction	CC Grade 3	Nil	Intact
11	45	215	Reconstruction	CC Grade 3	Nil	Intact
12	53	85	Reconstruction	Pain at port site, distortion of breast, CC Grade 3	Pre-op Radiotherapy	Intact
13	35	7	Reconstruction	Exchange for PI	Nil	Intact
14	35	7	Reconstruction	Exchange for PI	Nil	Intact
15	43	19	Reconstruction	Rippling	Nil	Intact
16	45	204	Reconstruction	Bilateral discomfort around implant	Nil	Intact
17	45	204	Reconstruction	Bilateral discomfort around	Nil	Intact

				implant		
18	60	110	Reconstruction	Symmetrisation	Nil	Intact
19	43	147	Reconstruction	Exchange for PI	Nil	Intact
20	33	86	Augmentation	Silicone granuloma Axilla	Nil	Ruptured
21	33	86	Augmentation	Contralateral revision	Nil	Intact

Appendix 2: PATIENT INFORMATION SHEET & CONSENT FORM.

Study title: Collection and Analysis of Retrieved Breast Implants and Surrounding Capsule Tissue.

Invitation

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends and relatives if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Thank you for reading this.

1. What is the purpose of the study?

Breast Implants are known to be associated with significant complications such as capsular contracture (severe scar tissue forming around implant) which can cause firmness, pain and distortion, implant leakage and rupture. This study is aimed to determine why breast implants fail by investigating the mechanical and chemical properties of retrieved breast implants as well as analysing the capsule (scar tissue) surrounding the implant to analyse the cells involved.

2. Why have I been chosen?

All patients waiting to undergo removal and/or exchange of breast implant(s) will be invited to take part in the study.

3. Can I refuse to take part in the study or withdraw from the study?

You do not have to take part in the study or you can withdraw at any stage. This will not affect your treatment in any way. If you refuse to participate in the study we will provide you with the standard treatment. No further data will be collected.

4. What will happen to me if I take part?

- After your Surgeon removes your breast implant(s) instead of being discarded, these will be collected for further analysis
- As part of routine care, the capsule surrounding the implant or scar tissue is removed and discarded. We will use some of that tissue for research purposes.

5. Will there be any changes to my treatment because of this research?

There will be no difference in the care that people who participate and do not participate receive.

6. What do I have to do?

There are no changes to your routine treatment or restrictions imposed on you from taking part in this research.

7. What are the possible benefits of taking part?

The information we get from this study may help us to understand why breast implants wear and why patients develop complications from breast implants. This will allow us to develop safer breast implants in the future

8. Will my GP be informed of this research?

We will not inform your GP routinely that you have participated in this research, as there will be no difference in the care that people who participate and do not participate receive, apart from this inflation and deflation of the blood pressure cuff.

9. Will my taking part in this study be kept confidential?

All information which is collected about you during the course of the research will be kept strictly confidential. Any information about you will stored using hospital numbers rather than using identifiable information names, address, and date of birth.

10. What will happen to the results of the research study?

The results from the study may be published in medical journals anytime between 6 to 12 months following completion of the study, but you will not be identified.

11. Who is organising and funding the research?

The study is being supported by the Department of Breast Surgery and Department of Plastic Surgery at the Royal Free Hospital. There are no additional payments either to the doctor or patients for being involved in the study.

12. Who has reviewed the study?

The study has been reviewed and approved by the Research Ethics Committee of the Royal Free Hospital.

13. Contact for Further Information

Prof Mohammed Keshtgar
Professor of Cancer Surgery

We thank you for reading this information sheet. Please keep a copy of this information sheet and the signed consent form for your records if you agree to participate in this study.

CONSENT FORM

Title of Project: Collection and Analysis of Retrieved Breast Implants and Surrounding Capsule Tissue

Ethics approval number:

Hospital number of the patient:

1. I confirm that I have read and understand the information sheet dated 20th August 2015 (version 1[1]) for the above study and have had the opportunity to ask questions. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐
3. I understand that sections of any of my medical notes may be looked at by responsible individuals or from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records. ☐
4. I agree to take part in the above study. ☐

_____	_____	_____
Name of patient	Date	Signature
_____	_____	_____
Name of person taking consent	Date	Signature
_____	_____	_____
Researcher	Date	Signature

1

Appendix 3: Presentations and Publications

Published Manuscripts.

- **Magill LJ,** Tanska A, Keshtgar M, Mosahebi A, Jell G. *Mechanical and surface chemical analysis of retrieved breast implants from a single centre.* J Mech Behaviour of Biomedical Materials. 2019 Mar;(91); 24-31

- **Magill LJ**, Robertson FP, Jell G, Mosahebi A, Keshtgar M. *Determining the outcomes of post-mastectomy radiation therapy delivered to the definitive implant in patients undergoing one- and two-stage implant-based breast reconstruction: A systematic review and meta-analysis*
  J Plast Reconstr Aesthet Surg. 2017 Oct;(70)10: 1329-1335.

Conference Presentations

- **Magill LJ**, M Keshtgar, A Mosahebi, G Jell. *Histological Analysis of Retrieved Silicone Breast Implant Capsules and Correlation with the Implant's Mechanical Properties*
  Accepted for poster presentation, Association of Breast Surgery Annual Conference, 18th -19th June 2018, Birmingham, UK.

- **Magill LJ**, Jell G, Keshtgar M. *Analysis of the Mechanical and Chemical Properties of Retrieved Breast Implants.*
  Poster presentation, TERMIS, 28th June – 1st July 2016, Uppsala, Sweden

- **Magill, LJ,** Faulker P, Mosahebi A, Ricketts K, Jell G, Keshtgar M. *Polyhedral Oligomeric Silsesquioxane Poly (Carbonate-Urea) Urethane (POSS-PCU) has superior mechanical properties compared to current breast implant silicone shells and shows promise as a next generation breast implant shell.* European Journal of Surgical Oncology, Volume 42, Issue 5, S48 - S49
  Poster presentation. Presented at the Association of Breast Surgery Annual Conference, 16th-17th May 2016, Manchester, UK.

- **Magill LJ**, Mosahebi A, Davidson T, Ghosh D, Hamilton S, Marsh D, Jell G, Keshtgar M. *An analysis of the mechanical strength properties of retrieved silicone breast implants in a single centre*. European Journal of Surgical Oncology, Volume 42, Issue 5, S46 - S47
  Poster presentation, Presented at the Association of Breast Surgery Annual Conference, 16th-17th May 2016, Manchester, UK.

- **Magill LJ**, Faulker P, Ricketts K, Mosahebi A, Jell G, Keshtgar M. *Treatment dose radiation therapy does not significantly weaken 5th generation silicone implant shells*. European Journal of Surgical Oncology, Volume 42, Issue 5, S49

Poster presentation. Presented at the Association of Breast Surgery Annual Conference, 16th-17th May 2016, Manchester, UK.