Expression of myofibroblasts and identification of angiotensin receptors in capsular contracture disease around mammary implants

Leila Touil (MBCHB, MRCS)

A thesis submitted in partial fulfilment for the requirements of the degree of MSc (by Research) at the University of Central Lancashire

September 2013
Declaration

I declare that this thesis has been composed by myself and that, whilst registered as a candidate for the degree of Master of Science by Research, I have not been registered as a candidate for any other award or by any other awarding body. All sources of information used have been acknowledged.

Signature: Leila Touil 20/08/2013
Acknowledgements

I would like to thank all the people who have made this research possible.
Firstly, I would like to thank my Supervisor, Dr Philip Roberts for his patience, encouragement and guidance during this MSc and preparation of my thesis, and for never giving up on me! I would also like to thank my Research Degree Tutor, Professor Jai Paul Singh for his unconditional support.
I would also like to acknowledge the clinical support of Mr Milind Dalal, Consultant Plastic Surgeon and thank him for his encouragement and sound advice and also Mr S.W McKirdy, Consultant Plastic Surgeon at Royal Preston Hospital. I would also like to thank Mr. Partha Vaiude, Senior Registrar in Plastic Surgery for his inspiration, support and direction.
I am also appreciative to the Pathology Department at Royal Preston Hospital for the provision of their facilities in the hospital laboratory. I would particularly like to thank Katherine Ashton, Biomedical Scientist at Lancashire Teaching Hospitals Trust for her endless support in the hospital laboratory which was paramount to this study. I will be eternally grateful for all that she has taught me. I would also like to acknowledge Dr Laxminarayam Gudur, Consultant Breast Histopathologist at Royal Preston Hospital for his kind support, his time and expert advice.
Finally, thank you to my colleague Mr Christopher Stephen, for his moral support and help in the lab!
Table of Contents

Abbreviations .................................................................................................................. 5
Abstract ............................................................................................................................ 8

Chapter 1 – Introduction
1.1. Breast Cancer ............................................................................................................. 10
1.2. Breast reconstruction ................................................................................................. 12
1.3. Capsular contracture ................................................................................................. 15
1.4. Wound healing .......................................................................................................... 21
1.5. The Myofibroblast .................................................................................................... 24
1.6. The Renin- Angiotensin system (RAS) ...................................................................... 27
1.7. Angiotensin- Converting Enzyme Inhibitors (ACE-Inhibitors) ............................... 32
1.8. Cell culture ................................................................................................................. 33
1.9. Immunohistochemistry ............................................................................................. 34
1.20. Gel contractions ....................................................................................................... 36
1.21. Working hypothesis ................................................................................................. 38
1.22 Aims of research ...................................................................................................... 38

Chapter II- Materials & Methods
Reagents and materials ..................................................................................................... 40
2.1. Cell culture ................................................................................................................ 43
2.2. Light microscopy and Immunolabelling ..................................................................... 47
2.3. Gel contraction .......................................................................................................... 50
2.4. Statistics .................................................................................................................... 51

Chapter III- Results
Study 1:
3.1. Studies into patient population with capsular contracture disease ......................... 53
Study 2:
3.2. Studies into breast capsule myofibroblasts ............................................................... 70
Study 3:
3.3. Studies into fibroblast cell line .................................................................................. 71
Study 4:
3.4. Studies into breast capsule architecture: immunohistochemistry ............................ 72
Study 5:
3.5. Angiotensin-II Type I receptor studies ..................................................................... 80
Study 6:
3.6. Angiotensin- II Type-2 receptor studies .................................................................... 90
Study 7:
3.7. Alpha-smooth muscle actin studies ................................................................. 100
3.8. Comparison of immunostaining in capsule specimens .................................... 106
3.9. Gel contractions .............................................................................................. 107

Chapter IV - Discussion
4.1. Discussion ...................................................................................................... 109

Chapter V - Conclusion
5.1. Conclusions .................................................................................................. 116
5.2. Scope for future work .................................................................................... 117
References ............................................................................................................. 119
Appendix .................................................................................................................. 132
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACE</td>
<td>Angiotensin Converting Enzyme</td>
</tr>
<tr>
<td>AGT</td>
<td>Angiotensinogen</td>
</tr>
<tr>
<td>Ang-I and II</td>
<td>Angiotensin I and II</td>
</tr>
<tr>
<td>ASPS</td>
<td>American Society of Plastic Surgeons</td>
</tr>
<tr>
<td>AT1</td>
<td>Ang-II type 1 receptor</td>
</tr>
<tr>
<td>AT2</td>
<td>Ang-II type 2 receptor</td>
</tr>
<tr>
<td>BBA</td>
<td>Bilateral Breast Augmentation</td>
</tr>
<tr>
<td>BMI</td>
<td>Body Mass Index</td>
</tr>
<tr>
<td>DIEP</td>
<td>Deep Inferior Epigastric Perforator flap</td>
</tr>
<tr>
<td>IL-1</td>
<td>Interleukin-1</td>
</tr>
<tr>
<td>KGF</td>
<td>Keratinocyte growth factor</td>
</tr>
<tr>
<td>LD</td>
<td>Latissimus Dorsi flap</td>
</tr>
<tr>
<td>MHRA</td>
<td>Medicines and Healthcare products Regulatory Agency</td>
</tr>
<tr>
<td>NICE</td>
<td>National Institute for Health and Clinical Excellence</td>
</tr>
<tr>
<td>PCT</td>
<td>Primary Care Trust</td>
</tr>
<tr>
<td>PF4</td>
<td>Platelet Derived Growth Factor-4</td>
</tr>
<tr>
<td>SIEA</td>
<td>Superficial inferior Epigastric Artery flap</td>
</tr>
<tr>
<td>SMC</td>
<td>Smooth muscle cells</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming Growth Factor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour Necrosis Factor</td>
</tr>
<tr>
<td>TRAM</td>
<td>Transverse Rectus Abdominis Myocutaneous flap</td>
</tr>
<tr>
<td>RAS</td>
<td>Renin-angiotensin system</td>
</tr>
<tr>
<td>REN</td>
<td>Renin</td>
</tr>
<tr>
<td>RER</td>
<td>Rough Endoplasmic Reticulum</td>
</tr>
</tbody>
</table>
Abstract
Abstract

Capsular contracture is both a physically and psychologically debilitating disease characterised by fibrosis and contraction of the breast capsule which can affect women with mammary implants. Incidence is higher in women undergoing breast reconstruction and radiotherapy following breast cancer. There is evidence that Angiotensin II (Ang II) is implicated in pathological wound healing and blockers of Ang II may help in preventing fibrosis. The hypothesis of the study was that angiotensin receptors may be involved in the aetiology and development of capsular contracture disease. Therefore, identifying and blocking these receptors with ACE inhibitors may prevent fibrosis around mammary implants, providing a potential pharmacological treatment of this disease. Capsules from 12 breasts excised from eight patients undergoing surgery for capsular contracture in the Department of Plastic Surgery at Royal Preston Hospital were harvested. Tissue from each patient was dissociated into myofibroblasts using collagenase and cultured to determine growth patterns. Expressions of myofibroblasts and AngII receptors (AT1 and AT2) within breast capsule tissue were also investigated using immunohistochemistry. Staining of paraffin sections in the first four patients was poor and therefore, cryostat sections were also prepared for the second half of the study. An in vitro cell culture model was also designed to endeavour in the measurement of fibrotic contraction of cultured breast capsule fibroblasts to allow possible therapeutic interventions to be investigated.

The results show that myofibroblasts were present in all capsules (n=12). AT1 and AT2 receptors were presented in 50% (n=6) of capsules (paraffin and cryostat sections). However, in the cryostat-only group, staining was positive in 100% (n=5) of capsules. The results also showed that expressions of these receptors were co-localised with α-sma uptake demonstrating the key components of the RAS are present within breast capsule myofibroblasts. A higher expression and wider distribution of AT2 receptors were also noted compared with AT1 receptors. Solidification of 3D gel collagen lattices was suboptimal for consistent measurements of contraction.

In conclusion, the results presented in this thesis have identified the presence of myofibroblasts in breast capsule tissue and have demonstrated that they are amenable to culture successfully. Moreover, these myofibroblasts contain both AT1 and AT2 receptors. Further experiments are required to study the role of blocking AT1 and AT2 receptors excitation-contraction coupling process of the myofibroblasts. This study may provide the basis for a safe and cheap therapeutic strategy with which to modify capsular contracture affecting women with mammary implants.
Chapter I

Introduction
Introduction

1.1 Breast cancer

1.1.1 Epidemiology

Worldwide, more than a million women are diagnosed with breast cancer every year. It is the most common cancer diagnosed in the UK, despite that it is rare in men. In 2009, 48,417 women and 371 men in the UK were diagnosed with breast cancer (Cancer Research UK, 2012).

Female breast cancer incidence is strongly related to age, with the highest incidence rates overall being in older women, supporting a link with hormonal status. Overall, female breast cancer incidence rates have increased for all age groups in Britain over the last 40 years. Nearly half (48%) of female breast cancer cases are diagnosed in the 50-69 age group therefore women aged 50-70 are invited to attend National Breast Screening (see Table 1)

**Table 1.1:** Estimated risk of developing breast cancer by age, females, UK, 2008 (Taken from Cancer Research UK).

<table>
<thead>
<tr>
<th>Estimated risk at birth up to and including:</th>
<th>UK (2008)</th>
</tr>
</thead>
<tbody>
<tr>
<td>age 29</td>
<td>1 in 2,000</td>
</tr>
<tr>
<td>age 39</td>
<td>1 in 215</td>
</tr>
<tr>
<td>age 49</td>
<td>1 in 50</td>
</tr>
<tr>
<td>age 59</td>
<td>1 in 22</td>
</tr>
<tr>
<td>age 69</td>
<td>1 in 13</td>
</tr>
<tr>
<td>Lifetime risk</td>
<td>1 in 8</td>
</tr>
</tbody>
</table>

Risk factors for breast cancer include early menarche, low parity, late menopause, exposure to exogenous hormones, previous breast cancer, family history (one in 450 women carry a BRCA gene mutation), increased Body Mass Index (BMI), alcohol consumption and smoking (Bissel et al., 2001).

Unlike most other cancers, prognosis increases with age, with lower survival rates for younger women diagnosed with breast cancer. It has been suggested that this is because the types of breast cancer diagnosed in pre-menopausal women tend to be more aggressive. The five-year relative survival rates for breast cancer in the UK from 2005-2009 ranged from 84% in the 15-39 year age group to a peak of 90% in those aged 50-69 years, then decreasing gradually to 69% in 80-99 year olds (Cancer Research UK, 2012).
Breast cancer is a complex and heterogeneous disease, comprising multiple tumour entities associated with distinctive histological patterns and different biological features. Invasive ductal carcinoma and invasive lobular carcinoma constitute the majority of breast cancer (Fig 1.1) (Langstein et al, 2003).

Figure 1.1: A photomicrograph of a Grade III invasive ductal carcinoma with prominent and pleomorphic cell nuclei and increased number of cell mitoses (kindly provided by Royal Preston Hospital histopathology department).

1.1.2 Breast cancer treatment

Treatment for breast cancer is guided by the tumour type, grade, stage, size, hormone receptor status and the patients’ general health. Treatments for breast cancer include surgery, radiotherapy, chemotherapy, hormonal therapy and biological treatments. Mastectomy remains an integral part of the treatment for breast cancer. With an understanding of the psychological benefits of breast reconstruction, breast and plastic surgeons have an integral role in the multidisciplinary treatment of women with breast cancer.
Figure 1.2: A photograph showing a scar following right mastectomy for breast cancer (the patient is also wearing a compression garment for lymphoedema secondary to her right axillary node clearance). All images were obtained from the department of medical illustration, Royal Preston Hospitals. All patients had previously given written informed consent for photography and for the use of images in research, teaching and publications.

1.2 Breast reconstruction

Approximately 40% of women with breast cancer will undergo a mastectomy (Fig 1.2) and, according to the National Institute for Health and Clinical Excellence (NICE, 2012), all should be offered the chance of breast reconstruction.

The first published breast reconstruction was performed by Czerny et al (1895) using a lipoma from a patient’s back to fill the defect after removal of a large benign breast mass. Breast reconstruction techniques have significantly advanced since with autologous pedicled flaps and microvascular free-tissue transfer, along with tissue expanders and breast implants, now forming the mainstay of 21st Century breast reconstruction (Wain and Srinivasan, 2011).

Immediate breast reconstruction during surgery for breast cancer is gaining in popularity. This is when breast reconstruction is performed during the same operation as the mastectomy. It is considered oncologically safe and can be performed in patients who require post-operative
radiotherapy (Langstein et al, 2003). Some women find that immediate reconstruction helps them cope with the loss of a breast.

Delayed reconstruction occurs anytime after four months from initial mastectomy. This allows patients to undergo, and recover from, adjuvant radiotherapy and/or chemotherapy prior to breast reconstruction (Wain and Srinivasan, 2011). The overall aims of breast reconstruction are to create a breast mound, to achieve a normal and symmetric silhouette, to limit patient morbidity, and to avoid the need for an external breast prosthesis.

1.2.1 Autologous breast reconstruction techniques

Breast reconstruction with autologous (patient’s own) tissue can involve pedicled and free tissue transfer, with or without an implant (Fig 1.3). Pedicled flaps remain connected to their native arterial and venous supply whilst the tissue is transferred to a new anatomical location whereas, free flaps are completely disconnected from their original blood supply and the vessels are anastomosed using micro-surgical techniques to recipient blood vessels (Saint-Cyr and Schaverien, 2008). Techniques include the ‘Latissimus Dorsi (LD) Flap’, the ‘Transverse Rectus Abdominis Myocutaneous (TRAM) Flap’, the ‘Deep Inferior Epigastric Perforator (DIEP) flap’ (Fig 1.3) and the ‘Superficial Inferior Epigastric Artery (SIEA) flap’ (Janis, 2007). Autologous tissue reconstruction (Fig 1.3) has the advantage of a more natural appearance and texture compared to implant-based reconstructions (Fig 1.4). Autologous reconstruction (Fig 1.3) is particularly beneficial in patients who require post operative radiotherapy, for those who prefer not to have a silicone implant, or in cases where implant reconstruction has been previously unsuccessful (Saint-Cyr and Schaverien, 2008).
Figure 1.3: A photograph to show a patient who has undergone autologous left breast reconstruction, following breast cancer, using a deep inferior epigastric perforator (DIEP) free-flap.

1.2.2 Prosthetic (implant) reconstruction techniques

A survey in 2009 of the American Society of Plastic Surgeons (ASPS) revealed that of 86,424 breast reconstructions performed, 66,075 (76%) were implant based (ASPS, 2009). Prosthetic techniques include fixed volume implants, variable volume expander-implants and tissue expanders (which can be inflated over a number of weeks to increase the size the pocket) and subsequent exchange for implant. Breast implants consist of a filler material, either silicone or saline, inside a silicone elastomer shell. The shell can be either smooth or textured. Breast implants are regulated as medical devices. The Medicines and Healthcare products Regulatory Agency (MHRA) is responsible for ensuring that breast implants meet appropriate standards of safety, quality and performance, and that they comply with the Medical Devices Directive (MHRA, 2012).

When planning implant-based reconstruction, consideration of the amount skin, subcutaneous tissue and muscle, and whether post-operative radiotherapy is required, are important as insufficient soft tissue coverage will give poor cosmetic results. Implant-based breast reconstruction is ideally suited to reconstruct small to moderate breasts with none or mild ptosis. Patients with a normal body-max index and intact pectoralis major muscle are ideal candidates. Prosthetic techniques are also useful for patients undergoing bilateral breast reconstruction and for those patients who are not suitable to undergo autologous tissue reconstruction (Wain and Srinivasan, 2011). The most frequent
complication associated with the reconstruction using a breast prosthesis, especially those filled with silicone, is the onset of fibrous capsular contraction (Fig 1.4).

1.3 Capsular contracture

As part of the physiologic response to a foreign material (antigen), the body also reacts by forming a fibrous capsule around the material (i.e. the breast implant) that is too large to be phagocytised and too inert to produce a toxic reaction (Embrey et al., 1999). Why this biologically normal capsule around a mammary prosthesis contracts in some women resulting becoming hard, tight and painful is yet to be determined (Shiffman, 2009).

Capsular contraction following implantation of breast prostheses occurs in 2–33% of patients undergoing breast augmentation (Singh-Ranger and Mokbel, 2004). Most surgeons quote a figure of 5-20% in pre-operative breast augmentation counselling (Shiffman, 2009). This complication can develop weeks or years after implantation of breast prosthesis however, approximately 60% of capsular contracture occurs within six months and 90% within 12 months postoperatively (Shiffman, 2009). Women who have undergone more complicated procedures, such as mastectomy with breast reconstruction, are reported to have higher rates of contraction than women who have undergone simple breast augmentation alone (Handel et al., 1995).

This complication is a huge problem for breast cancer patients undergoing reconstructive surgery. The condition can be debilitating for patients causing significant pain and distortion of the appearances of the reconstructed breast causing further emotional stress (Fig 1.4).
Figure 1.4: Two photographs showing two patients (A and B) with capsular contracture following breast reconstruction using implants. A: left-side Baker grade IV and right-side grade III capsular contracture. The capsule has contracted distorting the shape of the breast; B: asymmetry and severe deformity of the right reconstructed breast due to grade IV capsular contracture.
1.3.1 Classification of capsular contracture

The severity of capsular contracture is classified by Baker’s Classification as shown in Table 2. There are four grades of breast capsular contracture - Baker grades I through IV. For this system, a soft but visible implant (grade I) and an implant with mild firmness (grade II) are considered good or excellent outcomes. An implant with moderate firmness (grade III) may require treatment if symptomatic and class IV classification, with an excessively firm and symptomatic breast result in a significantly poor aesthetic result (Spear and Baker, 1995).

Table 1.2: Baker’s classification of capsular contracture.

<table>
<thead>
<tr>
<th>Baker Grade</th>
<th>Clinical Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade I</td>
<td>breast is normally soft and looks natural</td>
</tr>
<tr>
<td>Grade II</td>
<td>breast is a little firm but looks normal</td>
</tr>
<tr>
<td>Grade III</td>
<td>breast is firm and looks abnormal</td>
</tr>
<tr>
<td>Grade IV</td>
<td>breast is hard, painful, and looks abnormal</td>
</tr>
</tbody>
</table>

1.3.2 Aetiology of capsular contracture

The aetiology of capsular contraction is unclear. The pathogenesis clearly involves a localised tissue inflammatory response. The fibrous capsule may be a normal response to a foreign body but may be related to factors such as bacterial infection, excessive bleeding at the time of surgery, or ongoing inflammation and fluid accumulation. It is more common in women who undergo immediate breast reconstruction during surgery for breast cancer and tends to be more severe. Capsular contracture is also more likely if patients have radiotherapy before or after reconstruction with a breast implant (Embrey et al., 1999).

This process occurs over many months and years. Lack of knowledge of the cellular mechanisms governing this process makes pharmacological therapy difficult (Kamel et al., 2001). However, many theories have been proposed:
1.3.2.1  **Bacterial infection**

There is increasing evidence for the role of microorganisms in the pathogenesis of capsular contracture (Shiffman, 2009). Bacterial colonization or subclinical infection of the capsule around an implant may stimulate contraction through immune processes. Bacterial contamination has also been shown to stimulate fibroplasia and contraction in wounds (Embrey *et al.*, 1999; Shiffman, 2009).

Several authors have reported positive microbiology cultures, particularly coagulase negative *staphylococcus epidermis*, from breast pockets prior to the insertion of breast prosthesis and also from the majority of fibrous capsules submitted for culture. However, studies have not correlated cultures from capsules with the subjective assessment of clinical firmness (Embrey *et al.*, 1999; Shiffman, 2009). Peri-operative prophylactic antibiotics have been shown to reduce the incidence of implant infection further supporting this hypothesis (Becker *et al*, 1999).

1.3.2.2  **Placement of the implant**

The extensive literature on capsular contracture supports submuscular placement to decrease the incidence of capsular contracture. The exact mechanism is unknown however, the continuous massage provided by the muscle may keep the capsule soft and pliable (Shiffman, 2009). However, it is likely that the layer of muscle over the capsule also masks any capsular contracture as it is more difficult to appreciate. Also, with submuscular placement, there is decreased potential for haematoma formation and contamination of the implant due to decreased vascularity of this plane when compared with the subglandular plane (Becker *et al*, 1999).

1.3.2.3  **Type of implant**

It is thought that a smooth implant surface results in less bacterial adhesion than textured implants, reducing the potential for infection which is known to play a role in capsular contracture (Embrey *et al.*, 1999; Becker *et al*, 1999). Adjustable implants, used to over-expand the pocket followed by volume reduction several weeks later have also been associated with a lower incidence of contracture (Becker *et al*, 1999).

1.3.2.4  **Haematoma**

Haematoma (a collection of blood outside the blood vessels within the tissues) is known to predispose patients to forming a capsular contracture (Embrey *et al.*, 1999). Intra-operative bleeding
should therefore be minimised. Post-operatively compression dressings can be applied to reduce bleeding from small vessels once epinephrine-induced vasoconstriction wears off. Patients should avoid over-activity for at least two-weeks following surgery to prevent haematoma (Becker et al, 1999).

1.3.2.5 Smoking

Smoking is known to impair wound healing and is associated with a higher incidence of post-operative infection. Patients should be advised to stop smoking and refrain from using nicotine patches, for at least four weeks pre and post operatively (Becker et al, 1999).

1.3.3 Histological characteristics of breast capsules

Early basic histological studies of breast capsules suggested that they were simply laminated collagen with limited cellularity largely consisting of fibroblasts, myofibroblasts occasional mononuclear cells and lymphocytes (Baker, 1981). Contemporary analyses of capsules however, demonstrate a more sophisticated histological structure composed of three distinct layers (Shiffman, 2009). The internal layer, adjacent to the prosthesis is either single or multi-layered and consists of macrophages and fibroblasts. The middle layer is formed from loosely arranged connective tissue containing an internal vascular supply whilst the third layer is composed of dense connective tissue with an external vascular supply. In 60% of patients, an inner layer of synovial-like metaplasia consisting of mononuclear macrophages and multinucleated giant cells is also described. Chronic inflammation is present in 90% of capsules (Granchi et al., 1995; Shiffman, 2009).

Large amounts of silicone are associated with increased local inflammation (Prantl et al, 20007). Silicone is present in most capsules, usually confirmed by vacuolated macrophages containing refractory material. It has been identified in the capsules surrounding both silicone and saline-filled implants. The origin is thought to be frictional from shearing forces on the silicone elastomer implant envelope. It has been shown that the thickness of the capsule is positively correlated with increasing levels of free silicone (Granchi et al., 1995; Shiffman, 2009).

Prantl et al (2007) investigated the histological changes in capsules formed around silicone breast implants and their correlation with the clinical classification of capsular contracture defined by the Baker score. They found a positive correlation between capsule thickness and Baker score and also showed a trend toward greater capsular thickness in patients with a more severe inflammatory reaction.
1.3.4 Current treatment of capsular contracture

Once established, capsular contracture is difficult to treat. Treatment of capsular contracture usually requires surgical removal (Fig 1.5) or release of the scar around the implant and often replacement of the implant itself. The rate of recurrence of capsular contracture after surgery is high (Shestak, 2006). Treatment is often life-long and can involve multiple operations.

Conservative treatment modalities include breast compression exercises which may prevent contracture progression and help to keep the capsule soft and pliable. Breast massage, in particular quadrant massage, also helps to maintain a softer capsule and ultrasound from an external source has also been used, with little effect, to soften and reduce contractures (Shestak, 2006).

Surgical options for treatment of capsular contracture include capsulectomy and capsulotomy. ‘Ootomy’ means to release scar tissue in Latin. During ‘capsulotomy’ the breast capsule is simply opened with an electrocautery device to release the implant from contracture and allow for expansion. In capsulectomy, the entire constricting capsule is excised removed during the procedure (Fig 1.5). This is the most effective method of relieving all symptoms and preventing a recurrence (Broughton et al., 2006). Capsulotomy is only appropriate in some circumstances, such as for patients with minimal breast tissue in who complete excision of the capsule may result in visibility of the implant itself. However, this technique is generally less effective than full excision of the capsule. Closed capsulotomy uses manual pressure to break the contracture. This is a painful and dangerous procedure which is no longer advised.
1.4.1 The Process of Wound Healing

There are three phases of wound healing. Although these phases overlap they are biologically distinct. The initial inflammatory phase consists of haemostasis with formation of a clot, removal of devitalised tissue and prevention of infection. This is followed by the second proliferative phase during which the provisional wound matrix is formed. During the last phase ‘remodelling’ scar tissue matures and wound strength is maximised (Diegelmann, 2004).

1.4.1.1 Haemostasis and Inflammatory Phase (24-48hrs)

The first phase lasts from the first 24 to 48 hours following wounding. Tissue injury causes endothelial disruption and release of proteolytic enzymes. Haemorrhage ensues and a platelet plug adheres to exposed type II collagen following clotting cascades (Witte, 1997). Growth factors within the platelet plug (PDGF, TGF-\(\alpha,\beta\)) and inflammatory cytokines (fibrinogen, fibronectin, vWF) increase cell proliferation and migration to the area. Initial vasoconstriction is followed by vasodilation due to inflammatory mediators histamine, kinins and complement. This results in
vessel engorgement and plasma exudation into the damaged area. PDGF, secreted by platelets during thrombus formation, acts as a chemo-attractant for macrophages, which remove bacteria and debris from the wound. Macrophages also attract fibroblasts, which migrate into the wound by forming cell-matrix contacts with matrix proteins (fibronectin, vitronectin, and fibrinogen). This migration of fibroblasts signals the start of the proliferative phase. Growth factors and proteins stimulate fibroblasts to synthesise collagen and extracellular matrix components (ECM) (Witte, 1997; Diegelmann, 2004; Li, 2007). Within 12 hours following wounding, neutrophils and monocytes migrate into the wound. Initially neutrophils adhere to the vascular endothelial cell wall releasing elastase and collagenase, allowing migration through the basement membrane into the wound matrix where they kill bacteria by phagocytosis. This neutrophil response declines after a few days and monocytes are attracted by soluble mediators and bind the extracellular matrix via integrin receptors. Here, they differentiate into macrophages which function to phagocytose bacteria, devitalised tissue and depleted neutrophils; produce collagenase and elastase, facilitating proteolytic destruction of devitalised tissue and; release soluble mediators (TNF-α, TGF-β, PDGFs, IL-1, IL-6) which recruit and activate fibroblasts and promote angiogenesis (Diegelmann, 2004; Li, 2007).

### 1.4.1.2 Proliferative Phase: (Day 3 up to 4 weeks)

During the second stage of wound healing the provisional wound matrix is remodelled and replaced by scar tissue. This consists of collagen, proteoglycans and elastin fibres. During this phase, epithelial cells, fibroblasts and vascular endothelial cells migrate to the wound site and proliferate. Fibroblasts also actively produce type III collagen. The early collagen is disorganised and wound tensile strength is poor. Fibroblasts also lay down extracellular matrix to strengthen the wound, increasing its tensile strength. The process of angiogenesis occurs concurrently with fibroblast proliferation and granulation tissue, consisting of capillary loops and surrounding collagen fibres, is formed (Diegelmann, 2004; Li, 2007). The formation of granulation tissue into an open wound allows re-epithelialisation phase to take place. Epithelial cells migrate across the new tissue to form a barrier between the wound and the environment. Basal keratinocytes from the wound edges and dermal appendages such as hair follicles, sweat glands and sebaceous glands are the main cells responsible for the epithelialisation phase of wound healing. (Diegelmann, 2004; Li, 2007).

### 1.4.1.3 Remodeling Phase (21 days- 1 year)

The final, and longest, phase of wound healing starts from day 21 up to one year. It commences once the wound defect has been replaced by granulation tissue and re-epithelialisation has been
completed by migrating keratinocytes. The precise mechanisms by which this takes place are not fully understood however, it is proposed that during this phase collagen remodelling, wound contraction and, a progressive decrease in scar vascularity occur (Witte 1997). During collagen remodelling, type III collagen is slowly degraded and replaced by type I collagen (normal dermis contains mainly type I collagen). Collagen synthesis peaks at five to seven weeks, although increased synthesis and degradation continue for up to one year. There is also increase collagen cross-linking subsequently increasing the tensile strength of the wound. Wound contraction is produced by myofibroblasts (fibroblasts with actin filaments capable of matrix contraction).

1.4.2. Wound contraction

Following surgical injury, such as breast reconstruction, new connective tissue is synthesized and mesenchymal fibroblasts become activated. (Gabbiani, 2003). These wound fibroblasts, located at the site of injury, synthesise a skeleton matrix of collagen type III, glycosaminoglycans and fibronectin which are responsible for wound contraction.

Wound contraction, a process by which wound edges move together to minimise dead space, begins four to five days post wounding and continues for approximately 12-15 days at an average rate of 0.6-0.75 mm per day (Tejero-Trujeque, 2001). The healing of a wound is dependent on two key phenomena: i) epithelialisation; and ii) formation of granulation tissue (Gabbiani, 2003). Wound healing ends with scar formation as a result of permanent deposition of connective tissue. This is characterised by the presence of extracellular matrix components among which collagen is the most important, fibroblasts and small vessels (Gabbiani, 2003).

At the end of normal wound healing process, when epithelialisation has been achieved, granulation tissue disappears and the contractile and synthesizing activity of myofibroblasts is usually terminated. The cell number is dramatically reduced by apoptosis. (Desmouliere et al., 1995).

1.4.2.1 Pathological wound contraction

Wound contraction permits wound closure however, excessive contraction can produce functional and aesthetic problems. It is generally considered that fibrotic disease processes occur due to failure of the normal wound-healing response (Zinmann et al, 2007). In chronic pathological contracture diseases, the process of massive apoptosis is absent and the contractile and synthesising activity continues leading to soft tissue deformation (Gabbiani, 2003). In these pathological scarring processes, the excessive extracellular matrix deposition may continue for several years. (Gabbiani, 2003). These pathological phenomena can lead to life threatening fibrosis when affecting organs such as the heart lungs and kidneys.
1.5 The Myofibroblast

Gabbiani et al (1971) first described a certain type of fibroblast, the myofibroblasts, as the cell responsible for contraction of granulation tissue exhibiting an important cytoplasmic microfilamentous apparatus (Gabbiani, 2003). They defined the myofibroblast as a cell with the intermediate morphological characteristics of a fibroblast and smooth muscle cell as they contain smooth muscle actin (α-SMA) (Desmoulière et al., 2005).

The myofibroblast has been identified both in normal tissues, particularly in locations where there is a necessity of mechanical force development, and in pathological tissues, in relation with hypertrophic scarring, fibromatoses and fibro-contractive diseases (Klingberg et al., 1988).

The myofibroblast capacity for generating a contractile force is well documented. This contractile force generated by the myofibroblast during wound healing is beneficial for tissue remodelling however, it becomes detrimental when it becomes excessive such as in hypertrophic scarring, fibrotic disease and capsular contracture (Hinz, 2007).

1.5.1 Features of the myofibroblast

Three distinct structural components distinguish the activated myofibroblast from the inactive fibroblast.

- Bundles of contractile microfilaments
- Extensive cell-to-matrix attachment sites
- Intracellular adherans and gap junctions (Eyden, 2005; Hinz, 2007)

Fibroblast-to-myofibroblast differentiation represents a key stage during wound healing after injury and tissue repair (Gabbiani, 1992). This differentiation of fibroblasts into myofibroblasts is described as a two stage process:

1. Fibroblasts acquire a migratory phenotype by de novo and develop contractile bundles to repopulate damaged tissues. This phenotypic change occurs in response to changes in the composition, organisation and mechanical properties of the extracellular matrix and to cytokines released by inflammatory cells (Hinz, 2007). This ‘activated myofibroblast’ has previously been described as a ‘proto-myofibroblast’ (Tomasek et al., 2002).

2. The ‘proto-myofibroblasts’ develop into ‘differentiated myofibroblasts’ by expressing α-smooth muscle actin (Hinz, 2007). This expression is regulated by the action of growth factors including transforming growth factor (TGFβ1), ECM proteins and by the mechanical micro-environment (Tomasek et al., 2002). The contractile activity of fibroblasts is amplified as α-SMA is incorporated into stress fibres and this characterises the contractile
phase of tissue remodelling (Hinz et al., 2001). The contraction action of myofibroblasts during remodelling ceases when the tissue is repaired. The expression of α-SMA terminates and myofibroblasts are removed by apoptosis (Desmouliere et al., 1995).

Figure 1.6: Schematic diagram showing myofibroblast regulation (Taken from James et al., 2002).

1.5.2 Myofibroblast regulation

Two key factors have been implicated in the regulation of the myofibroblast: Transforming Growth Factor (TGF-1) and mechanical tension on a wound. Injection of TGF-1 in a rat wound healing model lead to formation of granulation tissue containing activated myofibroblasts (Katwa et al., 1998). Fibroblasts cultured under tension differentiate into myofibroblasts which produce smooth muscle actin bundles. However, when cultured in a relaxed state these actin bundles are absent which suggests wound tension has a role in regulating myofibroblast population (Hinz et al., 2001). After wound healing and re-epithelialisation occurs, there is mass myofibroblast apoptosis (Hinz, 2007). The exact mechanism for myofibroblast suicide is unclear although the mechanisms of cell release from stress and increased formation of specific cell-cell contacts have been advocated.
In animal models, myofibroblast apoptosis has been demonstrated to be inhibited by preventing the tension release of granulation tissue by splinting the wound. Myofibroblast apoptosis has also been induced by releasing fibroblast-attached collagen gels in vitro (Hinz, 2007).

1.5.3 Myofibroblast markers

Alpha-smooth muscle actin is the most widely recognised myofibroblast marker. However, its limitations are that it fails to differentiate between vascular smooth muscle cells (SMC) (elongated contractile cells that form the smooth muscles of the body) and myofibroblasts when the population is mixed (Hinz, 2007). Ronty et al (2006) proposed an innovative marker for myofibroblast differentiation: the 41 g isoform of the stress fibre protein paladin. However, this has also shown to be expressed by SMC therefore a cytoskeletal protein which can discriminate between SMC and myofibroblasts remains to be determined (Hinz, 2007). The myofibroblast also expresses specific proteins on its surface. These have importance for the targeting of specific drugs (Hinz, 2007). For example, the plasma membrane protein Thy-1 is present on the membrane of fibroblasts but absent in myofibroblasts. More recently, Hinz and Gabbiani (2003) suggested a novel approach to identify myofibroblasts by their expression of specific cadherins (transmembrane cell-cell adhesion proteins linked intracellularly to the actin cytoskeleton) (Hinz, 2007). These myofibroblast-specific proteins provide potential targets to modulate myofibroblast contractility to target fibrosis.

1.5.4 Role of the myofibroblast in capsular contracture

Several authors have investigated the significance of the myofibroblast in capsular contracture. Gabbiani et al (1971) described the presence of contractile myofibroblasts in granulation tissue and associated it with contraction processes. Zimman et al (2002), using electron microscopy, investigated the fibrous envelope around mammary implants and found the contractile myofibroblasts within collagen fibre inside the fibroblast cytoplasm (collagen fibre is produced normally in the extracellular space). Baker et al (1981) identified myofibroblasts in several breast capsules. They hypothesized that myofibroblasts execute contraction of the developing breast capsule by means of a ratchet-like mechanism composed of collagen and cross-linking. They also suggested that the contractile site is maintained by the adherent properties of the Extracellular Matrix (ECM), which provides the structural component of cells, in which a network of collagen fibres is imbedded. Hwang et al (2010) showed that the tensile strength of the breast capsule correlated with the degree of capsular contracture. They proposed that during capsule formation, myofibroblasts appear during an active phase of wound contraction and diminish when the wound
becomes matured. These data support the hypothesis that the myofibroblasts are a probable cause of capsular contracture.

1.5.5 The Extracellular Matrix (ECM)

The extracellular matrix (ECM) is the largest component of normal skin. It gives skin properties such as elasticity, compressibility and tensile strength. In acute wounds the provisional wound matrix, containing fibrin and fibronectin, acts as a scaffolding to direct cells to the site of injury as well as to stimulate cell differentiation and the production of new ECM. The synthesis of ECM is a key feature of wound healing (Frantz et al, 2010).

The constituents of ECM include polysaccharides, water, collagen and proteins. The two main classes of ECM molecules are:

1. Fibrous structural proteins, which give the ECM strength (collagens, elastin, laminin)
2. Proteoglycans, which consist of multiple glycosaminoglycan chains that branch from a linear protein core. These are large, highly hydrated molecules that help cushion cells in the ECM. (Schultz et al., 2005)

Enhanced ECM synthesizing activity significantly contributes towards tissue remodelling (Hinz, 2007). Myofibroblasts produce several components of the ECM such as collagen types I, III, IV and V (Schurch et al., 2006). Capsular contraction is characterized by excessive scarring resulting from excessive production and deposition of extracellular matrix (Zinmann et al., 2007).

1.6 The Renin-Angiotensin System (RAS)

Renin (REN) was first discovered as a pressor substance in 1898 (Lavoie and Sigmund, 2003). Angiotensin-II (AngII) is produced by an enzymatic cascade. Angiotensinogen (AGTN) is cleaved by REN, an aspartyl protease to form the decapeptide angiotensin I (Ang-I). Ang-I is then further cleaved by angiotensin-converting enzyme (ACE), a dipeptidyl carboxypeptidase, to produce Angiotensin II (AngII) the active biological octapeptide of the systemic renin-angiotensin system (Hikaru et al., 2004; Lavoie and Sigmund, 2003). This circulating humoral system is responsible for blood pressure regulation and salt-water homeostasis. Further degradation (or processing) by aminopeptidase A and N produce angiotensin III (Ang 2–8), and angiotensin IV (Ang 3–8) respectively (Lavoie and Sigmund, 2003).
The role of Angiotensin II (AngII), the physiologically active component and end-product of the RAS, in the control of systemic blood pressure and volume homeostasis is well described (Lavoie and Sigmund, 2003). The properties of Angiotensin II (Ang-II) include vasoconstriction, regulation of renal sodium and water absorption, and increasing thirst (Lavoie and Sigmund, 2003). These effects of AngII on blood pressure regulation were first thought to be mediated primarily through blood-borne angiotensin with actions in target tissue, known as the classical endocrine pathway. However, more recently, it has become appreciated that a local autocrine or paracrine RAS may also exist within tissues and that these may also play a significant role in blood pressure homeostasis (Lavoie and Sigmund, 2003).

Two major AngII receptors, namely Ang-II type I (AT1) and Ang-II type (AT2) have been identified (Timmermans et al, 1993). Ang II causes vasoconstriction both directly and indirectly by stimulating (AT1) receptors present on the vasculature and by increasing sympathetic tone and arginine vasopressin release (Lavoie and Sigmund, 2003). Ang-II also maintains blood pressure homeostasis by regulating renal sodium and water reabsorption directly by stimulation of AT-1 receptors in the kidney, and also indirectly by stimulation of the production and secretion of

---

**Figure 1.7:** Flow diagram demonstrating the REN-angiotensin cascade (Taken from Lavoie and Sigmund, 2003).
aldosterone from the adrenal glands and by activating the thirst centre in the central nervous system (CNS).

1.6.1 The ‘Classical’ role of Angiotensin

Ang II has a well described role in remodelling and repair in various pathophysiological situations. Angiotensin II (Ang II) plays a pivotal role in cardiac fibrosis (Yamagishi et al, 1996). In the myocardium, AngII causes left ventricular hypertrophy following myocardial infarction and in cardiomyopathy and arterial hypertension (Zhu et al, 2003). AngII also plays a key role in the progression of kidney diseases, contributing to renal fibrosis by up-regulation of pro-fibrotic factors, induction of epithelial mesenchymal transition and accumulation of extracellular matrix proteins (Lavoz et al, 2012). A large body of evidence also demonstrates that Ang-II and angiotensin receptors are required for the pathogenesis of experimental lung, liver and pancreatic fibrosis (Yamada et al, 2003).

1.6.2 Angiotensin in wound healing

For many decades, the renin–angiotensin system has been investigated solely with regard to its cardiovascular and renal homeostatic actions (Campbell, 1987; Carey, 2000; Ferder, 2003; Atlas, 2007) . In recent years however, the conventional role of AngII has been challenged with evidence to suggest that this active octapeptide hormone of the RAS, is not only manufactured locally within tissues but also plays a key role in tissue repair, regulation of the ECM and production of fibrous tissue (Katwa et al., 1998; Mckirdy et al., 2001).

Tissue RASs have been shown to be capable of the local generation of Ang II, which makes them independent of plasma-borne AngII and facilitates locally restricted effects (Steckelings et al, 2005). This tissue RAS, which acts independently of the circulating renin angiotensin system, has been demonstrated in models of cardiac, hepatic and renal fibrosis (Lavoie and Sigmund, 2003). However, compared to the cardiac models, the role of AngII in skin and subcutaneous tissue is far less documented. AngII stimulation has been shown to regulate proliferation of skin fibroblasts and production of extracellular matrix; two key processes in wound healing. AngII has been proposed to be involved in the stages of cutaneous wound healing including increasing vascular permeability, recruiting inflammatory cells, cell proliferation and migration, neovascularisation and fibrosis (Clark, 1996, Steckelings et al, 2005).

Evidence has also shown that AngII has fibrotic properties. It stimulates fibroblasts to synthesize the extracellular matrix components collagen I, fibronectin, laminin and elastin (Zhu et al, 2003; Sun and Weber, 1996; Sun et al., 1997). AngII has also been demonstrated to have a role in the
regulation of endothelial cell proliferation and differentiation and new vessel formation during neovascularisation (Walsh et al, 1997).

This duality of the RAS, working simultaneously in both local tissue and endocrine systems, has made the system extremely complex. It’s precise action and functions are yet to be determined. In the advent of new technologies, we will undoubtedly continue to learn more about how this complex system works in vivo.

1.6.3 **Angiotensin receptors**

Two distinct AngII receptor subtypes have been cloned: AT1 and AT2 (Kaschina et al., 2003; Steckelings et al., 2005). In binding studies and physiological experiments, these receptors can be distinguished using specific antagonists. Both AT1 and AT2 are cell surface receptors belonging to the large family of G-protein coupled seven-transmembrane receptors (Kaschina et al., 2003; DeGasparo et al., 2000). However, the pathways used are very different and the AT1 and AT2 receptors signal in apparent opposition. AT1 receptors mediate many of the well-known systemic stimulatory and physiological actions of Ang II including secretion of aldosterone, vasoconstriction, and renal sodium reabsorption responses whereas AT2 receptors are thought to mediate vasodilator responses (Lavoie and Sigmund, 2003).

AT1 and AT2 receptors also have a wide tissue-specific distribution. They are both present in the kidney, brain, and adrenal gland. In general, AT1 receptors are present in adult cardiovascular tissues, whereas AT2 is known to be highly expressed during foetal development (Kaschina et al., 2003; Steckelings et al., 2005).

In animal models, AngII receptor antagonism results in a reduction in the inflammatory response and fibrosis in these organs and tissues. This has been demonstrated in cardiac infarction, liver fibrosis and interstitial nephritis. In humans, there is also reduced fibrosis post myocardial infarction and diabetic renal disease following ACE-inhibitor therapy which is now used routinely to reduce the amount of collagen deposition.

Steckelings et al (2005) demonstrated an increased Ang II receptor expression in human cutaneous wounds using three different experimental approaches supporting the theory that angiotensin receptor expression is increased in other tissues undergoing repair and remodelling.

1.6.4 **Angiotensin Type 1 (AT1) receptors**

Pharmacological studies using specific antagonists have determined that most of the physiological actions of Ang-II are mediated by the AT1 receptor (such as vasoconstriction, cellular growth,
production of the extracellular matrix) (Timmermans et al., 1993). This receptor is also involved in the recruitment of inflammatory cells, angiogenesis, cell proliferation and extracellular matrix synthesis (Kaschina et al., 2003).

McKirdy et al (2001) identified the presence of AT1 receptors using immunohistochemistry in Dupuytren’s disease. They found that these receptors were co-localised with areas of myofibroblast expression, which has implications for the potential of pharmacological regulation using ACE-inhibitors to modulate contracture disease processes. Also, in AngII type 1-receptor (ATR1 knock-out-mice) wound healing was markedly delayed (Yahata et al, 2006).

1.6.5 Angiotensin type II (AT2) receptors

AT1 receptor function is well described in the literature. However, AT2 receptor function has not yet been fully determined. Studies have suggested that the AT2 receptor function may oppose the actions of the AT1 receptor with respect to blood pressure and cellular proliferation (Carey et al., 2000). It has also been suggested that AT2 receptor stimulation decreases renal tubular sodium reabsorption (Lo et al., 1995)

The actions and signal transduction mechanisms initiated by AT2 receptor stimulation are proposed to be unconventional and quite distinct from those described for AT1 receptor-coupled events. The ‘ying-yang hypothesis’ suggests that AT2 receptor-mediated actions counteract those of AT1 receptor-coupled actions. These opposing actions of AngII receptors predispose the RAS to take part in regulatory mechanisms (Steckelings et al., 2005). This phenomenon can be seen in the regulation of cell proliferation by AngII and also collagen synthesis which are both stimulation via the AT1 receptor and inhibited via the AT2 receptor (Steckelings et al., 2005)

1.6.6 AngII receptors in wound healing

In tissue repair and remodelling, changes in angiotensin receptor distribution have been described observed in a variety of organs (Nio et al., 1995; Busche et al., 2000). During wound healing, migration of keratinocytes and dermal myofibroblasts are stimulated by Ang II, mediated by the AT1 receptor. The AT2 receptor appears to have a counteracting effect on cell migration (Takeda et al., 2004). Previous studies have shown that skin wound healing is regulated by the balance of opposing signals between AT1 and AT2. Ang II-induced stimulation of collagen production was mediated via the AT1 receptor, whilst the AT2 receptor had an inhibitory effect on basal collagen synthesis in skin fibroblasts from AT1a receptor knockout mice (Min et al., 2004; Steckelings et al., 2005). It is thought that over-induction of AT1 signalling may contribute towards pathological fibrotic responses such as scarring.
Interestingly, the AT2 receptor, which is sparsely expressed in the vast majority of human tissues, is markedly upregulated during wound healing (Steckelings et al., 2005). In pathological states however, AT1 saturation with excess Ang II may lead to increased AT2 signalling which may contribute towards pathological fibrotic responses such as scarring.

1.7 Angiotensin-Converting Enzyme (ACE) Inhibitors

ACE is present in tissues composed largely of fibrillar collagen such as coronary arteries, adventitia of great vessels and intramyocardial coronary arteries as well as the scar that follows myocardial infarction (Katwa et al., 1995).

ACE inhibitors are hypotensive drugs that inhibit the converting enzyme peptidyl dipeptidase, which hydrolyses angiotensin I to angiotensin II and inhibits the degradation of a potent vasodilator, bradykinin, a mechanism which at least in part, is prostaglandin mediated (Ianello, 2006). There are several ACE inhibitors in clinical use at present. Captopril is a sulfhydryl-containing agent metabolised primarily to disulfide conjugates with other sulfhydryl-containing molecules, and is distributed to most body tissues and excreted chiefly by the kidneys. It has an inhibitory action on the renin-angiotensin system (Katwa et al., 1995; Ianello, 2006). Enalapril is a prodrug that is hydrolyzed in the body and converted by de-esterification to ACE inhibitor enalaprilat; it is a non-sulfhydryl-containing agent. Lisinopril is a lysine derivative of enalaprilat, the active metabolite of enalapril. Fosinopril and ramipril, next introduced, are slowly absorbed. On the other hand, Losartan is a nonpeptide angiotensin II antagonist (Katwa et al., 1995; Ianello, 2006).

Several reports have described the effects of ACE-inhibitors on myocardial infarction. Treatment with ACE inhibitors is known to cause reduction of left ventricular collagen content and to attenuate remodelling post myocardial infarct (consequently improving left ventricular function) (Ianello, 2006).

1.7.1 Rationale for testing the effects of ACE-inhibitors on capsular contracture

Angiotensin clearly has a role in organ fibrosis and in wound healing and there is significant evidence of the anti-fibrotic effect of ACE-Inhibitors. Previous laboratory work has consistently demonstrated a diminished fibrotic process in organs of animal models treated with ACE-Inhibitors or angiotensin II receptor antagonists (Yamagishi et al., 1996; Katwa et al., 1997; Toblli et al., 1999, 2004).

ACE-Inhibitors such as enalapril are commonly used in renal and cardiovascular diseases and prevent the expression of fibrotic mediators. Such pharmacological therapy with ACE-Inhibitors has already been demonstrated to reduce capsular contraction on mammary implants in rat models.
Zinmann et al (2007), using a rat model, demonstrated that Enalapril lowered the expression of fibrotic mediators around mammary implants significantly reducing the inflammatory process and fibrosis. Administration of AngII to skin wound models in rats also resulted in a reduction in healing times (McKirdy et al., 2003). Low dose ACE-inhibitors therapy has also been shown to improve post-operative cutaneous scarring in humans following abdominal surgery (Ianello, 2006). The present study aims to expand the findings of Zimman et al (2007) and McKirdy et al (2003) to establish whether the anti-inflammatory and anti-fibrotic ability of the renin-angiotensin system blockade, by an ACE-Inhibitor, can modulate the fibrotic process around mammary implants. This hypothesis may provide the basis for a safe and cheap therapeutic strategy with which to modify the capsular contracture that can affects women with mammary implants.

1.8 Cell culture

Cell culture, the technique to grow and maintain cells outside an organism or body, is practised extensively (Langdon, 2004). This technique is utilised for examination and manipulation of cell behaviour and to investigate fundamental processes of growth and development in both normal and abnormal including cell structure biochemical, genetic and reproductive activity; metabolism, functions, and reactions to physical, chemical, and biological agents (such as drugs and viruses).

There are two main types of cell culture:

- Primary: derived from a living organism.
- Cell lines: stable established cells which proliferate in culture.

Both types of culture employ similar practical sterile techniques and in order to grow cells in culture, a variety of conditions must be met. A sample of the tissue is spread on or in a culture medium of biological (e.g., blood serum or tissue extract), synthetic, or mixed origin. The culture medium must have the appropriate nutrients, temperature, and pH specific to the cells being incubated. The results are observed with a microscope, sometimes after treatment (e.g., staining) to highlight particular features (Langdon, 2004). Research employing tissue culture techniques have helped to identify infections, enzyme deficiencies, and chromosomal abnormalities, classify brain tumours and formulate and test drugs and vaccines. The present study employed this tissue culture technique to grow myofibroblasts from breast capsules.
1.9 Immunohistochemistry

Immunohistochemistry is the localisation of antigens or proteins in tissue sections by the use of labelled antibodies as specific reagents through antigen-antibody interactions that are visualized by a marker such as fluorescent dye, enzyme, or colloidal gold.

1.9.1 Antibodies

The pivotal reagent common to all immunohistochemical techniques is the antibody (Fig 1.8) (Kumar and Rudbeck, 1999). Antibodies belong to a group of proteins called immunoglobulins (Ig) that are present in the blood of immunised animals (Kumar and Rudbeck, 1999). The immunoglobulins comprise five major classes (in order of decreasing quantity found in plasma): IgG, IgA, IgM, IgD and IgE. Each is composed of two identical heavy chains and two identical light chains (Fig 1.8). IgG and IgM are the most utilised immunoglobulins for immunohistochemistry (Kumar and Rudbeck, 1999).
Fig 1.8. Schematic diagram showing the structure of an immunoglobulin molecule. It comprises two identical light chains and two identical heavy chains held together by disulphide bonds (Taken from www.cas.vanderbilt.edu bsci111b/immunology/supplemental.htm).

Polyclonal antibodies are a heterogeneous mixture of antibodies produced by different cells. They react with various epitopes of the antigen against which they are raised. The most frequently used animal for the production of polyclonal antibodies is the rabbit however they are also made in other mammals such as goats, pigs, guinea pigs and cows. The popularity of rabbits for this purpose is attributed to their ease of maintenance and relative rarity of human antibodies to rabbit proteins when compared to other mammals, such as goats (Boenisch, 1999; (Kumar and Rudbeck, 1999).

Monoclonal antibodies are a homogeneous population of immunoglobulin as they are the product of an individual clone of plasma cells. Antibodies from a given clone are immunochemically identical and react with a specific epitope on the antigen against which they are raised (Kumar and Rudbeck, 1999). Mice are the most commonly used animals for the production of monoclonal antibodies but may be produced in other species such as rat and camel (Harboe, 1983).
1.9.2 Direct Method

Direct method is a single-step staining method. It utilizes a labelled antibody which directly reacts with the antigen in tissue sections. The advantage of this single-step method is that it is more time efficient however; it is limited by poor signal amplification with weak sensitivity and is rarely used since the introduction of the indirect method.

1.9.3 Indirect Method:

Indirect method is a two-stage process which involves an unlabelled primary antibody (first layer), which reacts with the tissue antigen, and a labelled secondary antibody (second layer) which then subsequently reacts with primary antibody. In order to do so, the secondary antibody must be against the IgG of the animal species in which the primary antibody was raised. This method is now preferred as it has a higher sensitivity due to signal amplification through several secondary antibody reactions with multiple antigenic sites on the primary antibody. Indirect immunofluorescence method is similar to method described however in addition; the second layer antibody is labeled with a fluorescent dye such as FITC, rhodamine or Texas red (Buchwalow and Bocker, 2010). The present study used immunohistochemistry to identify the key receptors of the RAS, AT1 and AT2, in human breast capsule tissue.

1.20 Gel Contractions

Wound healing comprises of three processes: epithelialisation, connective tissue deposition, and contraction (Diegelmann, 2004). Tissue contraction is a dynamic event, believed to be mediated by myofibroblasts. There are several different culture models to study the ability of fibroblasts to reorganize and contract collagen matrices in vitro. Contraction models are produced from a polymerised collagen matrix containing cells. Mechanical tension develops during contraction, and cellular stress fibres assemble. This model allows the three dimensional cell matrix interactions and influence of specific agonists on the rate and extent of contraction to be investigated (Colgen, 2006). In the floating contraction model, a freshly polymerized collagen matrix containing cells is released from the culture dish and allowed to float in culture medium, and contraction occurs in the absence of external mechanical load and without appearance of stress fibers in the cells. In the attached model, a polymerized collagen matrix containing cells remains attached to the culture dish during myofibroblast contraction. Mechanical tension then develops during contraction and cellular stress fibers assemble. Lastly, the two-step model of gel contraction combines an initial period of attached matrix contraction leading to mechanical loading, followed by release of the matrices,
resulting in mechanical unloading and further contraction as mechanical stress dissipates (Cell Biolabs, 2004).

Three-dimensional collagen gels have been widely used in myofibroblast contraction studies and have also been used in the studies of integrin signalling, cell apoptosis and cytoskeleton reorganization. The collagen gel lattice contraction assay provides a model that takes advantage of the finding that cell-populated collagen-hydrogels contract over a period of time in a consistent manner (Colgen, 2006). The present study employed collagen gel contraction method to attempt to study the contractile behaviour of myofibroblasts from human breast capsule.
1.21 Working hypothesis

Angiotensin receptors may be involved in the aetiology and development of capsular contracture disease. Identifying and blocking these receptors with ACE inhibitors may prevent fibrosis around mammary implants, providing a potential pharmacological treatment of this disease.

1.22 Aims of Research

Due to lack of treatment for this physically and psychologically debilitating complication which is common in women having breast reconstructive surgery, this project was designed to investigate the potential for pharmacological modulation of this disease.

It has been proven in rodent models that:
- There are increased levels of AngII or ACE during wound healing;
- There is up-regulation of AT1 and AT2 receptors in wounded skin and;
- Topical administration of AngII accelerates skin wound healing and an increased level of AngII or ACE. (Liu et al., 2006).

Therefore, the ‘Specific Aims’ of the study were:
- To measure myofibroblast activity in vitro using tissue excised from surgery for capsular contracture disease.
- To test the hypothesis that there is a local Renin-Angiotensin System in breast capsule around mammary implants.
- To determine if AT1 or AT2 receptors are expressed in the fibrotic capsule around mammary implants.
- The study also focuses whether the anti-inflammatory and anti-fibrotic ability of the Renin-Angiotensin System blockade by an Angiotensin-Converting Enzyme (ACE) Inhibitor can modulate the fibrotic process around mammary implant tissue.
- The study may provide the basis for a safe and cheap therapeutic strategy with which to modify capsular contracture affecting women with mammary implants.
Chapter II

Materials and Methods
2.1. Reagents and Materials

Patients:
Twelve patients from Royal Preston Hospital

Tissues:
Isolated breast capsules from all 12 patients

Reagents:
Trypsin
Collagenase Solution (2000 digestion units / ml):
- 500 mg Collagenase Type 1A
- 31.25 ml Hanks Balanced Salt Solution (HBSS)

Antibodies:
- Anti-AT1 receptor antibody produced in rabbit (AT1 (TONI-1): sc-67036 (Santa Cruz Biotechnology, INC, CA, USA)
- Anti-AT2 receptor antibody produced in rabbit (AT2 (Anti-AGTR2: 53930904/ 1 in 4000 dilution/ Sigma Aldrich, UK)
- Anti-Actin, α-Smooth Muscle antibody produced in mouse (αSMA (M0851/ 1 in 50 dilution/ Dako, Cambridgeshire, UK)

Cell Culture Media:
DMEM, foetal calf serum, Ham’s F10

Chemicals:
Analar grade chemicals
Dimethyl Sulfoxide (DMSO)

Fixation and Immunohistochemistry:
Tris Buffered Saline (TBS):
- 500 ml of distilled water
- 6.08 g Trizma Salt
- 4.6 g Sodium Chloride
- 0.5 g Bovine Serum Albumin
• Mixed well on a magnetic stirrer. PH of solution was corrected to approximately 7.4 using 5M HCl

Vectastain® Universal Elite ABC Kit

Normal blocking serum:
• 1 drop (50 µl) of normal blocking serum stock added to 5 ml of Tris buffer pH 7.4 in mixing bottle.

Biotinylated Secondary Antibody:
• 1 drop (50 µl) of normal blocking serum added to 2.5 ml of Tris buffer pH 7.4 then one drop (50 µl) of biotinylated antibody stock added to same bottle and mixed well.

Vectastain Elite ABC Reagent:
• 1 drop (50 µl) of Reagent A and 1 drop (50 µl) of Reagent B added to 2.5 ml of Tris buffer pH 7.4 mixed immediately then allowed to stand for 30 minutes prior to use

Diaminobenzidene Solution (DAB)
• 2.5 ml frozen stock solution
• 22.5 ml TBS
• 25 µl Hydrogen Peroxide 30%

Collagen Gel Contraction:
• Glacial acetic acid 99.9%
• Sterile ELGA water
• 10 mg Collagen from Rat Tail (Sigma Aldrich C7661)
• 0.34M Sodium Hydroxide NaOH
• Culture well plates (35 mm²)
• 22 µm syringe-driven filter units
• 200 µl pipette tips
• Myofibroblasts

Drugs:
• Angiotensin II
• Lisinopril
• Penicillin
• Streptomycin
• Amphotericin
• Glutamine
- [Val⁵]-Angiotensin II acetate salt hydrate
- Irbesartan
- S-(-)-PD 123177 trifluoroacetate salt hydrate
- 1-Methylhydantoin
2.1.1 Tissue Collection

All experiments were approved by the National Ethics Service North West-8 Research Ethics Committee, Greater Manchester East (5th October 2009. Study number: 09/H1013/58 (Appendix). The study was also approved by the Research and Development Ethics Committee, Lancashire Teaching Hospitals NHS Foundation Trust, Preston (1st October 2009) (Appendix) and UCLAN.

Female patients due to undergo treatment for capsular contracture disease, under the care of the Department of Plastic Surgery, Lancashire Teaching Hospitals NHS Foundation Trust, Preston, were identified from the out-patient Plastic Surgery clinics and theatre Waiting-list Department. There were a total of 12 patients scheduled for this procedure during the research period. Patient information leaflets (appendix) were given to patients at least 24-hours prior to their operation and informed consent for tissue collection was obtained on the day of surgery (Appendix).

Tissue samples of breast capsule from twelve unilateral breasts (in eight female patients) were collected intra-operatively under sterile conditions from the operating theatres. Specimens were transported in 15 ml test tubes (Fisher Scientific, Loughborough, Leicestershire, UK) containing 10 ml Antibiotic Complete Growth Medium (100 ml of Complete Growth Medium [made from 500 ml of DMEM, 10 ml L-Glutamine solution and 50 ml Fetal Calf Serum] with 2 ml Penicillin and Streptomycin solution and, 1 ml of Amphotericin B solution).

2.1.2 Tissue processing

All experiments with human tissue were carried out under strict aseptic conditions within a laminar air flow hood (Holten HA2436BS, Fisher Scientific Inc, Loughborough, Leicestershire, UK) to maintain sterile conditions and avoid contamination.

Excess subcutaneous fat was removed from the capsule specimens before cutting into 1 cm² square pieces with a sterile blade. Dissected specimen blocks from each breast capsule were then stored initially at 4°C in a 15 ml test tube containing 10 ml of Complete Growth Media (500 ml of DMEM, 10 ml L-Glutamine solution and 50 ml Fetal Calf Serum) for cell culture. In preparation for immunohistochemistry, portions from each specimen were also fixed using 4% paraformaldehyde in phosphate-buffered saline overnight. A portion of each fixed specimen was processed into paraffin blocks for histopathology. As staining was weak with paraffin sections, for patients 5-8, portions were also immediately placed into liquid nitrogen as frozen sections for immunohistochemistry.
2.1.3 Isolation of breast myofibroblasts

The specimens for culture were stored in 10 ml Complete Growth Media at 4°C for 12-24 hours then transferred to a 100 X 15 mm Petri dish (BD Biosciences, Oxford, UK) containing 2 ml of Complete Growth Medium and dissected into small pieces using sterile forceps and scalpel prior to disaggregation. Dissected tissue was then transferred into a 15 ml centrifuge tube (VWR Inc, Lutterworth, Leicestershire, UK) containing 9 ml of Complete Growth Medium and 1 ml of Collagenase solution (2000 u/digestion Worthington Biochemicals, UK). The samples were incubated for 8-12 hours in a 5% carbon dioxide humidified incubator (Sanyo Biomedical, MCO - 18AIC UV, Loughborough, Leicestershire, UK) at 37°C, agitating approximately every hour to promote disaggregation.

Subsequently, preparations were pipetted several times to break down any large cell aggregates and the disaggregated cell suspensions centrifuged at 200 Xg (Heraeus Megafuge 16R, Fisher Scientific, Loughborough, Leicestershire, UK) for five minutes. The supernatant was carefully aspirated, leaving a small amount of medium above the pellet. The pellets were then re-suspended in 10 ml of Complete Growth Medium, centrifuged for a further five minutes at 200 Xg and the supernatant carefully aspirated above the dissociated cells.

Thereafter, the dissociated cells were re-suspended in 2 ml of Complete Growth Medium and mixed well to achieve a homogenous, evenly distributed cell suspension. This was transferred into a 25 cm² cell culture flask (Fisher Scientific, Loughborough, Leicestershire, UK) containing 8 ml of fresh Complete Growth Medium and incubated at 37°C in a humidified incubator with 5% CO₂ (Sanyo Biomedical, MCO - 18AIC UV, Loughborough, Leicestershire, UK).

2.1.4 Cell line

Foetal- lung fibroblast-like cell line (MRC-5, (PD 30) 05090501, Sigma, Aldrich, UK) was cultured as described in above in 10 ml of Complete Growth Media.

The cell line was maintained in 25 cm² initially, then 75 cm² cell culture flasks in a 37°C humidified incubator with 5% CO₂ (Sanyo Biomedical, MCO - 18AIC UV, Loughborough, Leicestershire, UK). For all experiments, the fibroblast cell line was used between passages two to four.
2.1.5 Subculture

Culture flasks were kept in the incubator and sub-cultured when 70-80% confluent. For the third passage cells were transferred to a 75 cm$^2$ culture flask.

The spent medium was aspirated from the culture flask and the flask washed with HBSS (2 ml for 25 cm$^2$ flask and 5 ml for 75 cm$^2$ flask). The media were then removed and 0.25% trypsin (Lonza, Slough, Berkshire, UK) was added to the culture flask (1 ml for 25 cm$^2$ flask and 2 ml for 75 cm$^2$ flask) which was subsequently incubated at 37$^\circ$C for five minutes.

Next, the culture flasks were gently tapped and viewed under the inverted light microscope (Olympus IMT-2) to ensure cells were adequately detached. The culture flasks were then sprayed with alcohol, wiped down and returned to the laminar flow hood (HA2436BS, Fisher Scientific Inc, Loughborough, Leicestershire, UK). A volume of 2 ml of Complete Growth Medium was added to neutralize the trypsin reaction and prevent unwarranted damage to cell membranes caused by trypsin exposure. The cell suspension was then pipetted several times to separate any remaining cell aggregates and transferred to a 15 ml centrifuge tube (Fisher Scientific, Loughborough, Leicester, UK).

The cell suspension was centrifuged at 200 Xg (Heraeus Megafuge 16R, Fisher Scientific, Loughborough, Leicestershire, UK) for five minutes and the supernatant was carefully aspirated, leaving a small amount of medium above the pellet. The pellet was then re-suspended in 2 ml of Complete Growth Medium and mixed well. This cell suspension was then added to new flasks containing 10 ml of Complete Growth Medium for re-culture and returned to the incubator at 37$^\circ$C.

Cell cultures were examined twice a week and the media containing unattached cells was aspirated and fresh Complete Growth Medium added. Cells from subsequent passages three to five were used for all experiments.
2.1.6 Cell counting

Once cells were trypsinized, 50 µl of cell suspension was pipetted into a sterile Eppendorf tube for cell-counting. A volume of 50 µl of Trypan Blue (Sigma, Aldrich, UK) was added to help distinguish live cells as this stain is only taken up by viable cells. Cover slips were attached onto the slide over the chambers of a Neubauer Haemocytometer. Approximately 20 µl of the cell and Trypan Blue suspension was pipetted out onto the haemocytometer and viewed under an inverted light microscope. The number of cells in 25 squares of one chamber was counted, and then the process repeated for the remaining chambers. The average number of cells in the centre grid was counted and this number multiplied by $2 \times 10^4$ to obtain the number of cells per 1 ml of cell suspension.

Cells were also counted using a Coulter- Counter (Beckman Coulter, High Wycombe, Buckinghamshire, UK) method. The Coulter Counter was flushed using Isoton II diluent. The particle size was set to 10 µm and a background count was performed initially using diluent only. A volume of 100 µl of cell suspension was then pipetted into an Accuvette II vial (Beckman Coulter, High Wycombe, Buckinghamshire, UK) and, 10 ml of Isoton II diluent was added. Each suspension was counted three times and an average cell count was calculated.

2.1.7 Cell freezing

Cells were passaged as previously described and harvested at 80-90% confluence. Cells were then counted as described.

The cells were centrifuged at 200 Xg for five minutes and the cell pellets re-suspended in the cell freezing media consisting of a ratio 90% media to 10% (v/v) of the cryoprecipitant Dimethyl Sulfoxide, (DMSO, Sigma, Aldrich, UK) to give a final cell concentration of $1 \times 10^6$cells per ml.

A volume of 1 ml of the cell suspension was aliquoted into cryovials (Greiner Bio One, Stonehouse, Gloucestershire, UK) and frozen at a cooling rate between 1-3 ºC/min using a programmable rate controlled freezer to prevent damage to the cells. The cells were kept in the freezer and once at -130 ºC the ampoules were transferred to a gas phase liquid nitrogen storage vessel the next day.
2.1.8 **Culturing cells from frozen cryovials**

For culturing cells from frozen cryovials, the respective media (10 ml Complete Growth Media was pre-warmed in a water bath to 37° C. The cryovials were removed from the liquid nitrogen and immediately immersed into the water bath at 37°C. Once de-frosted, the vials were wiped clean and sprayed with alcohol prior to the cells being cultured in a 75 cm² flask containing 10 ml of the pre-warmed fresh Complete Growth Media. The flasks were incubated overnight at 37° C then the media changed to remove any residual cryoprecipitant (DMSO).

2.2 **Light Microscopy and Immunolabelling**

2.2.1 **Staining of primary cells**

Myofibroblasts were trypsinized and 10³ cell suspension was pipetted into six-well culture chamber slides (BD Falcon, BD Biosciences, Oxford, UK) and grown in Complete Growth Medium to 50-60% confluence. The medium was aspirated from the chambers and the monolayers were washed three times with TBS.

Cells were then covered with a 1:1 ratio of methanol: acetone (pre-chilled to −20°C) and placed rapidly into a −20°C freezer for 10 minutes to fix. Next, fixative was removed from the chambers and the cells allowed to air dry. Cells were stored in a −20°C freezer prior to immunostaining.

Immunolabelling of specimens was carried out by a modified avidin-biotin-peroxidase complex technique (Vectastain ABC kit; Universal Elite, Vector Laboratories, Calif). Firstly, cells were rehydrated with three washes of Tris Buffered Saline (TBS) before the administration of the primary antibody. For AT1 receptor studies an AT1 receptor antibody was used (TONI-1: 67036/ 1 in 40 dilution/ Santa Cruz Biotechnology, INC, CA, USA). For AT2 receptor studies an AT2 receptor antibody was used (Anti-AGTR2: 53930904/ 1 in 4000 dilution/ Sigma Aldrich, UK).

A volume of 100 µl of primary antibody was pipetted into each chamber and the cells incubated with the lid replaced for 30, 60, 90 minutes and 12 hours respectively at room temperature. For each experiment, the primary antibody was omitted from one sequence for each specimen as a control study. Cells were then rinsed three times with TBS. Thereafter, the cells were incubated in 100 µl of the secondary antibody with the lid replaced for a further 30 minutes at room temperature. After 30 minutes any excess antibody was removed and the cells rinsed with three washes of TBS. A volume of 100 µl of ABC Reagent was subsequently pipetted into each chamber and cells incubated with
the lid replaced for a further 30 minutes at room temperature. Excess solution was removed and cells washed three times with TBS.

Diaminobenzidene (DAB) solution was mixed immediately prior to adding to each chamber using 0.1% diaminobenzydine (Peroxidase Substrate Kit (DAB) SK-4100; Vector Laboratories, Calif) and 0.2% hydrogen peroxide in 50 mM Tris buffer, pH 7.5. The cells were exposed to the DAB solution for five minutes then any excess was removed and the cells rinsed three times with distilled water.

Chamber slides (BD Falcon, BD Biosciences, Oxford, UK) were then counterstained by pipetting haematoxylin into each chamber for 15 seconds then rinsed several times with warm tap water to stain the nuclei. Cells were dehydrated by passing the chambered slides serially from water through graded concentrations of alcohol (50% ethanol, 70% ethanol, 90% ethanol and 100% alcohol). After rinsing, the chambered sections were removed from the slide and mounted in styrolite using a large cover slip.

Uptake was visualised by confocal microscopy and images captured with a 3.2 megapixel digital camera.

2.2.2 Staining of paraffin and frozen sections

Paraffin sections were de-waxed in xylene. Sections were then taken through decreasing concentrations of alcohol to water (100% alcohol, 90% ethanol, 70% ethanol, 50% ethanol then water). The microwave method, as first described by Shi et al. (1991), was used for high temperature antigen unmasking. The sections were then rehydrated with three washes of phosphate-buffered saline.

Frozen sections were allowed to de-frost overnight at room temperature. Sections (5–7 µm) of breast capsule, cut using a microtome (Rotary Microtome HM 325, Fisher Scientific, Loughborough, Leicester, UK) were then air dried onto glass slides (Surgipath X-tra Adhesive, Leica Biosystems, Newcastle Upon Tyne, UK).

Slides were then washed in running tap water then rinsed with TBS prior to placing on an immunostaining tray. Any excess moisture was carefully dried. Quenching of endogenous peroxidase activity was achieved by incubating the sections in Dako hydrogen peroxide blocking reagent for five minutes, with the lid replaced, at room temperature. The sections were then washed.
three times with TBS and any excess moisture dried. The sections were then incubated with normal blocking serum for 20 minutes with the lid replaced.

Without washing, next the 100 µl of primary antibody was added: AT1 receptor antibody (TONI-1: 67036/ 1 in 40 dilution/ Santa Cruz Biotechnology, INC, CA, USA) and AT2 receptor antibody (Anti-AGTR2, 53930904/ 1 in 4000 dilution/ Sigma Aldrich, UK). Kidney paraffin sections were used as a control for AT1 and AT2 receptor antibody staining. Smooth Muscle Actin was also quantified using monoclonal mouse anti human smooth muscle actin (M0851/ 1 in 50 dilution/ Dako, Cambridgeshire, UK). Small bowel paraffin sections were used for α-sma staining controls. For each experiment, the primary antibody was omitted from one sequence for each specimen as a control study.

Sections were then rinsed three times with TBS prior to incubation with the secondary antibody. 100 µl of secondary antibody was added to each slide using a pipette and incubated, with the lid replaced, for a further 30 minutes at room temperature. After three washes in TBS the sections were then incubated for 30 minutes with Vectastain Elite ABC reagent (Universal Elite, Vector Laboratories, Calif). A volume of 100 µl of streptABComplex/HRP solution was then added to each slide using a pipette. Slides were then incubated, with the lid replaced, for 30 minutes at room temperature. Excess solution was removed and slides washed three times with TBS and then exposed for five minutes to 0.1% diaminobenzydine (Peroxidase Substrate Kit (DAB) SK-4100; Vector Laboratories) and 0.2% hydrogen peroxide in 50 mM Tris buffer, pH 7.5 (mixed and used immediately). After five minutes excess DAB was removed and the slides rinsed three times with distilled water.

The slides were counterstained in haematoxylin and washed in hot running water until blue. Following this they were dehydrated in alcohol then taken through to xylene. Slides were mounted in styrolite and viewed using a Digital Microscope (Coolscope- Nikon, Kingston Upon Thames, Surrey, UK) to visualise the distribution of immunoreactivity and capture the images for analysis. Slides were also viewed by an independent histopathologist regarding distribution of immunoreactivity to exclude bias.
2.3 Gel contraction

Based on method described by Colgen (2006).

2.3.1 Preparation of collagen

Acetic acid solution (0.2 %) was prepared from 99.9 % glacial acetic acid and water. The solution was sterilised by passing through the syringe-driven filter units and then cooled to 4º C. Rat tail collagen (Sigma Aldich, UK) was mixed with the 0.2 % acetic acid solution under sterile conditions to a concentration of 6 mg/ml. The solution was mixed on a magnetic stirrer (Fisher Scientific, Loughborough, Leicestershire, UK) for two- five days in a refrigerator at 4ºC until the collagen was completely dissolved in solution. The collagen solution was then diluted to a concentration of 3 mg/ml and 0.1 % acetic acid with sterilized water, stirred for a further 24 hours and subsequently stored at 4ºC.

2.3.2 pH (NaOH) titration of collagen

A volume of 0.4 ml of Dulbecco’s Modified Eagle’s Medium was pipetted into eight individual Eppendorf tubes. Next, 0.2 µl of the prepared 3 mg/ml collagen solution and 1 µl of 1M NaOH were added to the first Eppendorf tube and the solution pipetted up and down to produce a collagen concentration of 1 mg/ml. This process was repeated with increasing concentrations (2-10 µl) of NaOH. The viscosity and colour of the gels were compared to determine the optimal volume of NaOH to produce a viscous gel with a neutral pH.

2.3.3 Preparation of cells for gel contraction

Myofibroblasts were trypsinized and centrifuged at 200 Xg for five minutes (Heraeus Megafuge 16R, Fisher Scientific, Loughborough, Leicestershire, UK). Once cells were trypsinized, 50 µl of cell suspension was pipetted into a sterile Eppendorf for cell-counting. The supernatant was aspirated and the cells re-suspended in Complete Growth Media. Cells were then counted using a Coulter-Counter (Beckman Coulter, High Wycombe, Buckinghamshire, UK). The remaining suspension was re-centrifuged at 200 Xg for five minutes and fresh Complete Growth Media (+/- experimental variable) was added to achieve a homogenous concentration of 1.5 x 10^5 cells per ml following the addition of the collagen solution.
Collagen gels were prepared under sterile conditions. A volume of 0.66 ml of cell suspension (+/- experimental variable) was added to a sterile 10 ml test tube. A volume of 0.33 ml of collagen solution (1 mg/ml) was added to the test tube followed immediately by the optimal volume of NaOH. The solution was pipetted to mix thoroughly then transferred to a 35 mm² culture well plate (BD Biosciences, UK) and allowed to set overnight at 37°C.

To investigate the RAS agonists and antagonists on the rate of gel contraction the following experimental variables were studied:

- [VAL⁵]-Angiotensin II acetate salt hydrate (AT1 receptor agonist - A2900 Sigma Aldrich)
- Irbesartan (AT1 antagonist - 12286 Sigma Aldrich)
- S-(+)-PD123177 trifluoroacetatesalt hydrate (Selective AT2 receptor antagonist - P5749 Sigma Aldrich)
- 1-methylhydantoin (AT2 receptor agonist M49887 Aldrich)
- Lisinopril (ACE- inhibitor- L2777 Sigma Aldrich)

Four of the wells were treated with different concentrations 5, 10, 20 and 40 µl/ml of the variable, a fifth was treated with 20 µl/ml of the respective antagonist and the sixth well was left untreated (control). Contraction rates were measured using a standard ruler for several days afterwards. Images were captured at 24, 48, 72 and 96 hours respectively.

**2.4 Statistical analysis of data**

All experiments were repeated at least three times to ensure that the experimental results were consistent and reliable. Histopathology slides were also reviewed by an independent Histopathologist to exclude bias. Values are presented as means ± SE. Statistical differences between the data were evaluated by the Student’s t-test, with P < 0.05 used as the requirement for significance. All analyses used the conventional two-sided 5% significance-level and were produced using Self-Propelled Semi-Submersible (SPSS) Version 15.
Chapter III

Results
Study 1

3.1 Studies into patient population with capsular contracture disease

3.1.1 Patient demographics and co-morbidities

All patients in the study were female, undergoing surgical treatment for capsular contracture disease. Four patients (50%) had bilateral breast capsule surgery and four patients (50%) underwent unilateral procedures. Therefore, tissue samples of breast capsule from a total of twelve single breasts in eight study patients were harvested. All patients were treated under the care of the Department of Plastic Surgery, Lancashire Teaching Hospitals NHS Trust, Preston, between October 2010 and March 2011. Mean patient age was 48.8 years (range 22-65 years ± 12.95). Mean patient body mass index (BMI) was 26.4 (range 22-30.5 ± 2.72). The average duration of breast implants was 51.5 months (range 6-120 months ± 38.2).

Co-morbidities amongst the study population included none (n=2, 25%), asthma (n=1, 12.5%), hypertension (n=2, 25%), reflex sympathetic dystrophy (n=1, 12.5%), osteoarthritis (n=2, 25%), hypercholesterolemia (n=2, 25%) and hypothyroidism (n=1, 12.5%). Three patients in the study (37.5%) had previously undergone surgical treatment and adjuvant radiotherapy for breast cancer. None of the patients had active breast cancer at time of treatment for capsular contracture and tissue harvest. Other previous surgical procedures included none (n=3, 37.5%) cholecystectomy (n=2, 25%), appendicectomy (n=1, 12) caesarean section (n=2, 25%), laparoscopic gastric bypass for obesity (n=1, 12.5%), post bariatric abdominoplasty (n=1, 12.5%), total abdominal hysterectomy and bilateral salpingoophorectomy (n=1, 12.5%). Three patients in the study were smokers (37.5%).

Regular medications taken by the patients in the study were of the drug classes beta-blockers (n=2, 25%), beta-2 adrenergic receptor agonist inhalers (n=1, 12.5%), anti-depressants (n=1, 12.5%), proton-pump inhibitors (n=1, 12.5%), diuretics (n=1, 12.5%), statins (n=2, 25%), anticonvulsants (n=1, 12.5%), thyroid hormones (n=1, 12.5%), and oestrogen-receptor antagonists (n=2, 25%). Three patients did not take any regular medications (37.5%). Interestingly, none of the patients in the study took ACE-inhibitors.
Table 3.1: Patient demographics and co-morbidities: age; body mass index (BMI); past medical history; past surgical history; smoking status and drug history.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>BMI</th>
<th>Past Medical History</th>
<th>Past Surgical History</th>
<th>Smoking Status</th>
<th>Drug History</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>41</td>
<td>26.5</td>
<td>Asthma</td>
<td>Cholecystectomy</td>
<td>Smoker</td>
<td>Salbutamol inhaler, Citalopram</td>
</tr>
<tr>
<td>2</td>
<td>49</td>
<td>28</td>
<td>Hypertension, Reflex sympathetic dystrophy</td>
<td>Laparoscopic gastric bypass, Post-bariatric abdominoplasty, Caesarean section</td>
<td>Non-smoker</td>
<td>Lansoprazole, Bendroflumethiazide, Gabapentin</td>
</tr>
<tr>
<td>3</td>
<td>55</td>
<td>27</td>
<td>Fit and well</td>
<td>Nil</td>
<td>Non-smoker</td>
<td>No regular medications</td>
</tr>
<tr>
<td>4</td>
<td>49</td>
<td>30.5</td>
<td>Grade II invasive ductal carcinoma right breast, Hypercholesterolemia</td>
<td>Nil</td>
<td>Non-smoker</td>
<td>Tamoxifen, Simvastatin</td>
</tr>
<tr>
<td>5</td>
<td>65</td>
<td>27</td>
<td>Osteoarthritis</td>
<td>Cholecystectomy, Total abdominal hysterectomy and bilateral salpingoophorectomy</td>
<td>Non-smoker</td>
<td>No regular medications</td>
</tr>
<tr>
<td>6</td>
<td>52</td>
<td>22</td>
<td>Grade II invasive ductal carcinoma left breast with ductal carcinoma in situ, Hypothyroidism</td>
<td>Nil</td>
<td>Smoker</td>
<td>Levothyroxine</td>
</tr>
<tr>
<td>7</td>
<td>22</td>
<td>26.5</td>
<td>Fit and well</td>
<td>Appendicetomy</td>
<td>Non-smoker</td>
<td>No regular medications</td>
</tr>
<tr>
<td>8</td>
<td>58</td>
<td>23.5</td>
<td>Grade III invasive ductal carcinoma right breast, Osteoarthritis, Hypercholesterolemia, Hypertension</td>
<td>Caesarean section</td>
<td>Smoker</td>
<td>Tamoxifen, Bisoprolol, Simvastatin</td>
</tr>
</tbody>
</table>
Figure 3.1: Bar chart showing the distribution of age of the study population. Mean age was 48.8 years (range 22 – 65 years ± 12.95).
3.1.2  Indications for initial breast augmentation

Indications for initial breast implant placement included breast reconstruction following breast cancer (n=3, 37.5%); correction of asymmetry following multiple lumpectomies for benign breast disease (n=1, 12.5%); correction of congenital asymmetry (n=1, 12.5%); augmentation post bariatric weight loss (n=1, 12.5%); and breast augmentation for cosmesis (n=2, 25%).
Figure 3.2: Pie chart showing the indications for initial breast augmentation amongst the study population.
3.1.3 Baker Grade of capsular contracture

Regarding the degree of capsular contracture (Baker’s classification) surgical interventions were performed for Baker Grade III and IV capsular contractures only in the study population (Grade I and II do not routinely require surgical excision unless symptomatic). Capsule samples were harvested from 12 breasts in the eight study patients. Seven (58%) implants had grade IV capsular contractures and, five (42%) implants had grade III capsular contractures.
Figure 3.3: Pie chart showing the Baker classification of severity of capsular contracture amongst the study population. (Grade I: breast is normally soft and looks natural; Grade II: breast is a little firm but looks normal; Grade III: breast is firm and looks abnormal; Grade IV: breast is hard, painful, and looks abnormal).
Table 3.2: Table showing the breast laterality of capsule specimens, indications for initial augmentation and, Baker Grade of capsular contracture for the study population.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Capsule Laterality</th>
<th>Specimen</th>
<th>Indication</th>
<th>Baker Grade</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Left Breast</td>
<td>A</td>
<td>Bilateral breast augmentation for cosmesis</td>
<td>III</td>
</tr>
<tr>
<td>2</td>
<td>Left Breast</td>
<td>B</td>
<td>Bilateral breast augmentation post bariatric</td>
<td>IV</td>
</tr>
<tr>
<td></td>
<td>Right Breast</td>
<td>C</td>
<td></td>
<td>III</td>
</tr>
<tr>
<td>3</td>
<td>Left Breast</td>
<td>D</td>
<td>Bilateral breast augmentation for cosmesis</td>
<td>III</td>
</tr>
<tr>
<td></td>
<td>Right Breast</td>
<td>E</td>
<td></td>
<td>IV</td>
</tr>
<tr>
<td>4</td>
<td>Left Breast</td>
<td>F</td>
<td>Reconstruction following wide local excision and radiotherapy for breast cancer right breast</td>
<td>III</td>
</tr>
<tr>
<td></td>
<td>Right Breast</td>
<td>G</td>
<td></td>
<td>IV</td>
</tr>
<tr>
<td>5</td>
<td>Right Breast</td>
<td>H</td>
<td>Reconstruction following multiple lumpectomies for benign breast disease</td>
<td>IV</td>
</tr>
<tr>
<td>6</td>
<td>Left Breast</td>
<td>I</td>
<td>Reconstruction following surgery and radiotherapy for breast cancer left breast</td>
<td>IV</td>
</tr>
<tr>
<td></td>
<td>Right Breast</td>
<td>J</td>
<td></td>
<td>III</td>
</tr>
<tr>
<td>7</td>
<td>Left Breast</td>
<td>K</td>
<td>Bilateral breast augmentation for congenital asymmetry</td>
<td>IV</td>
</tr>
<tr>
<td>8</td>
<td>Right Breast</td>
<td>L</td>
<td>Reconstruction following mastectomy and radiotherapy for breast cancer right breast</td>
<td>IV</td>
</tr>
</tbody>
</table>
The aetiological factors contributing to capsular contracture disease amongst the study group were recorded. Capsular contracture is recognised to have a higher incidence and, also be more severe, in women who undergo breast reconstruction following surgery for breast cancer (Handel et al, 1995). Three patients in the study initially had breast reconstruction following breast cancer and radiotherapy. They all developed more severe, Baker Grade IV, capsular contractures.

3.1.4 Previous surgical treatments for capsular contracture disease

Mean number of previous surgical interventions for capsular contracture was 2.75 procedures (range 1 – 9 ± 3.37). The results demonstrate the challenges associated with treatment of capsular contracture which often re-occurs despite surgical excision. Patient three was particularly prone to forming capsular contracture of her right breast (Specimen E). She had undergone six previous surgical procedures for treatment of recurrent capsular contracture disease prior to the study.
Figure 3.4: Bar chart showing the number of previous surgical interventions for treatment of capsular contracture disease.
3.1.5 Risk factors for capsular contracture disease

Risk factors for forming capsular contracture amongst the study patients included radiotherapy (n=3, 37.5%), post-operative haematoma (n=2, 25%), implant infection (n=2, 25%), implant rupture with silicone leakage (n=1, 12.5%) and smoking (n=3, 37.5%).

The results demonstrate that the aetiological factors thought to play a role in the formation of capsular contracture including radiotherapy, haematoma, infection, silicone leakage and smoking were all common findings amongst the study group, thus supporting these theories.
Figure 3.5: Bar chart showing the risk factors for capsular contracture disease amongst the study population.
Section 3.1.6 Surgical intervention performed for capsular contracture treatment

All operations were performed by a Plastic Surgery Consultant or a Senior Specialist Registrar in Plastic Surgery, under the supervision of the Consultant. Four patients (50%) had bilateral breast capsule surgery and four patients (50%) underwent unilateral procedures for the treatment of capsular contracture disease at the time of tissue harvest. Surgical interventions performed included capsulectomy (n=10 breasts, 83%) or capsulotomy (n=2 breasts, 17%).

All procedures were performed under general anaesthesia. Mean length of patient hospital stay following surgery for capsular contracture was 2.85 days (range one- five days ± 1.25). Prolonged hospital stay (four or more days) was either due to post-operative complications including haematoma (n=1, 12.5%) or excessive output from suction drains (n=2, 25%).
Figure 3.6: Bar chart showing the type of surgical intervention performed for treatment of capsular contracture disease at tissue harvest (i.e. capsulotomy or capsulectomy).
3.1.7  Breast implant analysis

Of the 12 breast implants removed at the time of surgical treatment for capsular contracture and tissue harvest 11 were silicone filled (92%) and only one implant (8%) (patient 7, specimen K) was saline filled. Mean implant volume (size) was 300 cubic centimetres (cc), (range 255-330cc).

Ten (83%) of the implants removed were smooth and only two (17%) had a textured surface (patient 3). 11 implants (91%) were intact with no evidence of silicone leakage. One implant (8%) demonstrated gel bleed (separation of small molecules of silicone from the surface of the implant) which is an indicator of implant degeneration (patient 6, specimen G).

The numbers of saline filled implants, and those with smooth textured surfaces were too small to assess whether implant filler type or surface type were associated with severity of capsular contracture.
**Figure 3.7**: Pie chart showing the type of implant filler material of the mammary prostheses removed from the study population at the time of surgery.
Figure 3.8: Pie chart showing the type of implant surface of the mammary prostheses removed from the study population at the time of surgery.
3.2 Studies into breast capsule myofibroblasts

3.2.1 Cell culture: growth curves of human breast capsule myofibroblasts

Myofibroblasts from breast capsule tissue were successfully isolated and grown in culture. Growth curves of the primary human breast myofibroblasts were constructed to show the total number of cells at different time points. Cells were counted as described in section 2.1.6.

Figure 3.9: Time course growth curves demonstrating total number of human breast myofibroblasts (Specimens D and I) over a period of ten days in subculture. The cells reached the peak of growth after 7.5 days. Cells reached a confluent level at 8.5 days, after which, the total cell number began to decrease as there was limited growing space due to cell density. There were no significant differences between the cultures. Data are the mean of three cultures.
3.3 Studies into fibroblast cell line

3.3.1 Cell culture. Growth curves of foetal lung fibroblasts

Growth curves of the foetal lung fibroblasts were constructed to show the total number of cells at different time points. Cells were counted as described in section 2.1.6.

Figure 3.10: Time course growth curves demonstrating total number of foetal-lung fibroblast-like cells (MRC-5, (PD 30) 05090501, Sigma, Aldrich, UK) over a period of seven days in subculture. The cells reached the peak of growth after five days. Cells reached a confluent level at 5.5 days, after which, the total cell number began to decrease as there was limited growing space due to cell density. Data are the mean of three cultures.
3.4 Studies into breast capsule architecture: immunohistochemistry

3.4.1 Haematoxylin and Eosin staining of tissues

All breast capsules consisted of dense fibrous tissue with chronic inflammatory infiltrates. There was evidence of contributing factors to the formation of capsular contracture in specimen G with foreign body giant cell reaction secondary to leaked implant material. The results of these capsule architectural studies suggest that tissue injury caused by the implant produces inflammatory mediator activation, enhancing the fibrotic process therefore increasing collagen matrix and the number of inflammatory cells.
Figure 3.11: Micrograph showing haematoxylin & eosin staining of human breast capsule, Specimen A (100 x magnification) demonstrating breast tissue with surrounding fibrosis at the capsule-breast tissue interface (breast duct centrally surrounded by two breast lobules). This is typical of three different experiments on similar tissues (n=3).
Figure 3.12: Micrograph showing haematoxylin & eosin staining of human breast capsule, Specimen G (100 x magnification). This section of dense fibrous breast capsule contains patchy chronic inflammatory cells, including histiocytes, which can be seen surrounding leaked foreign implant material (secondary to previous ruptured implant), in an attempt to remove it by phagocytosis. There is also foreign body giant cell reaction secondary to the leaked implant material. This chronic irritation and inflammation is thought to be a causative factor in the formation of capsular contracture. This is typical of three different experiments on similar tissues (n=3).
Figure 3.13: Micrograph showing haematoxylin & eosin staining of human breast capsule, Specimen H (100 x magnification). There is a thick fibrous hyaline breast capsule with focal calcium deposits suggesting a chronic inflammatory process. This is typical of three different experiments on similar tissues (n=3).
Figure 3.14: Micrograph showing haematoxylin & eosin staining of human breast capsule, Specimen I (100 x magnification) demonstrating a dense fibrous breast capsule. The elongated fibroblast cells produce collagen which has contracted to produce this uniformly dense mature fibrous scar tissue. This is typical of three different experiments on similar tissues (n=3).
3.4.2 Morphological characteristics of primary breast capsule myofibroblasts

As depicted below, the myofibroblast cells in breast capsule tissue were large and flat, with elongated processes protruding from the body of each cell, creating a spindle-like appearance. The nuclei in the body of the cells were oval. These cells can resemble normal fibroblasts however, immunostaining for α-SMA was subsequently performed to confirm that the cells were in fact myofibroblasts.
**Figure 3.15:** Light micrograph showing Specimen A, passage four. Subculture of human breast capsule myofibroblasts with cytoplasmic branches and elliptical speckled nuclei (100 x magnification). These active myofibroblasts forming collagen have abundant rough endoplasmic reticulum (RER). This image is typical of the appearances of the myofibroblasts in five different experiments on similar tissues (n=5).
**Figure 3.16:** Light micrograph showing Specimen F passage five. Subculture of human breast capsule myofibroblasts with fusiform morphology (100 x magnification). This image is typical of the appearances of the myofibroblasts in five different experiments on similar tissues (n=5).
3.5 Angiotensin-II Type-1 receptor studies

3.5.1 Immunostaining of primary myofibroblasts

There was no uptake of Angiotensin Type 1 (AT1) receptor antibodies in the primary cultured human breast capsule myofibroblast smears.
Figure 3.17: Micrograph showing breast capsule myofibroblast smear, Specimen A passage 5. AT1 in 40 (100 x magnification). No immunopositivity within the myofibroblast cells. Note the elongated projections of the myofibroblast cells. The negative control was also negative for immunostaining. This image is typical of 15 different experiments on similar tissues (n=15).
3.5.2 Immunostaining of paraffin sections

Figure 3.18: Micrograph showing paraffin section specimen G. AT1 1 in 40. Fibroblasts within this dense collagenous capsule show no staining. This image is typical of 15 different experiments on similar tissues (n=15).
**Figure 3.19:** Micrograph showing paraffin section, Specimen H. AT1 1 in 40 (10 x magnification) demonstrating weak staining within the vessel walls but also weak uptake within the stromal spindle myofibroblast cells. This image is typical of 12 different experiments on similar tissues (n=12).
Although AT1 and AT2 detection was low in the paraffin sections using polyclonal anti-AT\(_1\)R antibodies, this may have been because of the tissue fixation method used and/or a low detection efficiency. Similarly, lack of uptake in the fibroblast culture smears potentially due to an antigen retrieval deficiency limitation.
3.5.3 Immunostaining of cryostat sections

Figure 3.20: Micrograph to show frozen section of Specimen L. AT1 1 in 40 (20 x magnification). No immunopositivity is seen. Although AT1 and AT2 detection was low in the paraffin sections using polyclonal anti-AT1R antibodies, this may have been because of the tissue fixation method used and/or a low detection efficiency. Similarly, lack of immunostaining in the fibroblast culture smears potentially due to an antigen retrieval deficiency limitation. This image is typical of 12 different experiments on similar tissues (n=12).
Figure 3.21: Micrograph showing frozen section of Specimen I. AT1 1 in 40 (10 x magnification). Although there is a slight background staining, the weakly positive stromal myofibroblast cells are evident in this section. This image is typical of 12 different experiments on similar tissues (n=12).
Figure 3.22: Micrograph showing frozen section of Specimen J: AT1 1 in 40 (100 x magnification). Immunopositivity is seen in the stromal myofibroblasts and the cells of the synovial-like metaplasia of the breast capsule. This image is typical of 12 different experiments on similar tissues (n=12).
3.5.4 Control

In the kidney controls, for all experiments, AT1 and AT2 receptor staining uptake were positive in the endothelial cells of vessels including interlobular arteries, afferent and efferent arterioles, and glomerular capillaries, as previously reported (Tsutsumi et al, 1998).
Figure 3.23: Micrograph showing the kidney control. AT1 1 in 40 (50 x magnification). There is positive staining for the AT1 antibody in the cells lining the tubules. This image is typical of 12 different experiments on similar tissues (n=12).


3.6 Angiotensin-II Type-2 receptor studies

3.6.1 Immunostaining of primary myofibroblasts

There was no uptake of Angiotensin II- Type 2 (AT2) receptor antibodies in the primary cultured human breast capsule myofibroblast smears. This may be due to ineffective antigen retrieval or low levels of expression.
Figure 3.24: Micrograph showing breast capsule myofibroblast smear of Specimen B passage five, AT2 1 in 4000 (40 x magnification). No immunostaining is seen within the elongated myofibroblast cells. This image is typical of 15 different experiments on similar tissues (n=15).
3.6.2 Immunostaining of paraffin sections

**Figure 3.25:** Micrograph showing paraffin section of Specimen B. AT2 1 in 4000 (2 x magnification) No immunostaining is noted in the myofibroblasts. This section demonstrates the architecture of a dense mature fibrous capsule. This image is typical of 12 different experiments on similar tissues (n=12).
3.6.3 Immunostaining of cryostat sections

Figure 3.26: Micrograph showing frozen of Section Specimen H. AT2 1 in 4000. Strong immunopositivity is seen in the stromal spindle fibroblasts; the blood vessel walls provide an inbuilt internal control. This image is typical of 12 different experiments on similar tissues (n=12).
**Figure 3.27**: Micrograph showing frozen Section of Specimen I. AT2 1 in 4000 (10 x magnification) demonstrating strong immunopositivity within smooth muscle walls and spindle-cell shaped myofibroblast cells. This image is typical of 12 different experiments on similar tissues (n=12).
Figure 3.28: Micrograph showing frozen Section of Specimen J: AT2 1 in 4000 (50 x magnification). The stromal myofibroblasts in this section show strong immunopositivity. The blood vessels again provide an internal positive control for the stain. This image is typical of 12 different experiments on similar tissues (n=12).
**Figure 3.29:** Micrograph showing frozen Section of Specimen K. AT2 1 in 4000 (20 x magnification) showing moderate positive immunostaining within the spindle-shaped myofibroblast cells. This image is typical of 12 different experiments on similar tissues (n=12).
Figure 3.30: Micrograph showing frozen section of Specimen L. AT2 1 in 4000 (50 x magnification) demonstrating immunopositivity within the smooth muscle in vessel wall but also uptake of myofibroblast cells. This image is typical of 12 different experiments on similar tissues (n=12).
3.6.4 Control

Figure 3.31: Micrograph of kidney (control) AT2 1 in 4000 (100 x magnification). There is variable immunostaining in the tubular epithelial cells. A glomerulus which shows no staining is seen to the right of the centre. This image is typical of 12 different experiments on similar tissues (n=12).
**Figure 3.32: Control specimen:** Micrograph showing frozen section of Specimen L (100 x magnification) with the primary AT2 antibody omitted. Note no immune-positivity within myofibroblasts when compared to the same experiment where the primary antibody was added (**Fig 3.30**). This image is typical of 12 different experiments on similar tissues (n=12).
Study 7

3.7 Alpha-smooth muscle actin studies

3.7.1 Immunostaining of paraffin sections

Positive immunostaining for myofibroblasts were present in all capsule specimens (n=12). Interestingly, myofibroblasts were observed to be higher in less mature capsules, although not specifically counted in this study. The study also showed that in some capsules, the myofibroblasts were dispersed diffusely throughout the capsule, but in others the myofibroblasts were more localised on the outer layer of the capsule, contacting the implant.
Figure 3.33: Micrograph showing paraffin section of Specimen H. SMA 1 in 10 (50 x magnification). This concentration was too high therefore more dilute concentrations were used to find an optimum concentration. This image is typical of three different experiments on similar tissues (n=3).
Figure 3.34: Micrograph showing paraffin section of Specimen H: SMA 1 in 50. (40 x magnification). In this section, with a more optimum antibody concentration, the vascular smooth muscle is highlighted by positive alpha-SMA immunostaining. This image is typical of ten different experiments on similar tissues (n=10).
3.7.2 Immunostaining of cryostat sections

Figure 3.35: Micrograph showing frozen section of Specimen I. alpha-smooth muscle actin 1 in 50 (20 x magnification). The data show strong positive antibody uptake within stromal spindle-shaped cells. This demonstrates that the stromal cells that are staining with both alpha-SMA and AT2 are myofibroblasts. This co-staining was described by McKirdy et al, 2000. This image is typical of ten different experiments on similar tissues (n=10).
The results have clearly identified the presence of myofibroblasts in breast capsule tissue. Moreover, the expression of the AT1 and AT2 receptor antibodies was co-localised with the myofibroblast marker, for the first time demonstrating that in breast capsule, these cells contain both AT1 and AT2 receptors.
Figure 3.36: Micrograph showing small bowel (control). SMA 1 in 50 (100 x magnification). The smooth muscle component of the muscularis mucosae (short arrow) and the blood vessel (long arrow) walls is highlighted by the Alpha-Smooth Muscle Actin (alpha-SMA) antibody. This image is typical of 12 different experiments on similar tissues (n=12).
Comparison of immunostaining in capsule specimens

Table 3.3: Table summarising and comparing the uptake of polyclonal antibodies against Angiotensin II Type 1 receptors (AT1), Angiotensin II type 2 receptors and alpha-smooth muscle actin in breast capsule paraffin and frozen sections in this study
(Note: X indicates frozen section was not performed for these early specimens).

<table>
<thead>
<tr>
<th>Patient</th>
<th>Specimen</th>
<th>Baker Grade</th>
<th>Paraffin Sections</th>
<th>Frozen Sections</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>AT1</td>
<td>AT2</td>
</tr>
<tr>
<td>1</td>
<td>A</td>
<td>III</td>
<td>nil</td>
<td>nil</td>
</tr>
<tr>
<td>2</td>
<td>B</td>
<td>IV</td>
<td>very weak</td>
<td>nil</td>
</tr>
<tr>
<td>2</td>
<td>C</td>
<td>III</td>
<td>nil</td>
<td>nil</td>
</tr>
<tr>
<td>3</td>
<td>D</td>
<td>III</td>
<td>nil</td>
<td>nil</td>
</tr>
<tr>
<td>3</td>
<td>E</td>
<td>IV</td>
<td>nil</td>
<td>nil</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>III</td>
<td>nil</td>
<td>nil</td>
</tr>
<tr>
<td>4</td>
<td>G</td>
<td>IV</td>
<td>nil</td>
<td>nil</td>
</tr>
<tr>
<td>5</td>
<td>H</td>
<td>IV</td>
<td>weak</td>
<td>nil</td>
</tr>
<tr>
<td>6</td>
<td>I</td>
<td>IV</td>
<td>weak-moderate</td>
<td>nil</td>
</tr>
<tr>
<td>6</td>
<td>J</td>
<td>III</td>
<td>nil</td>
<td>nil</td>
</tr>
<tr>
<td>7-</td>
<td>K</td>
<td>IV</td>
<td>nil</td>
<td>nil</td>
</tr>
<tr>
<td>8-</td>
<td>L</td>
<td>IV</td>
<td>nil</td>
<td>nil</td>
</tr>
<tr>
<td>Control Kidney</td>
<td>n/a</td>
<td>strong positivity</td>
<td>strong positivity</td>
<td>n/a</td>
</tr>
<tr>
<td>Control Uterus</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>strong positivity</td>
</tr>
</tbody>
</table>

106
The results show that myofibroblasts were present in all capsules (n=12). AT1 and AT2 receptors were presented in 50% (n=6) of capsules (paraffin and cryostat sections) however, in the cryostat-only group, staining was positive in 100% (n=5) of capsules. Results also showed that expression of these receptors was co-localised with α-sma uptake demonstrating the key components of the RAS are present within breast capsule myofibroblasts. A higher expression and wider distribution of AT2 receptors was also noted compared with AT1 receptors.

3.8 Gel contraction

In this study, solidification of three-dimensional gel collagen lattices was suboptimal for consistent measurements of contraction to test the experimental variables. The NaOH titration stage of the gel contraction assay was repeated several times to explore more optimal amounts of NaOH to add to the collagen/media mixture. Increasing amounts of NaOH were investigated (2-10 µl). Increasing concentrations of cells were also investigated in attempt to set the gels. The mixture was also left to solidify for increasing amounts of time 20, 40 and 60 minutes. When solidification was still unsuccessful longer periods of three, six, and twelve hours were tested. However, in the described method by Colgen, they describe rapid solidification of the gels, albeit the method being difficult. Despite efforts, the consistency of the gels did not reach a substantial density in order to accurately measure the rate of myofibroblast contraction.
Chapter IV

Discussion
4.1 Discussion

4.1.1 Epidemiology of breast cancer in the Lancashire and Cumbria Cancer Network

More women are surviving breast cancer than ever before. Over 80% of patients survive for at least five years after diagnosis (Clay, 2008). The latest analysis of breast cancer incidence rates throughout the UK reports only modest variation between most cancer networks, but notably higher rates in parts of south England and south-east Scotland, and lower rates in parts of London (Cancer Research UK, 2012). Royal Preston Hospital is part of the Lancashire and Cumbria Cancer Network. Recent statistics have shown that women living in the former West Cumbria Primary Care Trust (PCT) have the highest survival rate within the Strategic Health Authority at 83.6%. Average out breast cancer survival within Cumbria is 81.7% placing it above the district average of 80.6%. However, women living in Preston (78.4%) and Blackpool (75.7%) have the lowest survival rates within the Cancer Network (Clay, 2008). Heredity, obesity and certain hormonal medications may contribute to a woman’s risk of developing breast cancer (Cancer Research UK, 2012). Approximately 40% of women with breast cancer will undergo mastectomy and as mentioned previously, all should be offered breast reconstruction according to NICE.

4.1.2 Capsular contracture disease in the study population

All patients in the study underwent surgical treatment for capsular contracture disease. The patients were generally otherwise well with no significant co-morbidities at the time of surgery. The patients in the study had a mean average of 2.75 previous procedures (range 1 – 9 ± 3.37) for treatment of capsular contracture. This clearly demonstrates the challenges associated with treatment of this disease which often re-occurs despite surgical excision. Capsular contracture is recognised to have a higher incidence and, also be more severe, in women who undergo breast reconstruction following surgery for breast cancer (Handel et al, 1995). Three of the patients underwent initial breast augmentation following previous surgical treatment and adjuvant radiotherapy for breast cancer. All of these patients developed more severe, Baker Grade IV, capsular contractures. Other aetiological factors proposed to play a role in the formation of capsular contracture including haematoma, infection, silicone leakage and smoking were all also common findings amongst the study group, thus supporting these theories.
4.1.3 Capsular contracture disease

All surgeons who perform augmentation mammoplasty agree that the most unpredictable and challenging problem associated with this popular procedure is the onset of capsular contracture. Encapsulation of a foreign body, such as breast implant, which is too large to be removed by phagocytosis, is a normal physiological phenomenon. However, the mechanisms as to why these biologically normal capsules contract in some patients remain poorly understood (Embrey et al., 1999).

This contraction of the capsule around an implant firms and distorts the appearance of the breast, causing significant pain and morbidity for women often undergoing this procedure following breast cancer surgery. Capsular contracture is also more likely if patients have radiotherapy before or after reconstruction with a breast implant. This problem occurs so frequently that most Breast and Plastic Surgeons consider capsular contracture to be an undesirable sequelae of augmentation than as a complication *per se* (Shiffman, 2009).

Successful prevention and treatment of capsular contracture disease are notoriously challenging. This potentially may be due to the lack of knowledge of the exact cellular mechanisms governing this phenomenon which makes targeting of pharmacological therapies difficult. Surgery is currently the only recognised treatment for capsular contracture disease.

4.1.4 The myofibroblast in capsular contracture

The cell thought to be implicated in the formation of capsular contracture is the myofibroblast (Rudolph *et al.*, 1978). First described in granulation tissue, myofibroblasts are responsible for wound contraction, disappearing once wound healing is complete (Gabbiani, 1992). These cells are histologically similar to fibroblasts but contain smooth muscle elements that are connected to the extracellular matrix. These connections are thought to be the driving force of the contractile activity of these cells. This actin also facilitates the identification of the myofibroblast on immunological stains (Foo *et al.*, 1992; Gabbiani 1992).

In pathological wound healing, the contractile activity of myofibroblasts persist which leads to abnormal fibrous tissue such as in hypertrophic and keloid scarring and palmar fibromastosis in Dupuytren’s disease (Hinz, 2007). Similar phenomena are likely to be responsible for the fibrotic and contractile processes in capsular contracture.

Rudolph *et al* (1978) found myofibroblasts in 20 of 25 firm contracted capsules, but also in 16 soft non-contracted capsules. They also found that myofibroblasts were more likely to be found in immature capsules, as is consistent with the regulation of the myofibroblast in other wounds. However, despite many experimental and clinical studies, the aetiology and pathogenesis of the
cellular mechanisms that regulate the contractile properties of the myofibroblast in capsular contracture remains poorly understood (Baker et al., 1981; Shiffman, 2009).

4.1.5 The Renin-Angiotensin-System

The renin-angiotensin-system (RAS), which was originally described in the context of homeostasis of blood pressure and water and electrolyte balance has also been shown to be a major regulator of the inflammatory response (Lavoie and Sigmund., 2003; Paul et al., 2006). Angiotensin II, the active end product of the RAS, has been implicated as a mediator of organ fibrosis and cutaneous repair (Sun et al., 1996b). Ang II stimulation has been shown to regulate proliferation of skin fibroblasts and the production of extracellular matrix, which are important processes in skin wound healing and fibrosis (Liu et al., 2004). However, few studies have examined the localization of RAS components, such as angiotensin II receptors, renin (REN), angiotensinogen (AGTN), and angiotensin-converting enzyme (ACE), in fibrotic breast capsular tissue.

4.1.6 Significance of angiotensin receptors

Angiotensin II exerts its important physiological functions through two distinct receptor subtypes, type 1 (AT1) and type 2 (AT2) receptors (Busche et al., 2000). AT1 receptors mediate all well-known effects of angiotensin II, ranging from vasoconstriction to tissue remodelling (Dudley et al., 1990). While the pro-inflammatory features of the angiotensin AT1 receptor are well established, the role of the AT2 receptor has been less well defined (Rompe et al., 2010). Most current evidence supports an anti-inflammatory role for AT2 receptor and expression of the AT2 receptor has been shown to be in injured tissues and organs undergoing remodelling. In some pathological situations, the effects of AT2-receptor activation are hypothesised to antagonise those mediated through AT1 receptors (Akazawa et al., 2012). A Ying-Yang hypothesis has been proposed in which AT1 and AT2 receptors have principally contrary functions (Rompe et al., 2012). These opposed actions of AT1 and AT2 predispose the RAS to take part in regulatory mechanisms, which may apply to wound healing and the inflammatory response (Steckelings et al., 2005).

However, this view that AT2 receptors exclusively exert beneficial effects has been recently challenged. In pathological models, the function of AT2 has been proposed to mimic that of AT1 receptors, for example, in cardiac models, inducing vasoconstriction and cardiac hypertrophy (Rompe et al., 2012). It could therefore be proposed that excess Ang II, which cannot bind anymore to the occupied AT1 receptors, then binds to the AT2 subtype and its function is no longer
beneficial. Given its up-regulation in various pathological conditions, the AT2 receptor remains a promising target for treatment.

4.1.7 Aims of study

Clinical studies were designed to review the aetiology and factors contributing to capsular contracture disease amongst the study group. The study was also designed to investigate whether myofibroblasts were present in the fibrous breast capsules. The aims of the study were to determine the presence of a local RAS in fibrotic capsular tissue around mammary implants. The expression of the fundamental components of the RAS, namely the AT1 and AT2 receptors were investigated in breast capsule tissue excised during surgery for capsular contracture disease. The expression of the RAS key receptors were compared with markers for smooth muscle actin to compare the localisation of the RAS activity with the primary cell involved in the formation of the fibrous capsule, the myofibroblast, and to explore the possible role of Ang II in capsule formation.

This study proposed to elucidate whether modulation of the RAS may provide the basis for a safe and cheap therapeutic strategy with which to modify capsular contracture affecting women with mammary implants.

4.1.8 Findings of the study

Histological examination of breast capsule specimens with haematoxylin and eosin stains showed dense fibrosis along with massive inflammatory cell infiltration. The study showed that myofibroblasts were present in all capsules (n=12) and the fibrotic and inflammatory processes were associated with myofibroblast uptake. The myofibroblast is recognised as the primary cell to express Ang II in other organs and its detection in all capsules supports the hypothesis that this cell is a key mediator of the fibrotic process in this context. Interestingly, myofibroblast levels were observed to be higher in less mature capsules, although not specifically measured in this study, suggesting that this cell may play a role in the earlier stages of capsular contracture formation and remodelling. Observations in the study also showed that in some capsules, the myofibroblasts were dispersed diffusely throughout the capsule but in others, the myofibroblasts were more localised on the outer layer of the capsule, contacting the implant.

Indirect immunofluorescence was performed on the primary cells and also on paraffin and cryostat sections. Anti-AT1 and anti-AT2 receptor antibodies were used as the primary antibodies for
detection of the RAS key receptors. In this present study, the presence of Angiotensin II Type I and Angiotensin II Type II receptors were identified within 50% (n=12) of all (paraffin and cryostat) sections and 100% (n=5) of cryostat only sections from primary capsule tissue of patients with capsular contracture disease. AT2 receptor expression was significantly enhanced compared to that of AT1. AT1 and AT2 receptor expression was also found to be co-localised with areas of myofibroblast expression, demonstrated by the corresponding uptake of alpha-smooth muscle actin. Positive expression of AT1 and AT2 in the endothelial cells of the capillary vessels within the tissue sections also provided an inbuilt internal antibody control.

Although detection of the AT1 and AT2 receptors in the paraffin sections, using polyclonal anti-AT1 and AT2 receptor antibodies was weak, this may have been because of the tissue fixation method and/or low detection efficiency. Similarly, lack of uptake in the fibroblast culture smears potentially due to an antigen retrieval deficiency limitation.

In the present study, although the level of angiotensin II was not determined, for the first time it has been demonstrated that the key AngII receptors, AT1 and AT2, of the RAS were expressed by the myofibroblast cells in breast capsular contracture tissue. These data provide strong evidence for a capsular RAS within the fibrosing breast capsule, and a linkage between the RAS and capsular fibrogenesis, suggesting that within the capsule, AngII may be generated and, in turn, activate myofibroblast cells to contract in the fibrosing diseased capsule.

The concept discussed in the literature that AT1 and AT2 receptors mediate opposed actions of Ang II could suggest that their up-regulation in this study does not necessarily augment the effects of Ang II (Steckelings et al, 2005). The results of the study could suggest that AT2 receptor expression is up-regulated in mature capsules to counteract the effects caused by the AT1 receptor during early capsule formation. The present study was not able to determine AT1 and AT2 receptor expression in the initial stage of capsular fibrosis as these patients do not require surgery. The expression of these receptors may change as the capsule undergoes maturation and remodelling and the results propose that the ratio of the expression of AT1 and AT2 receptors in the capsule tissue could potentially determine the overall result of Ang II action.

4.1.9 ACE inhibitors

Angiotensin-converting enzyme inhibitors are commonly used in renal and cardiovascular diseases. Enalapril, an ACE-inhibitor, prevents the expression of fibrotic mediators, TGF-1 and inflammatory markers, anti-ED1, and anti–collagen III (Zinmann et al, 2007). Through the same processes, such treatment has also shown to prevent capsular contraction on mammary prostheses that have been implanted in rats (Zinmann et al, 2007). Given that AT1 and AT2 receptors have now been
demonstrated in capsular contracture tissue, from these data, therefore, it could therefore be proposed that the development of capsular contracture may be modulated by inhibiting the RAS using ACE inhibitors and AT1 and AT2 receptor antagonists.

The use of AT-1 receptor blockers has been shown to decrease myocardial fibrosis (Busche et al., 2000). Data on the AT2-type receptor in inflammation are still rather sparse and are somewhat controversial. However, the results of this study propose the potential utilisation of AT2 receptor stimulation also as a novel pharmacological concept in anti-inflammation. Studies are required to know more about the AT1 and AT2 receptors in this context and there potential application in drug development.
Chapter V

Conclusion
5.1 Conclusions

Capsular contracture is a disorder of unknown aetiology characterised by fibrosis. Several studies have demonstrated that the RAS plays a key role in wound healing and fibrosis. Therefore, therapies targeting the RAS may represent a promising paradigm for the prevention and treatment of capsular contracture in the setting of breast reconstruction.

The goal of this study was to investigate the presence of a local RAS and the profibrotic potential of Ang II in capsule tissue. The study employed primary tissues isolated from twelve breast capsules in eight patients with capsular contracture disease. Myofibroblasts from each patient were successfully cultured over a period of 7-10 days to produce normal growth curves. Following culture, myofibroblasts were studied morphologically. Paraffin and cryostat sections were subsequently treated with specific antibodies for the identification of angiotensin type 1 (AT1) and type II (AT2) receptors.

AT1 and AT2 receptors were present in 50% (n=12) of all capsules (paraffin and cryostat sections) and 100% (n=5) of the cryostat-only group. Receptors were co-localised within myofibroblasts. The AT1 and AT2 receptors were also present within the smooth muscle walls of blood vessels within the tissue sections which provided an inbuilt internal antibody control. A higher expression and wider distribution of AT2 receptors were identified in the tissue compared to AT1 receptors. Solidification of 3D gel collagen lattices was suboptimal for consistent measurements of contraction despite numerous attempts.

In conclusion the results of this study have identified the presence of myofibroblasts in tissues of patients with capsular contracture disease and have demonstrated that they are amenable to culture successfully. Moreover, these myofibroblasts contain both AT1 and AT2 receptors. This co-localisation of Angiotensin II receptors and myofibroblast expression has implications for potential pharmacological regulation of capsular contracture disease which, until now has not been responsive to safe, effective pharmacological therapy. Further experiments are required to study the role of blocking AT1 and AT2 receptors excitation-contraction coupling process of the myofibroblasts.
5.2 Scope for future work

1. Breast capsule myofibroblasts can be cultured as has been demonstrated. The molecular mechanism of myofibroblast differentiation and function needs to be clearly understood in order to eradicate this key cell in pathological tissue contractures. Novel strategies and pharmacological drug therapies are required to target the actions of the myofibroblast.

2. It is also possible to study the morphology of the primary tissues and the myofibroblasts using phase contrast and electro-microscopy. Further studies into the myofibroblasts in breast capsule are required, particularly pattern of localisation and myofibroblast count in relation post-operative age of capsule (i.e. maturity), thickness and Baker’s classification.

3. Little is known regarding the RAS components within breast capsule. The localisation and expression levels of other components of RAS: REN, AGTN, and ACE, are yet to be determined in the fibrosing capsule, and to what extent the system is involved in abnormal capsule formation.

4. The presence of AT1 and AT2 receptors has been clearly demonstrated in breast capsule in this study. Future work should determine what consequences alterations in receptor expression may have in this context and whether the expression of AT1 and AT2 receptors are affected by administration of ACE-inhibitors.

5. It may also possible to employ binding studies to identify angiotensin receptors in the tissues and cells using radio-labelled angiotensin. The levels of such receptors in capsules of different maturity could be investigated using flow cytometry.

6. In the literature it has been possible to measure contraction in the myofibroblasts using different physiological methods. A video-edge system in conjunction with three-dimensional collagen gel can be used to measure contraction. The cells can be treated with different drugs and their respective antagonists to study their effects on contraction. If the effects of angiotensin II are blocked using an angiotensin-converting enzyme inhibitor, the inflammatory and fibrotic processes may be attenuated, which may be demonstrated by a reduced rate of contracture.

7. A histological classification for capsular contracture is also needed to describe precisely the morphological changes in capsular contracture such as age of the collagen fibres, presence of synovia-like metaplasia on the inner surface of the capsule, number of histiocytes, giant cells, and other inflammatory cells, silicone levels and foreign body granulomas. Such a classification would allow a more accurate comparison of techniques and therapeutic interventions then current subjective clinical classifications.

8. Measure intracellular calcium in myofibroblasts to investigate the role of calcium in the process of contraction in the myofibroblast.
References


Appendix
Appendix

1. National Ethics Service North West-8 Research Ethics Committee, Greater Manchester East (5th October 2009. Study number: 09/H1013/58)
2. Patient information leaflets
3. Written patient informed consent forms
4. Summary of study patients previous breast summary
7.1 Patient Information Sheet

PATIENT INFORMATION SHEET

STUDY TITLE: THE EFFECTS OF ANGIOTENSIN CONVERTING ENZYME (ACE)-INHIBITORS ON CAPSULAR CONTRACTURE AROUND MAMMARY IMPLANTS.

Simplified Title: Study to test the effect of drugs on scar tissue (capsular contracture) around breast implants.

You are being invited to take part in a research study. Before you make a decision on whether you would like to participate 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

Please ask us or your consultant plastic surgeon 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 and discuss it with others if you wish. Please keep this information sheet for future reference.

Thank you for reading.

What is the purpose of the study?

Capsular contraction around breast implants is very difficult to prevent or treat. Treatment of capsular contracture usually requires surgery to remove the ‘capsule’ as there are currently no medicines to treat this condition.

Angiotensin-Converting Enzyme (ACE)-Inhibitors is the medical name for drugs commonly used in heart and kidney disease to reduce scarring and fibrosis. Our study aims to determine whether these drugs can also be used to prevent scarring and fibrosis around breast implants. The study aims to try to develop a medicine to treat capsular contracture affecting breast implants that are commonly used for women having breast reconstruction or enlargement.

What does taking part involve?

We are asking for patients to donate scar tissue that is normally removed during treatment for capsular contracture. We would use this tissue for the research to test the drugs. NO extra tissue will be taken for the study, only tissue which would be removed as part of your routine surgery would be used. Taking part in this study would not mean any changes to your routine treatment.

What will happen to the samples?

The samples will be taken to laboratories at Royal Preston Hospital and the University of Central Lancashire where we will conduct experiments to tests the effects of drugs. The researcher and their clinical supervisors will have access to the samples. NO tissue will be stored and any tissue not used will be disposed of according to hospital policies. The samples will not be used for any other research.

Do I have to take part?

It is entirely your decision whether or not to take part. If you do wish to take part you will be given this information sheet to keep and will be asked to sign a consent form (you will also be given a copy of the signed consent form to keep). You are still free to withdraw at any time without giving a reason. If you decide not to take part or withdraw at any time this will not affect the standard of care you receive.

What will happen to me if I take part?

133
Taking part in the study will not alter your routine surgery in any way. You WILL NOT be required to have any extra hospital visits or have any extra investigations. The study only involves tests on the samples being donated.

**What is being tested?**

Angiotensin-Converting Enzyme (ACE)-Inhibitors such as Enalapril are drugs commonly used in kidney and heart disease to prevent scarring and fibrosis. Our study aims to determine whether these drugs could also be used to treat capsular contracture.

**What are the disadvantages or risks associated with taking part?**

There are NO disadvantages or risks specifically related to taking part in the study. The risks associated with your routine surgery will have been discussed separately with you by your consultant.

**What are the possible benefits of taking part?**

There is no medical benefit from taking part in the study and it will not affect the outcome of your surgery. The study aims to develop a drug treatment for capsular contracture affecting breast implants that are commonly used for women having breast reconstruction or enlargement.

**Will my taking part in the study be kept confidential?**

All information which may be collected will be kept strictly confidential. Any information about you which leaves the hospital will have your name, age, address and all personal information (including patient/hospital/NHS number) removed so that you cannot be recognised from it.

**What will happen to the results of the study?**

The results of the study will be submitted for publication in scientific journals. You may receive information about the results of the study upon request.

**Who is funding the research?**

The research will be funded by the Rosemere Cancer Foundation.

**Who has reviewed the study?**

All research in the NHS is looked at by an independent group of people, called a Research Ethics Committee, to protect your interests. This study has been reviewed and given a favorable opinion by: North West 8 Research Ethics Committee- Greater Manchester East and Lancashire Teaching Hospitals Trust Ethics Committee.

**What arrangements will be made for insurance/ indemnity?**

Patients will not be exposed to any risks associated with participation in this research however, NHS indemnity will apply and in the event that something does go wrong and you are harmed during the research and this is due to someone's negligence then you may have grounds for a legal action for compensation against (Lancashire Teaching Hospitals, NHS Trust,) but you may have to pay your legal costs.

**What happens if there is a problem or I am unhappy about the study?**

If you have any questions or concerns about the study you can speak to us (the researchers) who will do our best to answer your questions. Hopefully, we will be able to sort out your concerns very quickly. However, if you remain unhappy and wish to make a formal complaint related to the study you may do this in writing. Your letter should be addressed to the Chief Executive of Lancashire
Teaching Hospitals NHS Foundation Trust at the following address: Royal Preston Hospital, Sharoe Green Lane, Fulwood, Preston, PR2 9HT. This will not affect your treatment in any way. You could also seek advice from the Patient Advice and Liaison Services (PALS) at Royal Preston Hospital which provides support to patients, their families and visitors (contact details for PALS are listed below).

*Thank-you for reading.*

Contacts for further information:

**Dr Leila. Touil (Researcher)**  
Plastic Surgery Department  
Royal Preston Hospital  
Sharoe Green Lane  
Fulwood,  
Preston  
PR2 9HT  
Tel: 01772522847  
Email: Leila.Touil@lthtr.nhs.uk

**Patient Advice and Liaison Services (PALS)**  
Royal Preston Hospital, Sharoe Green Lane, Fulwood, Preston, PR2 9HT  
Tel: 01772 522972
7.1.3. Study consent form

CONSENT FORM

Centre Number:

Study Number: 09/H1013/58

Patient Identification number for this study:

Title of Project: THE EFFECTS OF ANGIOTENSIN CONVERTING ENZYME (ACE)-INHIBITORS ON CAPSULAR CONTRACTURE AROUND MAMMARY IMPLANTS.

Simplified Title: Study to test the effect of drugs on scar tissue (capsular contracture) around breast implants.

Name of Researcher: Dr Leila Touil

Please initial box

1. I confirm that I have read and understand the information sheet dated September 2009 (Version 2) for the above study. I have had the opportunity to consider the information, ask questions, and have these questions answered properly.

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 relevant sections of my medical notes and data collected during the study, may be looked at by the researchers, from regulatory authorities or from the NHS trust, where it is relevant to my taking part in this research. I give permission for these individuals to access my records.

4. I agree to take part in the above study.

Name of Patient         Date         Signature
Name of Person taking consent         Date         Signature

When completed: 1 for participant, 1 for researchers site file original to be kept in medical notes
<table>
<thead>
<tr>
<th>Patient</th>
<th>Previous Breast Surgery</th>
<th>Complications</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1997 Bilateral Breast Augmentation</td>
<td>1997 Left breast haematoma returned to theatre for evacuation</td>
</tr>
</tbody>
</table>
| 2       | 1998 Bilateral breast augmentation  
1999 Bilateral exchange of implants and capsulectomies  
1999 Left side implant replacement  
2002 Right side implant replacement and capsulectomy | 1999 Left implant infection returned to theatre for removal of implant (pus in cavity) and washout of pocket  
2001 Right side implant infection treated with intravenous antibiotics  
2002 Further right implant low grade infection managed surgically with right side washout, implant replacement and capsulectomy |
| 3       | 1984 Bilateral breast augmentation  
1987 Bilateral closed capsulotomies  
1988 Exchange of bilateral implants  
1991 Exchange of bilateral implants  
1992 Capsulotomy right breast  
1995 Subtotal capsulotomy right breast  
1997 Capsulotomy right breast  
2006 Bilateral exchange of implants and capsulectomies | 1988 Right side implant infection returned to theatre for exchange of implants |
| 4       | 2006 Wide local excision and axillary node clearance right breast  
2006 Adjuvant radiotherapy  
2007 right side breast augmentation  
2007 removal of right ruptured implant  
| 5       | 1980 Multiple lumpectomies right breast (histology showed benign disease)  
1982 Bilateral breast augmentation  
1999 Bilateral mastopexies  
2001 Bilateral exchange of implants | |
| 6       | 2004 Wide local excision left breast (incompletely excised)  
2004 Further wider excision  
2004 Adjuvant radiotherapy  
2006 Bilateral breast augmentation | |
| 7       | 2007 Bilateral breast augmentation for congenital asymmetry  
2011 Insertion larger left breast implant for asymmetry  
2011 Evacuation left breast haematoma | 2011 Post op haematoma left breast returned to theatre for evacuation. Implant replaced. |
| 8       | 2006 Right breast skin sparing mastectomy and immediate implant reconstruction  
2006 Adjuvant radiotherapy | |

**Figure 4.1:** Previous breast surgery including treatments for capsular contracture and complications.