University of the Witwatersrand

School of Clinical Medicine

Department of Internal Medicine

Analysis of three genes that contribute to fibrosis in South African systemic sclerosis patients

Jacqueline Michelle Frost

DECLARATION

I declare that this work is my own work. It is being submitted for the degree of Master of Science in Medicine, MSc (Med), at the University of the Witwatersrand. It has not been submitted for any other degree at any other University.

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Jacqueline M. Frost
03 May 2010

"The best scientist is open to experience and begins with romance -

the idea that anything is possible"

Ray Bradbury, author of 'Fahrenheit 451' and 'Something wicked this way comes', amongst others.

ACKNOWLEDGEMENTS

ABSTRACT

Introduction: Systemic sclerosis (SSc) is a complex autoimmune disease characterised by autoantibody release, leading to microvascular injury, fibroblast activation and increased production of collagen. The genetics of SSc is complex with many genes implicated in the development and maintenance of the extracellular matrix (ECM). The main aim of this study was to test for differential expression of *matrix metalloproteinase 1 (MMP1), tissue inhibitor of metalloproteinase 1 (TIMP1)* and *hepatocyte growth factor (HGF)* in SSc patients compared to healthy control individuals and to assess whether the differential expression of these genes could have an impact on clinical features of the disease.

Methods: Two skin biopsies were analysed for each of 16 black SSc patients, one from clinically involved skin (lateral forearm) and one from clinically uninvolved skin (back). One skin sample was obtained from 15 ethnically matched control individuals. The differential expression of *MMP1, TIMP1* and *HGF* in the clinically involved and uninvolved patient samples would be compared to control individuals using relative quantification polymerase chain reaction (qPCR). The gene expression profiles were then compared to specific clinical features to deduce whether any of the gene expression profiles is correlated with the manifestation of specific clinical features.

Results: *MMP1* gene expression was significantly decreased in SSc patients for both involved (p=*0.0004*) and uninvolved skin (p=*0.0004*) compared to controls. Conversely, *TIMP1* gene expression was significantly increased in SSc patients at both sites compared to controls (p=*<0.00001* for both comparisons). A trend of significance was observed for the difference in *TIMP1* expression between the involved an uninvolved skin within the patients (p=*0.05*) with a greater increase in involved skin. *HGF* had increased gene expression in the patients compared to controls for involved and uninvolved skin (p=*0.002* and *0.004,* respectively). The difference in gene expression between the involved and uninvolved biopsies was not significant for either *MMP1* or *HGF* (p=0.87 and 0.83, respectively). The only correlates that may have a biological significance are *HGF* in involved skin correlated with disease activity (r=0.60; p=0.013) and *HGF* in uninvolved skin

correlated with skin score (MRSS) with r=0.50 and p=0.048. With regards to the categorical data, two marginally significant observations were found, once again with *HGF*, which was found to be associated with gender in involved skin (p=*0.037*) and renal disease in uninvolved skin (*0.031*).

Conclusion: The relative under expression of *MMP1* and over expression of *TIMP1* reflect the pro-fibrotic state of scleroderma skin. The over expression of *HGF* suggests that *HGF* may play a compensatory anti-fibrotic role, although this is not sufficient to overcome the pro-fibrotic state of the skin. This study provides supporting evidence to debunk the myth of uninvolved skin in SSc patients. The altered expression of *MMP1*, *TIMP1* and *HGF* in the clinically uninvolved skin of SSc patients suggests that all subcutaneous tissue is affected, although to a greater extent in the clinically involved skin of the patients.

ABBREVIATIONS

A	adenine
ACR	American College of Rheumatologists
ACA	Anti-centromere antibody
ANA	Anti-nuclear antibody
ATA	Anti-topoisomerse antibody
BAL	broncheoalveolar lavage
bp	base pairs
C	cytosine
°C	degrees celcius
C4	complement 4
cDNA	complementary deoxyribose nucleic acid
c-Met	c-mesenchymal-epithelial transition factor
CP	Crossing point in qPCR (also known as CT or Cq)
CpG	cytosine and guanine separated by a phosphate
CRP	C-reactive protein
CTGF	connective tissue growth factor gene
DAS	Disease activity score
dcSSc	diffuse cutaneous systemic sclerosis
ddH$_2$O	deionised distilled water
dH$_2$O	distilled water
DNA	deoxyribose nucleic acid
ddNTP	dideoxyribonucleoside triphosphate
dNTP	deoxyribonucleoside triphosphate
DLCO	diffusing capacity of the lung for carbon monoxide
ECM	extracellular matrix
ESR	erythrocyte sedimentation rate
G	guanine
g	gram
GFP	green fluorescent protein

HATs	histone acetyltransferases
HDACs	histone deacetylases
HGF	hepatocyte growth factor gene
HLA	human leukocyte antigen
IL	interleukin
ISH	*in situ* hybridisation
kb	kilobases
kDa	kilo Dalton
lcSSc	limited cutaneous systemic sclerosis
MgCl$_2$	magnesium chloride
mg/L	milligram per litre
ml	millilitre
µl	microlitre
miRNA	micro ribose nucleic acid
mRNA	messenger ribose nucleic acid
MMP	matrix metalloproteinase
mmol/L	millimoles per litre
mmHg	millimetres of mercury
mm/hour	millimetres per hour
MRSS	modified Rodnan skin score
NAC	no-amplification control
NTC	no template control
ng	nanograms
PCR	polymerase chain reaction
PDGF	platelet derived growth factor gene
PTN22	Protein tyrosine phosphatase, non-receptor type 22 gene
qPCR	quantitative real time PCR
RA	rheumatoid arthritis
RNA	ribose nucleic acid
RNAi	RNA interference
rt-PCR	reverse transcription PCR
SAGE	serial analysis of gene expression

Scl-70	Scleroderma 70 antigen (Anti-topoisomerase 1)
SSc	systemic sclerosis
SLE	systemic lupus erythematosus
SNPs	single nucleotide polymorphisms
T	thymine
Taq	DNA polymerase isolated from *Thermus aquaticus*
TGFβ	transforming growth factor beta gene
TIMP	tissue inhibitor of matrix metalloproteinase
Tsk	tight skin mouse
USA	United States of America
3'UTR	3' untranslated region
vs.	versus

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Chapter 1

Introduction

1.1. Definition

Systemic sclerosis or scleroderma (SSc) is an autoimmune systemic rheumatic disease characterised by vascular damage, subcutaneous and visceral fibrosis and circulating autoantibodies (Vlachoyiannoploulos, 2001).

1.2. History of SSc

Cases of skin diseases that have similar descriptions to SSc can be found in very ancient writings, such as that of Hippocrates (460-370 B.C.). It is however, difficult to say whether these ancient alludings are truly examples of the disease, as the descriptions were vague and inexact (Coyle, 1988). The first modern published account of SSc is generally attributed to the Italian, Curzio, in 1753 (reviewed in Roberts-Thomson and Walker, 2006). The patient was a 17-year-old woman, who was assigned to Dr. Curzio when she was admitted to hospital. As described by the doctor, her symptoms were: hardness of the skin (differing in degree from place to place), tightness around the mouth, and hardness around the neck. Curzio's student, J.L Alibert, claimed to be the first to recognise the disease, calling it "Sclermia Circumscripia" (Coyle, 1988).

There was little mention of the disease in medical literature until 1847, when Dr. Grisoll in Paris rediscovered the disease and opened areas of discussion. In 1854 Erasmus Wilson described the lesions of one of his patients as "en coup de sabre" or, "the scar of the sword wound". In popular literature during the same century, Sir Arthur Conan Doyle, the creator of Sherlock Holmes used SSc as the theme for his book "The Adventure of the Blanched Soldier" (Coyle, 1988).

By 1900, more than 500 cases of SSc had been reported and discussed in the medical literature (Coyle, 1988). The modern "father" of SSc was undoubtedly, Carwille LeRoy, who was a prolific figure in the field of rheumatology and was one of the first rheumatologists to apply modern scientific methods to study fibroblasts from SSc patients. In so doing, he was able to show that these fibroblasts had the ability to synthesise and secrete excess collagen *in vitro*. He was also among the first to show that vascular endothelium is a primary target in SSc (Reviewed by Silver, 2002).

1.3. Epidemiology
1.3.1. Prevalence

Approximately 75 000 to 100 000 people are affected with SSc in the United States, where the disease is found more frequently in African Americans than in Caucasians (Bogatkevich et al., 2007). The disease has a female:male ratio of between 3:1 and 8:1 after child-bearing years and a peak age of 45 to 55 years (Dumith et al., 2007). The prevalence of SSc in full-blooded Choctaw Indians from Oklahoma was reported to be at least 469/100 000, which is the highest in the world to be reported thus far (Arnett et al., 1996). These figures compare with an estimated prevalence of SSc in the USA of 26 cases/100 000 (Herrick and Worthington, 2002). Thai patients have a higher incidence of anti-topoisomerase 1 antibodies and are more likely to have diffuse disease compared with Australian Caucasians (Chen et al., 2003). African Americans and Hispanics are more likely to have diffuse skin involvement and pigment changes than Caucasians, who have more facial telangiectasia (visibility of small, dilated blood vessels near the surface of the skin) (Chen et al., 2003). The disease has a worldwide distribution and is found to be more frequent in females than in males, as much as an 80% higher incidence in females has been reported (Selmi et al., 2006). Different populations have been found to have differing susceptibility, which can be due to genetic and/or environmental agents. Large cohort studies from Australia and the USA have reported that SSc occurred in one or more first-degree relatives in 1.4% and 1.6% of the families of patients with SSc. The disease is generally considered rare in sub-Saharan Africans (Silman et al., 1996), although there is a considerable variation in the prevalence of the disease across the African

continent. In 1980, only two cases of SSc were reported in a teaching hospital in Nigeria (Somorin and Mordi, 1980) whereas the disease is widely reported in South Africa. The incidence of the disease in South African goldminers is approximately 2.5 fold more than the general population (7.3-8.1/100 000 vs. 0.33/100 000) (Cowie and Dansey, 1990; Tager and Tikly, 1999).

1.3.2. Environmental risk factors

Cases have documented the co-existence of silicosis and progressive SSc in Europe, Japan the USA and in Africa (Sluis-Cremer et al., 1985). SSc was uncommon in early studies from Africa. Only one case was documented in Nigeria and six in Zimbabwe over a 10-year period (Greenwood, 1968; Lutalo, 1985). Erasmus (1957) described the first association between gold mining and SSc in South Africa. His study was concerned with the high prevalence of progressive SSc in the Johannesburg area where gold miners were exposed to dust containing a high percentage (>30%) of free silica. Subsequent studies in the late 1980s documented cases of SSc in both white and black gold miners (Cowie, 1987). Pudifin et al. (1991) reported the largest series of SSc in Africa, where over 10 years 106 black and 60 Indian patients were seen. Antinuclear antibodies (ANA) and antitopoisomerase antibody (ATA) were more common amongst black patients with 54% being ANA positive and 32% being ATA positive.

A disease that appeared in Spain in 1981, affecting approximately 20 000 people, was named toxic oil syndrome as it was linked to the consumption of contaminated rapeseed oil sold in bulk in the industrial belt of Madrid. Patients presented clinically with edema, contractures, Raynaud's phenomenon and fibrosis of the dermis, suggesting a scleroderma-like syndrome (Alonso-Ruiz et al., 1986). In the United States during the early 1990s an association was made between ingesting tryptophan and a syndrome characterized by scleroderma-like skin abnormalities (fasciitis and eosinophilia), which developed within 1 to 18 months after the start of tryptophan therapy for insomnia, depression, or obesity. All had histopathological changes in the dermis and subcutaneous tissue typical of scleroderma (Silver et al., 1990).

1.3.3. Genetic risk factors

The genetics of the disease is complex and there are several lines of evidence that support a genetic component to susceptibility to SSc. Twins studies show a low disease concordance rate of less than 5% that is similar between mono and dizygotic twin pairs, although cases of twins with the disease are extremely rare (Feghali-Bostwick et al., 2003). Cohort studies, however, suggest that a positive family history is the strongest risk factor for SSc identified to date, with an increased relative risk among first-degree relatives. It is unclear however, if the increased risk is due to genetic influences or shared environmental factors (Chen et al., 2003). Due to the extreme variability of the disease phenotypes, epigenetic modifications have been suggested as additional factors, which can modify the SSc phenotype (Selmi et al., 2006). Evidence for the role of epigenetic modifications in SSc includes studies in which the addition of epigenetic suppressors normalised the expression of collagen within SSc fibroblasts (Wang et al., 2006).

Genetic association studies are generally regarded as a useful tool to study the molecular aetiology of complex diseases. Complex diseases are likely to be caused by several genes, each with a minor contribution to the overall relative risk. It has been proposed that combinations of common genetic variants, in particular single nucleotide polymorphisms (SNPs), not only influence the susceptibility to complex diseases but also are associated with particular aspects of the disease phenotype. The literature is littered with studies reporting relationships between various genes and SSc susceptibility or severity. Such genes include the HLA and non-HLA genes (Gilchrist et al., 2001; Gladman et al., 2005), tumor necrosis factor-α (Sato et al., 2004), endothelin (Fonseca et al., 2006) and fibrillin (Tan et al., 2001). All of these studies are able to provide potential insight into the pathogenesis and aetiology of the disease. Many studies have shown strong association between polymorphisms and the presence of antibodies, which are hallmarks of SSc, such as ATA or anti-centromere (ACA) reactivity (Fanning et al., 1998). The information generated by genetic association studies could prove to be valuable in diagnosis or therapy, but the results have to be interpreted with caution. Most limitations to these studies are connected to poor study design and statistical analysis. Underpowered studies

are able to provide interesting results, but with no reproducibility. A good example of this problem is the results from studies on polymorphisms in the *PTPN22* gene in SSc patients that cannot be replicated or that are even contradictory (Balada et al., 2006; Gourh et al., 2006).

1.4. The Clinical Triad

The disease is a multi-system disease, which is highly heterogeneous and has an unknown aetiology (Varga and Abraham, 2007). Progression of the disease is known to involve three main systems including the immune system, the vasculature system and the extracellular matrix (ECM) (Whitfield et al., 2003). Clinically the disease can be separated into diffuse cutaneous systemic sclerosis (dcSSc) and limited cutaneous systemic sclerosis (lcSSc). The clinical manifestation of each of these sub-groups is widespread skin thickening and skin thickening that is limited to the face and distal extremities, respectively (Steen, 1998).

The most common manifestations of SSc are the presence of specific autoantibodies with more than 90% of patients having antinuclear antibodies in their serum. Anti-topoisomerase 1 antibodies are almost exclusively present in sera from patients with the diffuse form of SSc, with a higher prevalence of these autoantibodies seen in African American patients (Maul et al., 1989). Anti-centromere antibodies are more commonly present in the sera of patients with the limited form of the disease. These two autoantibodies are mutually exclusive, coexisting in the same patient only in rare instances. Although autoantibodies are very common in SSc, they are not directly involved in the clinical manifestations of the disease and it is generally accepted that their titres do not correlate with disease activity or clinical severity (Derk and Jimenez, 2003).

The majority of SSc patients suffer from primary Raynaud's phenomenon, which is an episodic event, characterised by pallor and pain in the peripheries in response to cold stress (Young-Min et al., 2001). This symptom is caused by micro-vascular abnormality leading to reduced blood flow resulting in digital ulcers and, in some cases, gangrene

(Kuwana, 2006). Other symptoms of SSc include muscle weakness (myositis), problems of the digestive system and pulmonary arterial hypertension (Clements, 2000).
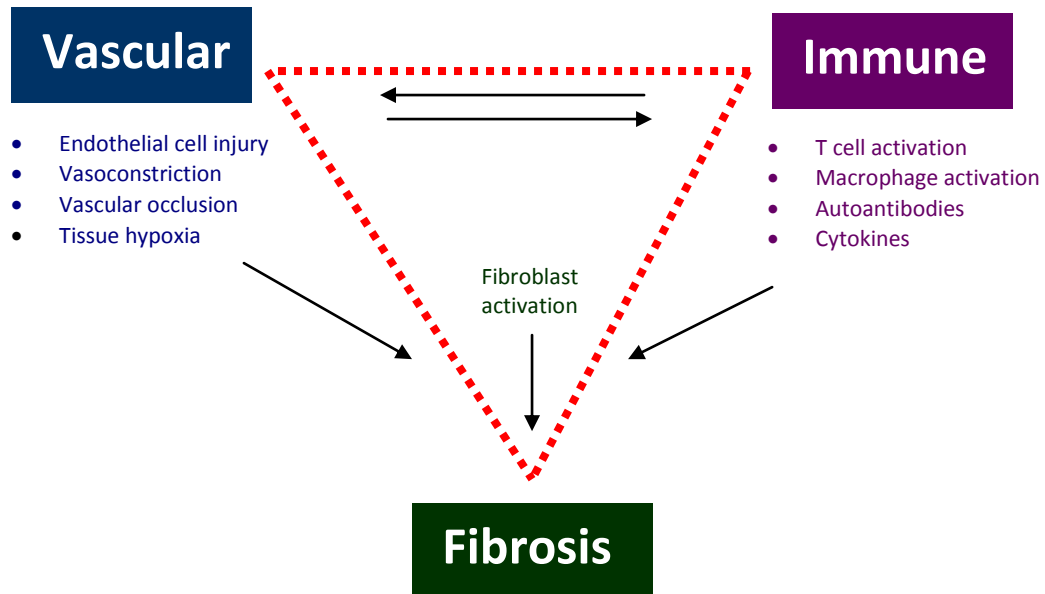
Figure 1.1. Schematic diagram of the SSc clinical triad.

The hallmark symptom of the disease is the gradual thickening and tightening of the skin. Often ulcers are found, particularly on the fingertips, which are slow to heal due to poor circulation. Other changes in the skin include pigmentation alteration and calcium deposits under the skin (Clements, 2000). Fibrosis is especially significant in the diffuse form of the disease. Basic remodelling of the ECM occurs causing the architecture of the skin to become disrupted and this leads to fibrosis of not only the skin, but also many internal organs. The lungs, gastrointestinal tract and heart are the worst affected by the fibrotic process. Much morbidity of the disease is due to fibrosis of the internal organs (Yazawa et al., 2000).

1.5. Prognosis and Treatment

Clinical outcomes have improved considerably in the last decade, presumably due to better management of the complications. The disease is however, still considered incurable and the diffuse cutaneous form of the disease carries the highest risk of fatality

within the connective tissue diseases, carrying a 55% survival rate at 10 years (Varga and Abraham, 2007). Many treatments are being tested for use in SSc patients to alleviate the symptoms of the disease. Drugs that are prescribed for treatment of the fibrosis include methotrexate, cyclophosphamide and d-penicillamine. Experimental treatments include intravenous gamma globulins, collagen tolerance induction and anti-TGF-beta antibodies (Bournia et al., 2009). The immunosuppressive drug, rapamycin, has been found to exert an antifibrotic effect, which could be caused by the drug's ability to directly target ECM deposition (Poulalhon et al., 2006). Although these treatments alleviate some of the symptoms of the disease, they tend to not be very effective, thus highlighting the need for research into an effective therapeutic intervention and on-going clinical, pharmaceutical research. To date there is no effective method of treatment for fibrosis.

1.6. Pathogenesis

There has been considerable research toward investigating and elucidating the pathogenic processes involved in the development of SSc. Factors affecting susceptibility and the initiating event remain largely unknown, however as this is a multifactorial disease, there are genetic as well as an environmental components. The sequence of events leading to the development of SSc is illustrated in Figure 1.2.
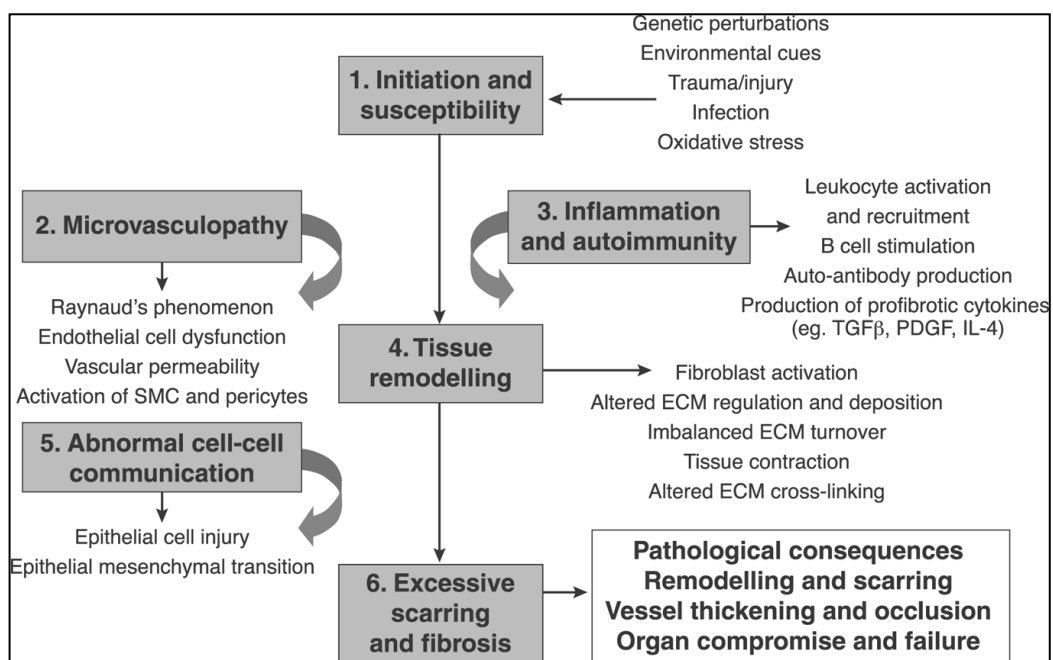
Figure 1.2. The sequence of pathological events that lead to the development of SSc (Abraham and Distler, 2007).

1.6.1. Endothelial cells

The endothelium is a highly specialised and complex organ involved in many important functions. It enables a compatible interface facilitating blood circulation, inhibits excessive platelet aggregation and coordinates vascular tone, which serves to inhibit ECM deposition. The endothelium has many prominent endocrine functions, the most important of which is the control of inflammation (Abraham and Distler, 2007).

Healthy functioning of the endothelium is crucial for remodeling of blood vessels during times of tissue growth and repair. Dysfunction of the endothelium is an important component of many common human diseases, including those characterized by inflammation and fibrosis (Figure 1.3). Endothelial dysfunction contributes to inflammatory and fibrotic connective tissue diseases, particularly SSc and the dysfunction that is observed results from endothelial cell injury leading to the generation of an inflammatory process and endothelial cell activation (Ross, 1999).
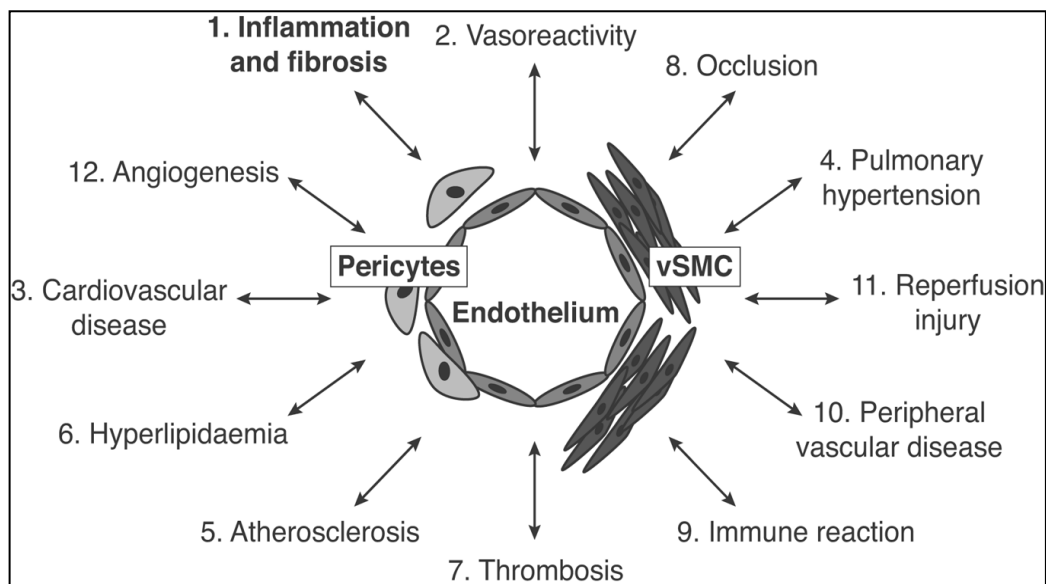
Figure 1.3. The many mechanisms of endothelial dysfunction, all of which are evident as symptoms in systemic sclerosis (Abraham and Distler, 2007).

1.6.2. Immunocytes (T-cells)

Immunocytes, or T cells, play a pivotal role in SSc although their precise role has not been fully elucidated. It is likely that T cell-derived cytokines contribute to the development of fibrosis. A study by Fuschiotti et al. (2009) focused on the ability of CD4+ and CD8+ T cells to produce cytokines following *in vitro* activation. They concluded that dysregulation of IL-13 production by CD8+ T cells is important in the pathogenesis of SSc and appears to be critical in the predisposition to more severe forms of cutaneous disease. Abnormalities in the cellular immunity of SSc patients consist of chronic mononuclear cell infiltration of tissues and the dysregulation of lymphocytes, growth factor and the production of autoantibodies. The expansion and activation of CD4+T cells as an early immune event is considered important in the development and maintenance of SSc (Besliu et al., 2009). T cells in the skin exhibiting high interleukin-4 (IL-4) production and T cells in the blood that express chemokine receptors associated with TH2 functions are found in SSc patients with active disease. Dermal fibrosis and capillary loss typical of systemic sclerosis can be reversible after high-dose immuno-suppression and haematopoietic stem cell transplantation (Chizzolini, 2008).

1.6.3. Fibroblasts

Fibroblasts play an important role in the metabolism of the ECM connective tissues within the skin. Fibrosis of the skin is due to an increase in the deposition of the ECM, including microfibrils and collagens (Lemaire et al., 2006). The ECM functions as a reservoir for many growth factors including *TGFβ*, *CTGF* and a number of other multicellular proteins. Excessive connective tissue accumulation is due to an overproduction by fibroblasts, which are activated by cell-cell or cell-ECM interactions. Collagens are by far the most abundant ECM components and have crucial roles in organ development, growth and differentiation. The collagen genes have been studied extensively and have been found to harbour highly conserved regulatory regions that are recognised specifically by DNA-binding transcription factors. Any alterations that occur in these interactions will contribute towards persistent fibroblast activation in systemic sclerosis (Varga and Abraham, 2007).
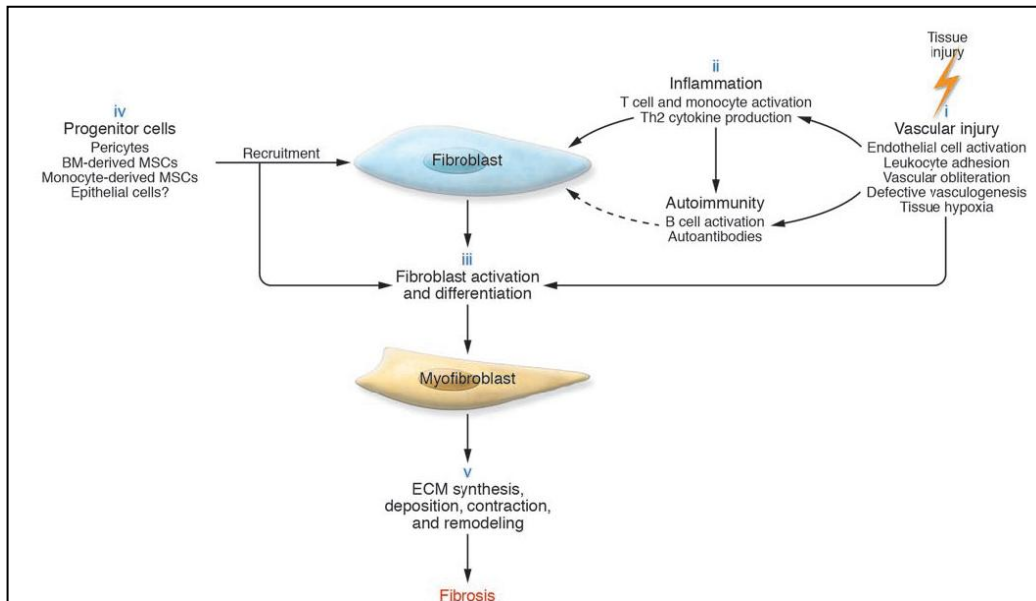
Figure 1.4. Vascular and immunological processes leading to fibrosis in SSc (Varga and Abraham, 2007).

There are many molecular effectors and determinants of fibrosis, the master regulator of these is considered to be *TGFβ* (Ihn, 2002). This gene is considered a potent pro-fibrotic stimulus and regulates cell growth, death, differentiation and synthesis of the ECM (Ihn, 2008). An increase in the number of *TGFβ* receptors has also been linked to the development of fibrosis (Yamane et al., 2002) although fibroblasts from SSc patients have been found to produce *TGFβ* at levels equivalent to that of normal fibroblast cells (Needleman et al., 1990).

CTGF (connective tissue growth factor) functions as an adaptor molecule that connects cell surfaces to the ECM. The gene encoding CTGF is expressed in relatively low levels in the tissue of normal individuals whilst showing markedly increased expression in SSc patients and in animal models such as the tight skin (*Tsk*) mouse (Ihn, 2002). *CTGF* over expression is considered a molecular marker of fibrosis. Hepatocyte growth factor (*HGF*) has been found to attenuate *CTGF* expression and collagen accumulation in mouse models with fibrosis (Bogatkevich et al., 2007).

1.7. Mediators of collagen metabolism

Collagen is the most abundant proteins on earth and accounts for 30% of the total protein in the human body. Collagen provides normal tissue with strength, integrity and structure and when tissue is injured, collagen repairs and restores structure and thus function. Too much collagen at a wound results in fibrosis and conversely, if insufficient amounts of collagen are deposited, the wound is weak and may rupture (Burgeson, 1987). Pivotal in the turnover of the ECM is the matrix metalloproteinase (MMP) family who have the ability to degrade not only collagen, but also most proteins that constitute the ECM. Conversely, the tissue inhibitors of metalloproteinases (TIMPs) protect the integrity of the ECM by inhibiting MMP activity (Hall et al., 2003). Hepatocyte growth factor is known to have anti-fibrotic properties and its ability to break down collagen has been reported in many studies (Bogatkevich et al., 2007; Esposito et al., 2009). Figure 1.5 illustrates the effect of growth factors (*TGFβ* and *CTGF*), matrix metalloproteinases, tissue inhibitors of metalloproteinase and hepatocyte growth factor on collagen metabolism, and consequently fibrosis and their role in ECM synthesis or degradation.

Pro-fibrotic = ↑ Collagen

↑*TIMP1*
↓*MMP1*
↑*TGFβ*
↑*CTGF*

↑HGF
↑*MMP1*
↓*TIMP1*

Anti-fibrotic = ↓ Collagen
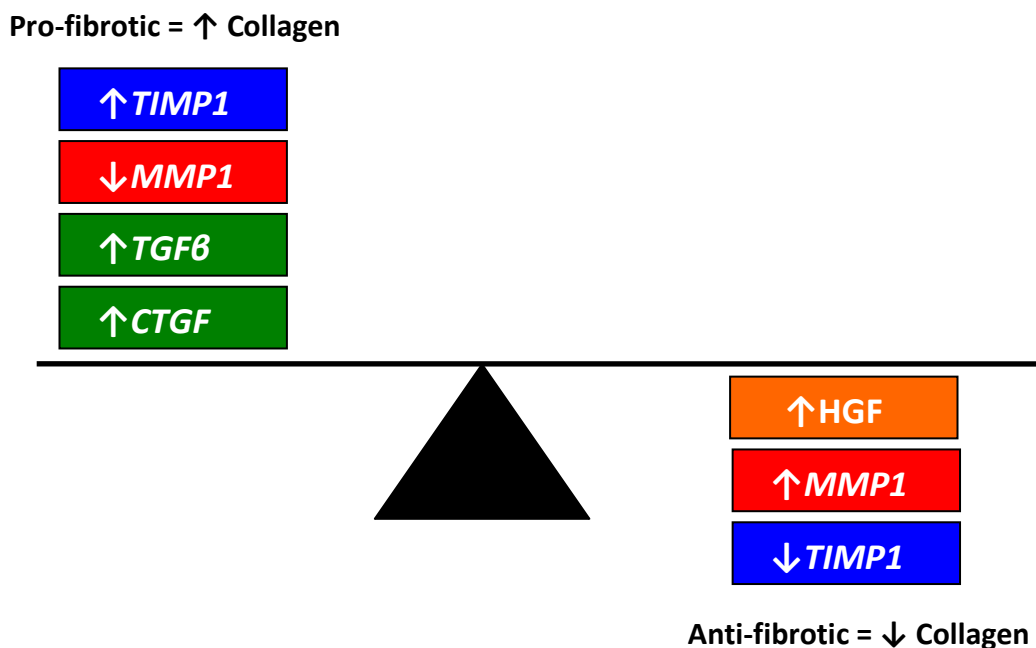
Figure 1.5 Diagrammatic representations of the key modulators of fibrosis in SSc.

1.7.1. Matrix metalloproteinase 1

The first of the metalloproteinases (MMPs), interstitial collagenase 1 (*MMP1),* was discovered by Gross and Lapiere in 1962 in the tail of a tadpole. The MMPs are all involved in normal tissue remodelling processes, such as embryonic development, postpartum involution of the uterus, ovulation and wound healing as well as in important disease processes such as joint destruction in rheumatoid arthritis (RA) (Curry et al., 2003; Salmela et al., 2004; Woessner, 1991; Pap et al., 2005). The accumulation of ECM is dependent on the balance between the synthesis and degradation of the metalloproteinases (Matilla et al., 1998).

The chief characteristics of genes of the metalloproteinase family are, their dependency on zinc for their activation, the cDNA sequences all show homology to that of collagenase, the enzymes cleave one or more components of the extracellular matrix and finally, the activity of the proteinases is inhibited by one of the tissue inhibitors of metalloproteinases (TIMPs) (Toubi et al., 2002). *MMP1* is by far the most widely studied metalloproteinase and was found to degrade fibrillar collagen at a neutral pH (Young-Min et al., 2001).

In SSc, as in other rheumatic diseases such as RA and systemic lupus erythematosus (SLE), certain cells including fibroblasts are exposed to pro-inflammatory cytokines. These cytokines, IL-1, IL-6 and *TNFβ*, are responsible for the resultant overproduction of MMPs (Toubi et al., 2002). Antibodies specific to both *MMP1* and *MMP3* have been reported specifically in the serum of SSc patients (Arnett, 2006). These antibodies have been proposed to result in a failure to degrade ECM components that cause accumulation of fibrotic material (Arnett, 2006).

1.7.2. Tissue inhibitor of metalloproteinase 1

Tissue inhibitors of metalloproteinases (TIMPs) are secreted from fibroblasts and act locally to specifically block the active MMPs and prevent matrix digestion. Four TIMPs

have been described to date of which *TIMP1* and *TIMP2* are the most widely studied and are produced in soluble forms. The expression of *TIMP1* is also differentially regulated and production of the gene product is enhanced by growth factors, especially TGFβ (Matilla et al., 1998).

Many previous *in-vitro* studies have shown that skin fibroblasts from SSc patients produce elevated amounts of *TIMP1* when compared to control samples (Matilla et al., 1998). *TIMP1* also appears to be increased in serum levels of SSc patients who have active disease (Kikuchi et al., 1995). This brings to the fore that it is the balance between matrix degrading enzymes and their inhibitors that contribute to the development and pathogenesis of fibrosis in SSc (Toubi et al., 2002). A study by Susol et al. (2000) indicates that increased expression of *TIMP1* may be a susceptibility marker in males who develop SSc. There was a lack of association with the *TIMP1* marker and female SSc patients, which may indicate that the aetiology of the disease is different in males and females (Susol et al., 2000). Systemic sclerosis cell lines have been found to synthesize elevated levels of *TIMP1* and these high levels could play an important role in the fibrotic process by inhibiting collagen degradation (Kirk et al., 1995).

In a study conducted by Kikuchi et al. (1995) an increased level of *TIMP1* in serum was correlated with an increased incidence of lung fibrosis. It has also been suggested that the *TIMP1* level reflects the severity of the disease in patients with SSc. Fibroblasts from SSc patients have shown decreases in collagenase activity and production, with no observable differences seen in the levels of collagenase mRNA, which suggests that collagenase is altered at the level of translation (Takeda et al., 1994).

1.7.3. Interaction between *MMP1* and *TIMP1*

TIMPs form very tight complexes in a 1:1 ratio with the MMPs (figure 1.6), although with some low selectivity, for example *TIMP1* does not always have to bind to *MMP1*, it can bind to many of the other MMP molecules. Mutational analysis has shown that changes made to the interaction site of the TIMPs can drastically change the affinity for different MMPs (Iyer et al., 2007). Investigating the roles of *MMP1* in physiological processes could

form part of a potential route for new therapies in diseases that are associated with excess MMP activity (Iyer et al., 2007).
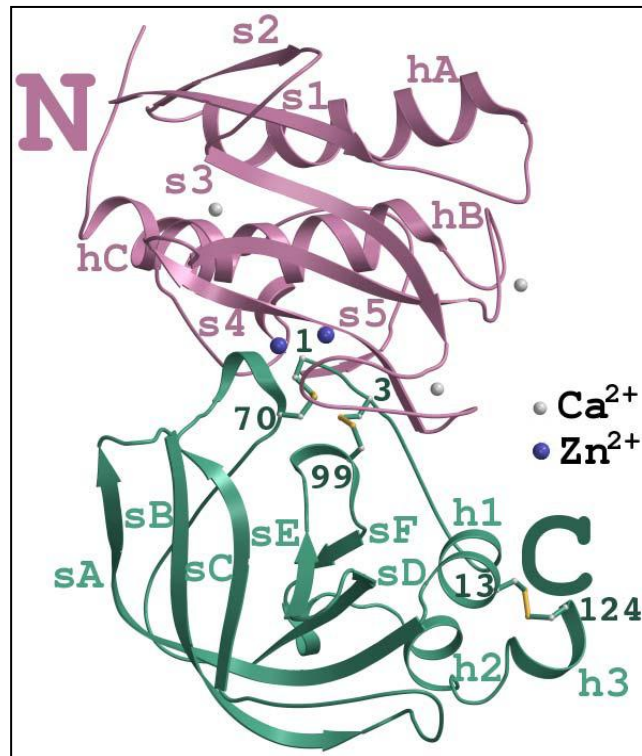
Figure 1.6. Ribbon three-dimensional structure of the N-terminal domain of *TIMP1* (green) in complex with the catalytic domain of *MMP1* (pink) (Iyer et al., 2007).

1.7.4. Hepatocyte growth factor

Hepatocyte growth factor (HGF), a potent mitogen also known as "scatter factor", shows mitogenic and morphogenetic properties in a variety of cells (Jinnin et al., 2005) and is involved in tissue regeneration after organ injury (Bogatkevich et al., 2007). Much evidence has accumulated which indicates that HGF has an important role *in vivo* as a hepatotrophic factor during regeneration of livers that have been injured by hepatectomy or hepatotoxin treatment (Kawaguchi et al., 2002). HGF targets many cell varieties including mesenchymal cells such as fibroblasts and renal epithelial cells. The many regulating effects of the gene product include cell proliferation, migration, morphogenesis, development and regeneration (Kawaguchi et al., 2002).

HGF has recently been shown to reverse the process of fibrosis by successfully inhibiting ECM deposition and reducing the amount of fibrillar collagen (Motone et al., 2004). This function of HGF has been studied extensively in an animal model but its effects in humans are not fully understood. One of the anti-fibrotic mechanisms of HGF however involves the induction of *MMP1* expression. The effects of HGF in SSc fibroblasts have not been studied extensively and in one study the investigators were interested in the direct effect of HGF on the expression of type 1 collagen (Jinnin et al., 2005). The study found that HGF functions like a cytokine that mediates tissue remodelling in the liver and skin. The study concluded that HGF has a direct effect on the deposition of collagen in fibrotic conditions (Jinnin et al., 2005).

Bogatkevich et al., (2007) undertook a study, which compared cytokine composition in bronchoalveolar lavage (BAL) samples in African Americans and Caucasians with SSc. They observed that *HGF* was the only cytokine that caused lower collagen expression in the lung samples. Further study showed that *HGF* was diminished in the lung fibroblasts isolated from the African American patients only, due to limitations in c-Met receptor phosphorylation.

1.8. Methods for studying gene expression

There is an array of methods that can be used for studying the expression of genes, each with advantages and disadvantages. Although relative quantitative polymerase chain reaction (qPCR) was the method of choice for this study, this and a few other methods are addressed along with some advantages and disadvantages.

Using reporter genes such as green fluorescent protein (GFP) can reveal where and when a gene is expressed. The expression of a gene is controlled by regulatory sequences, which can be manipulated to drive the expression of a reporter gene, by replacing the target gene's coding region with the reporter gene. The level, timing, and cell specificity

of reporter protein production reflect the action of the regulatory sequences that belong to the original gene (Chalfie et al., 1994).

Northern blotting allows the detection of low-resolution expression patterns by hybridising a gene or cDNA probe to total RNA or poly (A)$^+$ RNA extracts prepared from different tissues or cell lines. Because the RNA is size-fractionated on a gel, it is possible to estimate the size of transcripts (Alwine et al., 1977).

In situ hybridisation (ISH) is a technique that enables the morphological demonstration of specific DNA or RNA sequences in specific cells or tissues. It is the only method that allows for the identification of specific DNA/RNA sequences in a heterogeneous cell population, and thereby provides information on whether a gene is expressed in low or high levels in the cells. When this technique was introduced in 1969, it was developed for the easy localisation of DNA sequences, since then it has also been applied to viral DNA, mRNA and entire chromosomal regions (Simmons et al., 1989).

Immunohistochemistry is a technique that localises proteins in tissue sections by exploiting the principle of antibodies binding to antigens in biological tissues. Since immunohistochemistry involves specific antigen-antibody reaction, it has apparent advantage over traditionally used special enzyme staining techniques that identify only a limited number of proteins, enzymes and tissue structures (Ramos-Vara, 2005).

Serial analysis of gene expression (SAGE) is a method for multiplex gene expression screening which uses short sequence tags, which are located within transcripts. SAGE is one of the most accessible methods for studying gene expression, as it does not require sophisticated equipment to track gene expression. SAGE provides a measure of transcript quantification since genes that are highly expressed in a specific tissue will frequently be recovered as sequence tags. An advantage of the method is its speed (Velculescu et al., 1995).

In recent years, studying gene expression by conventional real time PCR (qPCR) has become the standard technique for various reasons. The advantage of PCR-based methods for studying gene expression is the speed, sensitivity and simplicity of the technique as well as providing a rough quantification of the expression of a particular gene. The sensitivity of PCR means that this method can also be used to study expression in single cells. In addition, qPCR can be useful for identifying and studying the expression of different isoforms of an RNA transcript (Heid et al., 1996).

Developed in the 1990s, DNA microarrays have revolutionized the way in which gene expression is analysed by allowing the RNA products of thousands of genes to be monitored at once. The ratio of two different fluorochromes reflects the relative expression of the genes on the slide. DNA chip technology has revolutionised the study of gene expression and has become standard practise in many gene expression studies but the most challenging part of any microarray experiment is the analysis of the data that is produced (Schena et al., 1995).

1.9. Microarray studies for gene expression in SSc

Studies that have analysed the gene expression in SSc fibroblasts using microarray, although few, have shown that there are consistent differences in the gene expression patterns between tissue samples from controls and patients. These differences were even detected in skin that was not clinically affected at the time the biopsy was taken (Whitfield et al., 2003). In this same study DNA microarray showed that *TIMP1* is up regulated in patients with SSc compared to control individuals. This up regulation was up to eight times higher in some patients.

In another study by Milano et al. (2008) it was found that gene expression profiling on a genome-wide basis demonstrated that the heterogeneity of SSc can be quantitatively measured using DNA microarrays. Such diverse gene expression shows that there are multiple and distinct gene expression profiles specific to the skin of SSc patients. But the interesting and perhaps most important finding in this study was that the gene expression

data shows that there are at least two very distinct patient sub-groups within patients with the diffuse disease. The authors also report on a 177-gene signature that is associated with the severity of the skin in the diffuse disease.

1.10. Factors controlling the expression of genes

Aberrant gene expression could be caused by a number of different mechanisms, each one unique and exerting it's affect in a different manner. The change observed in the patients with respect to the expression of particular genes could be caused by polymorphisms in the promoter sequences of the genes, epigenetic modification, abundance of transcription factors, hormones, other physiological differences between males and females as well as medication.

1.10.1. Polymorphisms

There are currently few candidate gene single nucleotide polymorphism (SNP) association studies for SSc, and in these the investigators use small sample sizes and the reproducibility of the results is low. Interestingly, several studies have implicated promoter polymorphisms suggesting that these may affect gene expression. Some examples of SNP associations found in SSc include *TNFα* polymorphisms −238A and +489A, and a *MCP1* promoter polymorphism, −2518 (G/G genotype is associated with SSc) (Assasi and Tan, 2005).

A SNP in *MMP1,* where a G is inserted into the promoter region was found to up regulate the transcription of this gene by creating a transcription factor binding site. This increases the transcription of the gene, resulting in irreversible degradation of collagen (Rutter et al., 1998). In a study by Indelicato et al. (2006) three polymorphisms were analysed in *TIMP1* (-19A>G, +261C>T and +372T>C). They found that the C allele of the +372T>C polymorphism was observed in higher frequencies in male SSc patients than controls while there was no difference in the frequency of the allele between female SSc patients and controls. They suggested that this polymorphism might predispose men to SSc. Three

SNPs within *HGF*, -1652C>T in the promoter region, +44222C>T in intron 9, +63555G>T in exon 15 were genotyped in SSc patients. The +63555 T/T genotype was correlated with marginally decreased gene expression in SSc patients, compared with healthy controls (p=*0.02*). Of 141 SSc patients whose disease duration was > 10 years, 17 (12%) developed lung disease, which was found to be more prevalent in patients with the −1652 T/T genotype than in those without (p=*0.002*). It was concluded that these SNPs in *HGF* could be used to predict lung disease in SSc patients (Hoshino et al., 2008). Further analysis of these genes, as a whole, need to be done in order to deduce if functional polymorphisms contribute as causative agents in the development of the disease. Most recently polymorphisms have been found in the IRF5 region (Ito et al., 2009) and the hypoxia-inducible factor 1A (*HIF1A*) gene (Wipff et al., 2009). These polymorphisms are associated with SSc in patients of Japanese and European ancestry respectively.

1.10.2. Epigenetic Modifications

Epigenetics refers to the heritable changes in gene expression that do not involve changes to the DNA sequence. The epigenetic regulation of gene expression is a dynamic process that plays a pivotal role in normal cell growth and differentiation (Strietholt et al., 2008). There are three main epigenetic mechanisms, DNA methylation, histone modification and RNA interference (RNAi) (Cheng et al., 2008).

DNA is highly organised within the chromatin. Euchromatin is uncondensed and transcriptionally active whereas heterochromatin is condensed and transcriptionally inactive rendering the genes in that area silenced. In order to achieve these states of transcriptional activity, the chromatin is dynamically modified by either histone acetyltransferases (HATs) or histone deacetylases (HDACs). HATs catalyse histone acetylation and this hyperacetylation of the N-terminus of the histones is associated with the opening of the chromatin and thus enhancing the rate of gene transcription and therefore expression (de Ruijter et al., 2003). On the other hand, HDACs remove the acetyl group from the histones resulting in hypoacetylation, reducing the space between the chromatin and the DNA. This hinders transcription factors from binding to the DNA,

leading to the silencing of affected genes (Briggs et al., 2002). HDAC inhibitors have been found to be therapeutically beneficial in certain cancers (Kim et al., 2006) as well as animals models of autoimmune diseases such as multiple sclerosis (Gray and Dangold, 2006) and SLE (Mishra et al., 2003). In SSc, the knockdown of HDAC7 in skin fibroblasts and treatment of skin fibrosis in mice with the HDAC inhibitor "trichostatin A" have both been shown to markedly reduce the accumulation of ECM protein and therefore the development of fibrosis (Nasu et al., 2008).

DNA methylation involves the methylation of deoxycytosine bases in CG dinucleotide pairs often within CpG islands and as this is the major form of modifying eukaryotic DNA, it has a profound effect on gene expression. DNA methylation is responsible for inhibiting gene expression and does so in several ways (Li et al., 2009). Methylation of promoter sequences inhibits the ability of certain transcription factors binding to the DNA. Several DNA methyltransferases (Dnmt) are responsible for catalysing the methylation process, of which Dnmt1 is the most abundant. Insertion of a methyl group into DNA by a Dnmt results in changes in chromatin structure and thus silencing of an affected gene. Methyltransferases are also susceptible to inhibition by chemicals and are sensitive to environmental influences which both have an effect on gene expression patterns (Richardson, 2008). Recently, mutations in Dnmt3 have been found to cause abnormalities in the immune system (Richardson, 2008). The role of DNA methylation in regulating retroviral elements responsible for the reactivation of suppressed sequences could contribute to autoimmunity (Richardson, 2008).

Recently, a new class of post-transcriptional regulators has emerged. These are small, endogenous, single-stranded, non-coding RNAs called micro RNAs (miRNAs). These have been estimated to account for between 2% and 3% of the human genome and regulate approximately 30% of human genes (Lewis et al., 2003). There is rapidly increasing evidence that miRNAs are involved in many physiological and pathological processes, including an important role in immune responses and the development of autoimmunity (Cobb et al., 2006; Rodriguez et al., 2007; Li et al., 2007; Pauley et al., 2009). Recent studies have found that altered expression and function of miRNAs could be involved in the pathogenesis of rheumatoid arthritis (RA) (Stranczyk et al., 2008) and SLE (Dai et al.,

2007). In RA patient samples it was found that miR-155 expression was higher than in control samples and it is suggested that a possible target of this miRNA is matrix metalloproteinase 3 (*MMP3*). This may be a mechanism to modulate downstream tissue damage. Figure 4.1 is a summary of the consequences of irregular miRNA regulation in immune functions.

The detailed role of miRNA regulation and dysregulation in autoimmune diseases still needs to be elucidated. However, miRNAs are emerging as a potential target for new therapies in the treatment and prevention of autoimmunity.
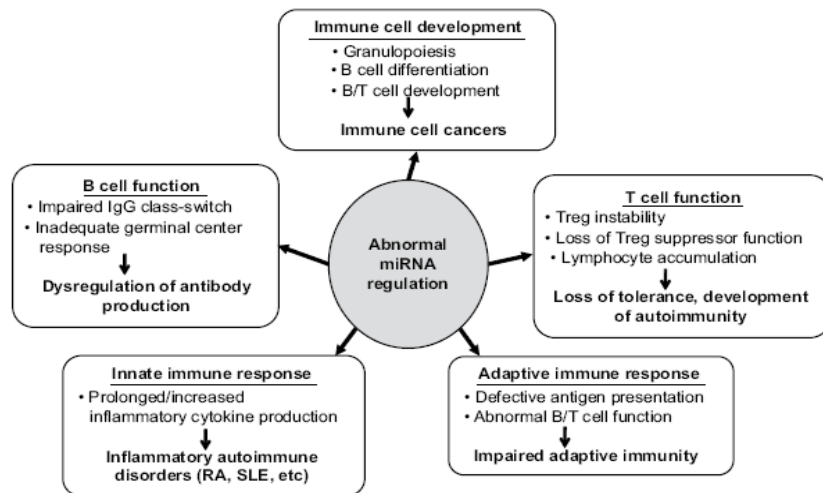
Figure 1.7. Consequences of abnormal miRNA regulation in autoimmunity (Pauley et al., 2009)

1.11. Study Aim and objectives

The predicted outcome of the study is that both *TIMP1* and *HGF* will have higher expression levels in the clinically involved SSc skin compared to both the control and clinically uninvolved SSc skin. Conversely, *MMP1* is expected to have lower expression in the clinically involved SSc skin compared to the control and clinically uninvolved SSc skin. The clinically uninvolved skin from the SSc patients is expected to have similar expression levels for *MMP1*, *TIMP1* and *HGF* to the control skin.

The aim of this study was to test for differential expression of *MMP1, TIMP1* and *HGF* in SSc patients compared to healthy individuals and to determine if expression profiles correlated with clinical features. There were three objectives to achieve this aim.

Objective 1: To elucidate the gene expression profiles of *MMP1, TIMP1* and *HGF* in skin biopsy samples from SSc patients and healthy controls using quantitative real time PCR (qPCR).

Objective 2: To elucidate the gene expression profiles of *MMP1, TIMP1* and *HGF* in clinically involved SSc skin compared to clinically uninvolved SSc skin using quantitative real time PCR (qPCR). This objective will refute or support the myth of uninvolved skin in SSc patients.

Objective 3: To correlate the gene expression profiles of the patients with clinical features of the disease.

Ethics clearance for collection and use of these biological samples was obtained from the Human Research Ethics Committee (Medical) of the University, ethics approval number M070820 (Appendix A).

Chapter 2

Subjects and Methods

2.1. Subjects

2.1.1. Patients

Consenting black South African patients from Chris Hani Baragwanath Hospital who fulfilled the 1980 American College of Rheumatology (ACR) preliminary criteria for classification of Scleroderma (Masi et al., 1980), had diffuse cutaneous disease (Leroy et al., 1988) and who had less than 6 years disease duration were included in the study. Originally 17 patients were recruited but one was excluded due to long disease duration and thus only 16 patients were used in the study.

Clinical and laboratory features that were documented at the time of taking biopsy samples were:

1. <u>Modified Rodnan skin score</u>:

Bedside clinical assessment of subcutaneous skin thickening based on a scale from 0 (normal) to 3+ (severe) at 17 anatomic areas; the maximum score is 51 (Furst et al., 1998). The assessment was done by a single trained medical professional to avoid inter-observer variability.

2. <u>Interstitial lung disease</u>:

Bilateral respiratory fine crackles, radiograph revealing reduced lung volume and bilateral, reticulonodular opacities. FEV1 (forced expiratory volume in one second) and FVC (forced vital capacity) both diminished (Harrison et al., 1991).

3. <u>Pulmonary hypertension</u>:

Right systolic ventricular pressure of >40mmHg on echocardiogram (Gaine and Rubin, 1998).

4. Renal involvement:

Proteinuria levels at +1 protein or more on a urine dipstick. Renal dysfunction is indicated by serum urea levels of >8mmol/L and/or creatinine levels of >100mmol/L.

5. Myositis:

Symmetrical proximal muscle weakness with at least 2 of the following three features: 1) typical electromyelogram changes, 2) raised creatine kinase, 3) muscle degeneration or necrosis (Bohan and Peter, 1975).

6. C-reactive protein:

The CRP test indicates acute inflammation by assessing the amount of the protein in blood (normal range between 1.0 and 3.0 mg/L, high >10 mg/L). To perform the test, patient serum is added to a CRP/latex solution onto a glass slide. Positive and negative controls should be done simultaneously. After slight agitation the results are read under an indirect oblique light source. A positive reaction is indicated by agglutination.

7. Erythrocyte sedimentation rate:

The ESR test is a measure of the settling of red blood cells per hour. This rate is an indication of inflammation (High >30mm/hour). To perform the test, anticoagulated blood is placed in an upright Westergren tube, and the rate at which the red blood cells fall is measured and reported in mm/h. Although, since the introduction of automated analysers into the laboratory, the ESR test has been automatically performed.

8. Serum C4:

Serum levels of C3 and C4 are determined antigenically by a turbidimetric procedure. Turbidity testing determines the cloudiness of a solution and measuring the loss of intensity of a light beam that passes through that solution. Levels of between 0.2 and 1.0 are considered low.

9. Global disease activity:

This is calculated using scores that are assigned to certain parameters (MRSS, scleredema, skin, digital necrosis, vascular, arthritis, DLCO, ESR>30mm/hour, hypocomplementemia) with maximum scores of individual parameters ranging from 0.5 to 2 giving a maximum total of 10. The disease is considered active if the score is equal to or greater than 3 (Valentini et al., 2001).

2.1.2. Controls

Control samples were obtained from fifteen consenting (Appendix B), otherwise healthy individuals undergoing reconstructive plastic surgery at the Charlotte Maxeke Johannesburg Academic Hospital.

2.2. Laboratory Methods
2.2.1. Collection and transportation of samples

Two 4mm punch skin biopsies were taken from each patient. A biopsy for clinically involved skin was taken 4cm proximal to the ulna styloid (Whitfield et al., 2003) on the dorsum of the left forearm and the biopsy for clinically uninvolved skin was taken from the upper back. Skin samples from control individuals were collected from the forearm area, except from one individual where it was taken from the groin. The skin samples for both patients and controls were placed in a cryotube and immediately immersed in liquid nitrogen so that the sample is flash frozen to ensure that no RNA degradation occurs.

2.2.2. RNA extraction

RNA was extracted from skin biopsies that had been flash frozen in liquid nitrogen within a few days of collection using the commercially available RNeasy Micro Plus kit (Qiagen) (Appendix C). The tissue sample was cut in half and approximately 1-2mg of the tissue sample was used for RNA extraction. The RNA was quantified on the Nanodrop spectrometer, showing that the average yield was 1µg of total RNA. Purity of RNA isolated with RNeasy Kits was evaluated by determining the ratio of absorbance readings at 260 nm and 280 nm (A260/A280). This ratio provides an estimate of the purity of RNA with respect to contaminants that absorb in the UV range, such as protein. Pure RNA has an A260/A280 ratio of 1.8-2.1, the RNA isolated in this study averaged a ratio of 1.8 , which is considered pure enough to continue with qPCR experiments (Wilfinger et al., 1997).

The RNA was used directly for reverse transcription polymerase chain reaction (rt-PCR). All the RNA samples were stored at -70°C. These samples were found to be stable for up to one year.

2.2.3. Reverse transcription polymerase chain reaction (RT-PCR)

According to nomenclature published in the MIQE (minimum information for publication of quantitative real-time PCR experiments) (Bustin et al., 2009), reverse transcription PCR is abbreviated to RT-PCR, qPCR refers to quantitative real-time PCR and Cq should be used as to describe the fractional PCR cycle used for quantification as CT and CP becomes confusing. The MIQE abbreviations will be used throughout this dissertation.

Total RNA extracted from the skin samples was reverse transcribed into cDNA using the ImProm-II Reverse Transcription kit (Promega). This kit uses an oligo (dT) reverse transcription primer, which will bind to the poly-A tails of the mRNA fragments. All the reagents and enzymes within the kit were extremely temperature sensitive and thus had to be thawed on ice. Incubation steps in this protocol had to be performed using a heating block or a PCR machine without a heated lid. It is important to note that both a no-template control (NTC) as well as a no-amplification control (NAC) was used during reverse transcription runs. An NTC should be run to rule out cross contamination of reagents and surfaces. This control includes all of the RT-PCR reagents except the RNA template, substituted for with nuclease-free water. No product should be synthesised, if a product is amplified it is an indication that one or more of the RT-PCR reagents is contaminated with the amplicon. The NAC is a minus-reverse transcriptase control, which contains all the RT-PCR reagents, including RNA, except the reverse transcriptase. If a product is seen in the NAC, it probably indicates that contaminating cDNA is present in the sample.

Firstly, 4µl target RNA was added to 1µl of 0.5µg/µl Oligo (dT) primer and incubated at 70°C for five minutes. The samples were then centrifuged, during which time the other reagents were combined in the amounts given in Table 2.1. The reagents were then

added to the RNA/primer mix. The PCR conditions using a GeneAmp 2720 Thermal cycler are: anneal at 25°C for five minutes, extend at 45°C for 1.5 hours and inactivate at 70°C for 15 minutes. The cDNA samples were then quantified using the Nanodrop ND1000 spectrophotometer. The cDNA could be stored at 4°C until required.

Table 2.1. Standard reverse transcription PCR mixture

Component	Concentration	Amount (µl)
Nuclease-free ddH$_2$0		3.7µl
ImProm-II buffer	5x	4µl
MgCl$_2$	25nM	4.8µl
dNTP Mix	10mM	1µl
RNAse inhibitor	40U/µl	0.5µl
ImProm-II Reverse Transcriptase enzyme		1µl
TOTAL		**15µl**

2.2.4. Primer design

Gene specific primers and total cDNA were used for qPCR (Appendix D). Primer pairs were designed so that they contained sequences from two adjacent exons ensuring that amplification of genomic DNA is reduced. The primers were then aligned against human cDNA reference sequences using the nucleotide BLAST algorithm in NCBI (http://www.ncbi.nlm.nih.gov). The primer sequences and annealing temperatures are listed in Table 2.2. The sequences of the primers for the reference gene, *GAPDH,* were found in the literature (Kapadia et al., 2003). All primers were purchased from Whitehead Scientific, manufactured by IDT (San Diego, United States of America).

Table 2.2. Primer sequences to amplify cDNA samples using Real Time PCR

Gene	Sequence (5' → 3')	Annealing temp (˚C)	Product size (bp)	Changes to protocol
MMP1 (F)	GAGCAAACACATCTGAGGTACAGGA	52	185	None
MMP1 (R)	TTGTCCCGATGATCTCCCCTGACA			
TIMP1 (F)	CCGACCTCGTCATCAGGGCCAAG	56	103	Added 4µl Betaine to each sample
TIMP1 (R)	AACCCTTTATACATCTTGGTCA			
HGF (F)	GAAGCTTGCCAGGCCTGCTGTC	57	135	Added 1µl primer
HGF (R)	CCATCATAGTTGATCAATCCAG			
GAPDH (F)	GAAGGTGAAGGTCGGAGTC	52, 56, 57	210	None
GAPDH (R)	GAAGATGGTGATGGGATTTC			

2.2.5. Quantitative real time PCR (qPCR)

Optimisation of any qPCR assay is key to its success and to ensure increased sensitivity, specificity and reproducibility (Appendix E). Primer concentrations in the reaction mix must be optimised to improve the assay sensitivity (Nolan et al., 2006). The use of melting curves when optimising is a powerful tool that provides accurate identification of amplified products and distinguishing them from primer dimers, as the short primer dimers will melt at lower temperatures. Lastly, the addition of PCR enhancers, such as DMSO or Betaine, has been known to greatly improve a real time assay. To optimise the qPCR assays in this study each of the above mentioned variables, as well as altering the number of PCR cycles during the qPCR run, were optimised to improve PCR sensitivity.

The Roche LightCycler® 2.0 was used for the gene expression analysis. The assays were set up using the standard protocol for qPCR with slight modifications for each gene (Table 2.3). Preparation was done in a pre-PCR room, where no PCR products are allowed, to ensure minimal risk of contamination. Before starting PCR set-up, an enzyme was added to the SYBR Green DNA 1 (Roche) Master Mix Tube as per the supplier's recommendations. This mixture had to be handled in a way that avoided light and heat exposure. The PCR mix was then placed in the well of the capillary tubes followed by the cDNA, the capillaries were then centrifuged and placed in the LightCycler®. Water blanks

were added as negative controls during the q-PCR runs. The standard assay protocol is shown in Table 2.4.

Table 2.3. Standard qPCR mix when using the Roche LightCycler® 2.0 using the Roche SYBR Green DNA 1 kit

Component	Concentration	Amount
cDNA	500ng/µl	2µl
ddH$_2$0		14µl
SYBR Green Master Mix	10x	2µl
Forward Primer	10µM	1µl
Reverse Primer	10µM	1µl
TOTAL		**20µl**

Table 2.4. Standard qPCR assay protocol for the Roche LightCycler® 2.0

Program	Analysis mode	Temperature and duration	Slope	Acquisition mode
Hot Start (1 cycle)	None	95°C → 10 mins	20	None
PCR (45 cycles)	Quantification	95°C → 10 secs	20	None
		Annealing temp → 10 secs	20	None
		72°C → 10 secs	20	Single
Melting Curve (1 cycle)	Melting curve	95°C → 0 secs	0	None
		65°C → 1 min	20	None
		95°C → 0 secs	0.1	Continuous
Cool (1 cycle)	None	40°C → 30 secs	20	None

After each qPCR run the products were run on a 3% agarose gel in order to ensure that the product being amplified was the correct size and that there were no spurious bands being amplified.

Relative qPCR measures the level of expression of a candidate or target gene to a reference gene, normally a ubiquitously expressed housekeeping gene. In this study the reference gene chosen was glyceraldehyde-3-phosphate-dehydrogenase (*GAPDH*) as this gene has been used as the endogenous control for many other SSc gene expression

studies (Renzoni et al., 2004; Nishijima et al., 2004; Wang et al., 2006; Milano et al., 2008). The results obtained from relative qPCR are measured as a Target gene/Reference gene ratio, which is calculated by the software (version 4.05) on the LightCycler® 2.0 instrument using the differences between the threshold crossing-points (Cq) of the samples. Three replicates were done per patient and control sample for each gene. Triplicate runs are the gold standard as this allows the investigator to check the reproducibility of the assay and the validity of the results obtained (Ahmed, 2002).

2.2.6. Analysis of qPCR

Analysis was based on the assumption that the concentration of DNA at a sample's crossing point (Cq) was the same for every sample containing the same target DNA. The Cq is the DNA concentration necessary for the LightCycler® fluorimeter to detect a signal. It is important that a Cq value differ by no more the 0.5 for any sample (Ahmed, 2002). To mathematically assess the reproducibility of the Cq values of the assay, the coefficient of variation (CV) was calculated. In order to calculate this value, the mean and the standard deviation and the Cq values for each data set were calculated. Previously published data on what the CV values should be to show high reproducibility of the assay varies between publications, but generally lie in the range of 0.5% - 16%. Tables containing the CV values, the mean expression ratios and the standard deviations calculated for *MMP1*, *TIMP1* and *HGF* are found in Appendix F. It is evident from the low CV values that the qPCR assays had a high degree of reproducibility between the triplicate real time PCR samples. This high reproducibility can also be visualised using graphs (Appendix G).

Depending on the initial concentration of DNA in the sample, the samples require a different number of cycles to reach the crossing point. Analysis uses the sample's Cq, the efficiency of the reaction and the number of cycles completed to determine how much the DNA concentration must have increased for each sample by the end of the amplification. The software (Roche LightCycler® software version 4.05) then uses these values to calculate, compare and generate the ratios. The efficiency value for an

experiment is calculated by using an external standard curve or by setting the efficiency value (equal to two) as default in the LightCycler® software.

Relative quantification can be calculated using either the $2^{-\Delta\Delta Cq}$ method or the variation of this, the ΔCq method using a reference gene (Livak and Schmittgen, 2001). Both methods assume that target and reference genes are amplified with efficiencies near 100% and within 5% of each other. Since qPCR assumes a linear relationship between initial template quantity and the Cq value obtained during amplification, optimisation of the qPCR assay is essential for accurate and reproducible quantification. There are three hallmarks of an optimised qPCR assay, these are; a linear standard curve with $R^2 > 0.980$, a high amplification efficiency of between 90 – 105% and consistency across replicate reactions (Appendix H).

The ΔCq method uses a reference gene, and although simpler to perform than the Livak method, gives essentially the same result. This method uses the difference between the reference Cq and the target gene Cq for each sample. A hypothetical example of a calculation is:

$$2^{(CP\ (GAPDH)-CP\ (target))} = Expression$$

Patient sample:
GAPDH CP=15.9
Gene X CP=12
Thus for patient gene expression,
Expression = $2^{(15.9-12)}$ = 14.9

2.3. Statistical analyses

Data were captured in an Excel spreadsheet and analysed using the statistical programs *SAS* (SAS Institute Inc.), *STATA* (StataCorp), *Statistica* (Statsoft Inc.) and *R* (R development core team). The non-parametric Mann-Whitney U (Wilcoxon) and one-way ANOVA tests were applied to compare gene expression between groups. Correlations between

continuous clinical variables and gene expression ratios were calculated using Spearman's correlation test. Statistical significance levels were set at p<0.05.

Chapter 3

Results

3.1. Clinical description

3.1.1. Patient demographics and clinical features

The demographic, clinical and laboratory data for the patient group are summarised in Table 3.1. The clinical data are summarised in Appendix I. Of note is that the vast majority of the patients recruited for the study were female (n=14), all patients were black South Africans and the mean disease duration was about 2 years. It is important to note that one patient (patient 10) was excluded from analysis since the patient had disease duration of 8 years, thus only 16 patients were used in data analysis.

Table 3.1. Demographic, clinical and laboratory data for the patient group

Clinical feature	Patients (n=16)
Female: Male ratio	7:1
Age of patient in years (*mean, SD*)	35.6 (10.6)
Disease duration in months (*mean, SD*)	24.7 (17.2)
Disease activity (*mean, SD*)	3.4 (1.7)
MRSS (*mean, SD*)	22.5 (8.5)
Involved biopsy skin score (*mean, SD*)	2 (0.57)
Interstitial lung disease	3/15 (20%)
Renal disease	2/15 (13%)
Myositis	4 (25%)
CRP (*mean, SD*)	32.5 (88.6)
Elevated ESR	8 (50%)
ANA positive	16 (100%)
ATA positive	4 (25%)
Low C4	3 (18.75%)

MRSS - modified Rodnan skin score, CRP - C-reactive protein, ESR - erythrocyte sediment rate, ANA - anti-nuclear antibodies, ATA - anti-topoisomerase 1 antibodies

3.1.2. Control samples

Fifteen control samples were collected from the Charlotte Maxeke Johannesburg Academic Hospital from black adult patients undergoing reconstructive plastic surgery. The mean age of the control samples was 30 years. The skin samples were mainly taken from around the hand, wrist or forearm, with only one sample being taken from the groin area. The ratio of males to females was 7.5:1. Table 3.2 is a summary of the control samples.

Table 3.2. Control sample information

Control	Age	Site of skin sample	Gender
1	34	Left hand	M
2	33	Left hand	M
3	31	Skin graft – groin	M
4	58	Left hand	F
5	20	Left forearm	M
6	19	Left forearm	M
7	30	Right hand	M
8	22	Right hand	M
9	52	Left hand	M
10	31	Left hand	M
11	19	Right wrist	M
12	29	Left forearm	F
13	20	Left hand	F
14	32	Left hand	M
15	21	Right forearm	M

3.2. Gene expression

Initially the ratios of gene expression were used to compare the differences in the gene expression between the patients and controls and between the two samples (clinically involved and clinically uninvolved skin) taken from each patient.

3.2.1. Matrix Metalloproteinase 1

The differences in *MMP1* gene expression are illustrated in Figure 3.1. The boxplot clearly shows a difference in gene expression ratio when comparing the patient group to the control group. *MMP1* expression in the clinically uninvolved and involved skin of the SSc group was significantly lower than in the control group (p=*0.004,* for both comparisons). In contrast, there was no statistically significant difference in *MMP1* expression between clinically involved and uninvolved skin (p=0.876). Figure 3.2 illustrates the expression levels of *MMP1* in patient samples. *MMP1* was calculated to have lowered expression by 30-90% in SSc skin compared to that of the control samples. In the majority of patients (n=9/16) the relative gene expression of *MMP1* was higher in the uninvolved skin and in 4 cases expression was higher in the involved skin.
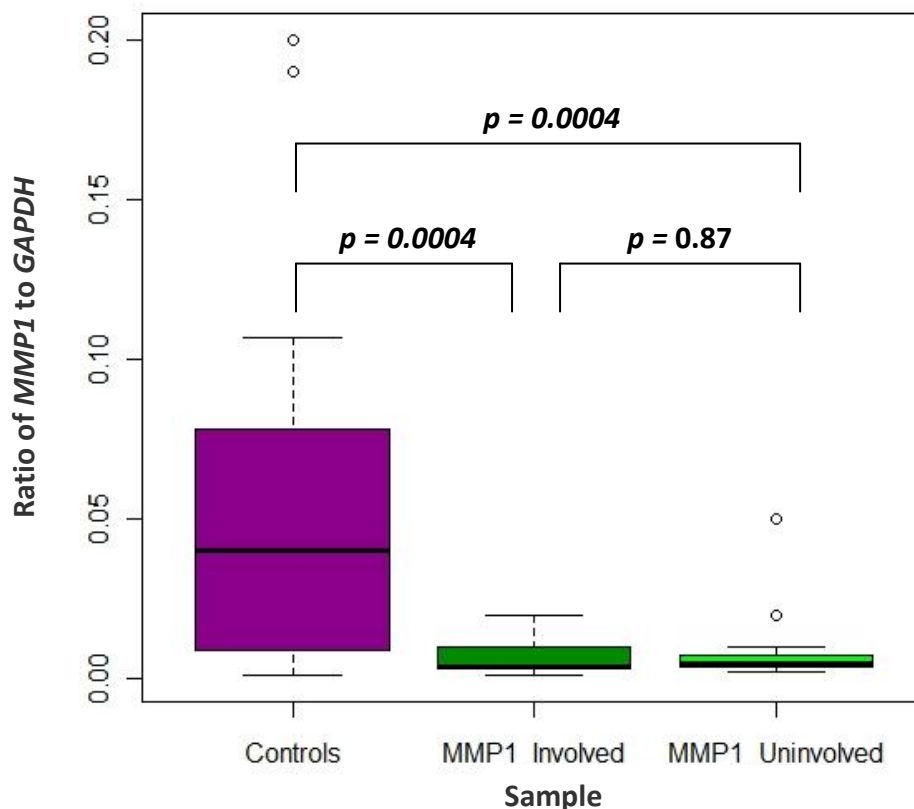
Figure 3.1. Boxplot illustrating differences in expression ratios of *MMP1* in the patient clinically uninvolved skin samples, patient clinically involved skin samples and control samples.
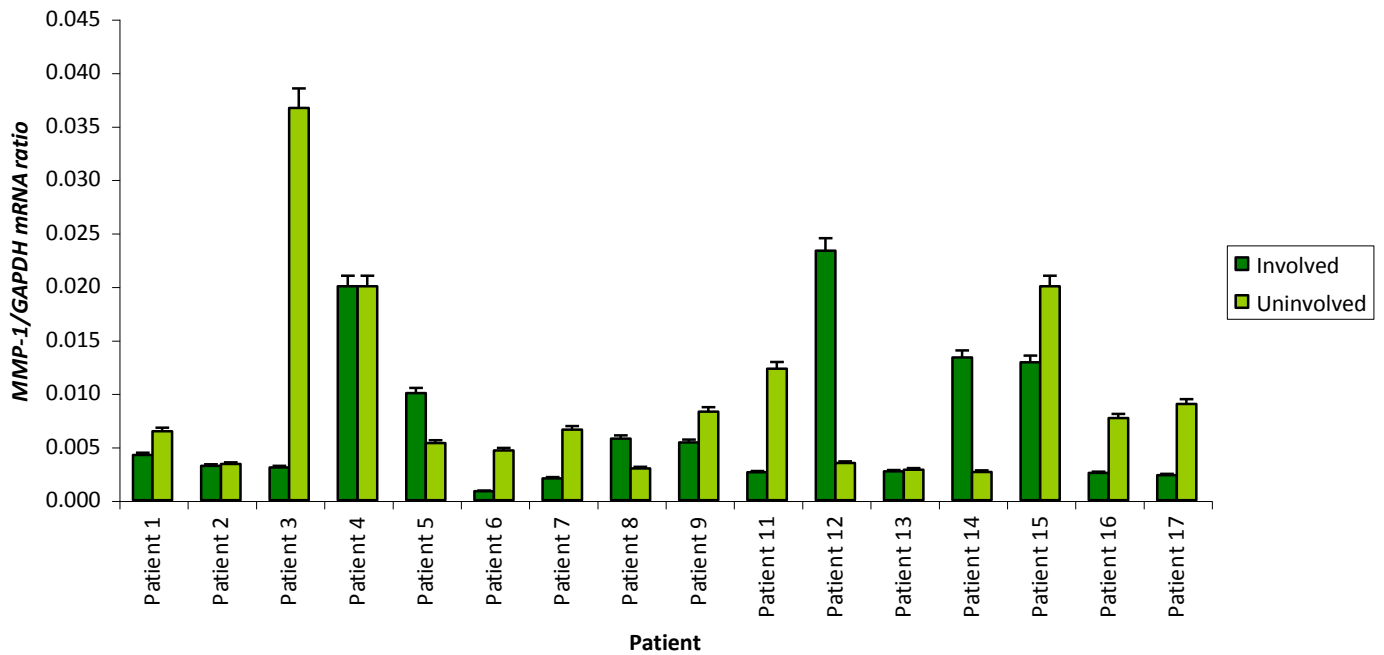
Figure 3.2. Means (±SEM) of normalised ratios of *MMP1* to *GAPDH* mRNA from the triplicate runs for patient samples.

3.2.2. Tissue inhibitor of metalloproteinase 1

The expression ratios of *TIMP1* in the controls, patient clinically uninvolved skin and clinically involved skin samples are illustrated in Figure 3.3. *TIMP1* gene expression is very low in the control samples and relative to this, the patient samples have higher expression, especially in the clinically involved skin samples. *TIMP1* expression in clinically involved and uninvolved SSc skin was significantly higher than the control group (*p<0.00001,* for both comparisons). A significant difference was also observed for *TIMP1* expression in the uninvolved skin compared to involved skin (p=*0.05*).

The patient group had an overall increased expression of *TIMP1* (Figure 3.4). This graph shows the expression levels of *TIMP1* calculated according to the ΔCq method. It is clear that *TIMP1* expression in SSc skin is expressed between 5 and 100 times more than in control skin. In 11 of the 16 patients, involved skin demonstrated higher levels of expression than uninvolved skin.
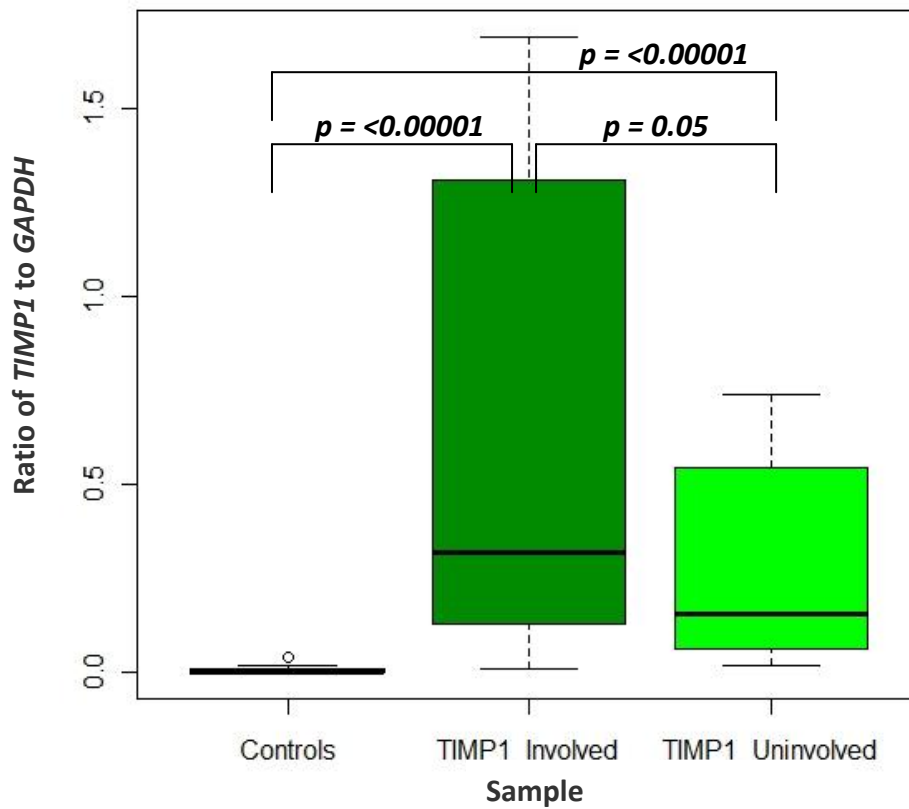
Figure 3.3. The expression ratios of *TIMP1* in the different sample groups clearly show the increased expression in the patient samples compared to the controls.
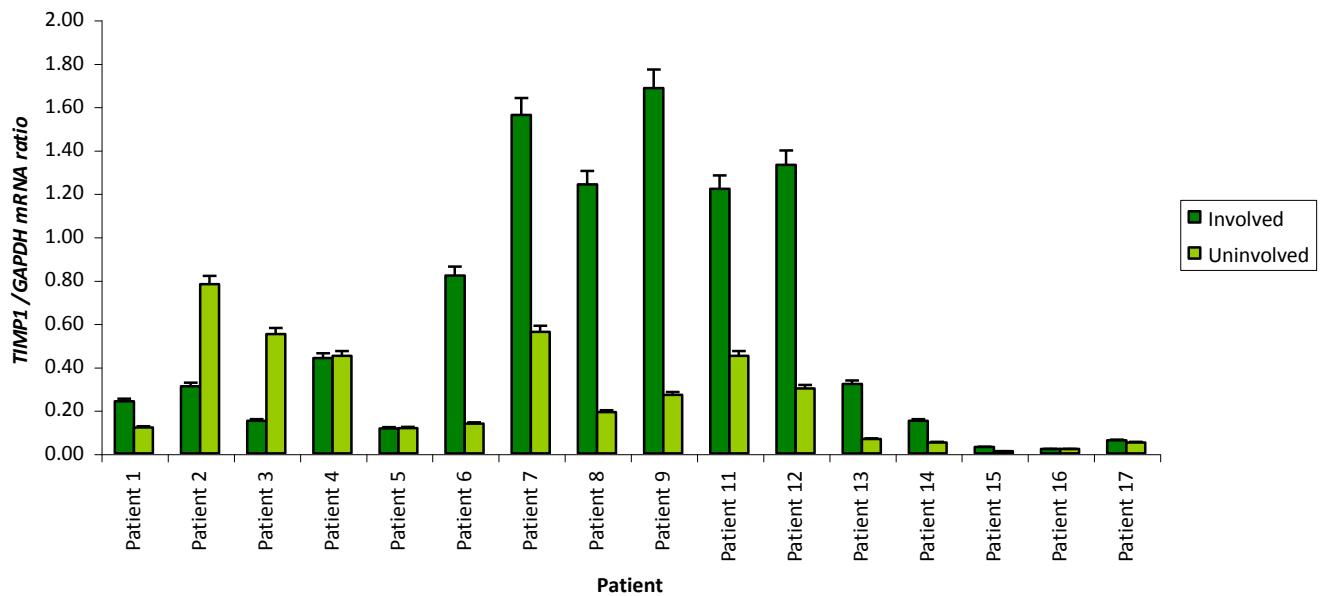
Figure 3.4. Means (±SEM) of normalised ratios of *TIMP1* to *GAPDH* mRNA from the triplicate runs for patient samples.

3.2.3. Hepatocyte growth factor

The relative expression ratios of *HGF* are shown in Figure 3.5, is clear from this boxplot that *HGF* has a higher expression in the patient samples compared to the controls. *HGF* expression in the uninvolved and involved SSc skin samples was significantly higher than in the control group (*p=0.002 and p=0.004 respectively*). In contrast, *HGF* expression reveals no significant difference between the clinically uninvolved and the involved skin (p=0.83). Figure 3.6 illustrates the expression levels of *HGF* in patient samples, and it is clear from this graph that this gene is expressed between 0.5 and 9 times more in SSc skin compared to normal skin.
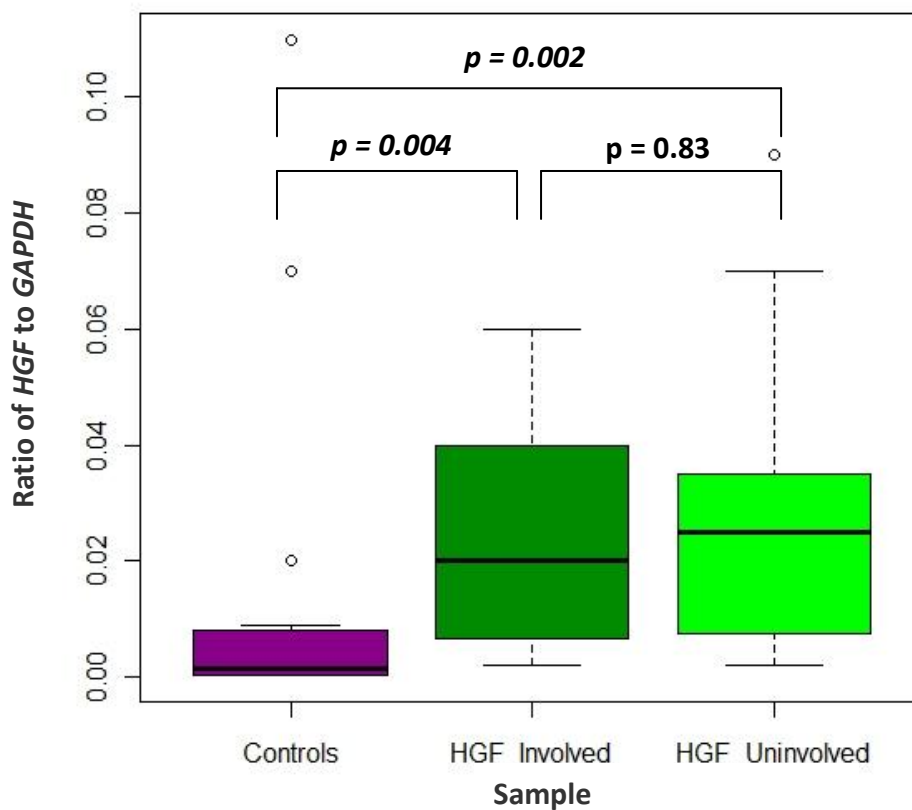
Figure 3.5. Boxplot showing the average relative expression of *HGF* in the patient samples and control samples.
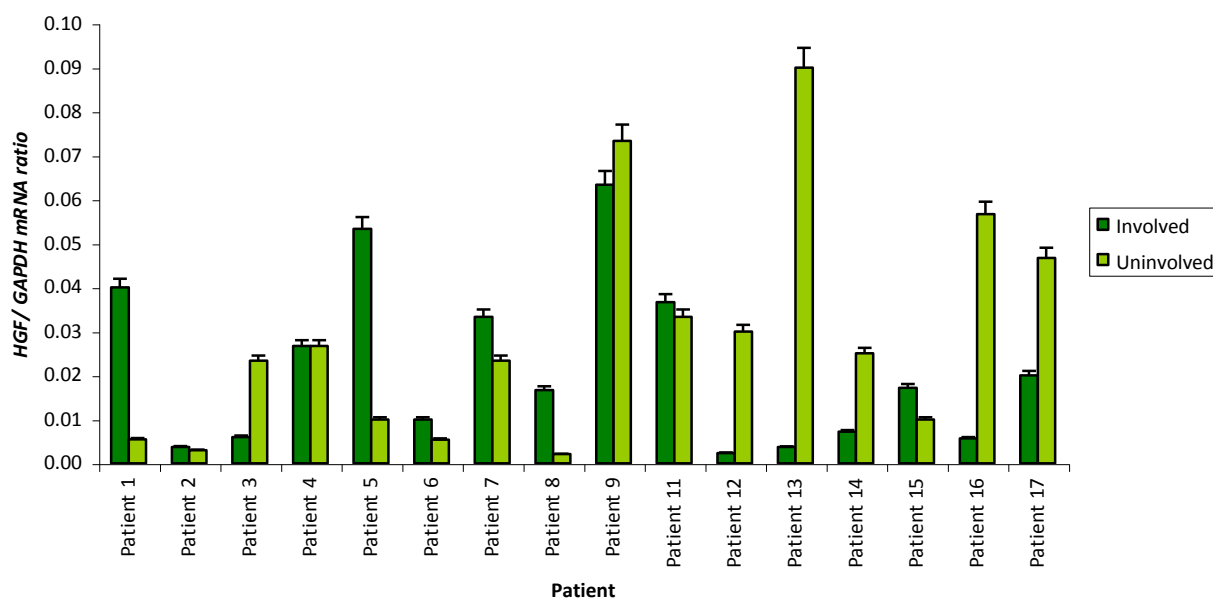
Figure 3.6. Means (±SEM) of normalised ratios of *HGF* to *GAPDH* mRNA from the triplicate runs for patient samples.

3.3. Correlations of clinical features with gene expression profiles

To calculate the associations of the clinical data with the gene expression profiles, the data were categorised depending on whether it was a categorical variable or continuous data. Continuous data (age, modified Rodnan skin score (MRSS), disease duration, involved skin biopsy skin score and disease activity) was analysed using the Spearman's correlation coefficient (Table 3.3). The correlation plots for the two statistically significant relationships were generated by *SAS* (Figures 3.7 and 3.8). Both of these relationships show a positive correlation with r values greater than 0.50. Analysis of the gene expression data with respect to specific categorical clinical data revealed that *HGF* expression in uninvolved skin was showing a trend in correlation with renal disease (n=4, *p=0.031*). HGF in involved skin also showed a trend in significance when correlated with the gender of the patients (*p=0.037*).

Table 3.3. *Correlations between continuous variables and gene expression*

		Involved skin			Uninvolved skin		
		MMP1	*TIMP1*	*HGF*	*MMP1*	*TIMP1*	*HGF*
Disease duration	r	-0.128	0.306	-0.060	-0.375	-0.028	-0.034
	p	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*
Age	r	0.101	-0.070	-0.2216	0.021	-0.064	0.414
	p	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*
MRSS	r	0.031	0.053	0.395	0.479	-0.225	0.500
	p	*NS*	*NS*	*NS*	0.060	*NS*	0.048
Involved skin score	r	-0.495	0.073	0.196	-	-	-
	p	0.061	*NS*	*NS*	-	-	-
Disease activity	r	0.292	0.096	0.600	0.276	-0.166	0.219
	p	*NS*	*NS*	0.013	*NS*	*NS*	*NS*
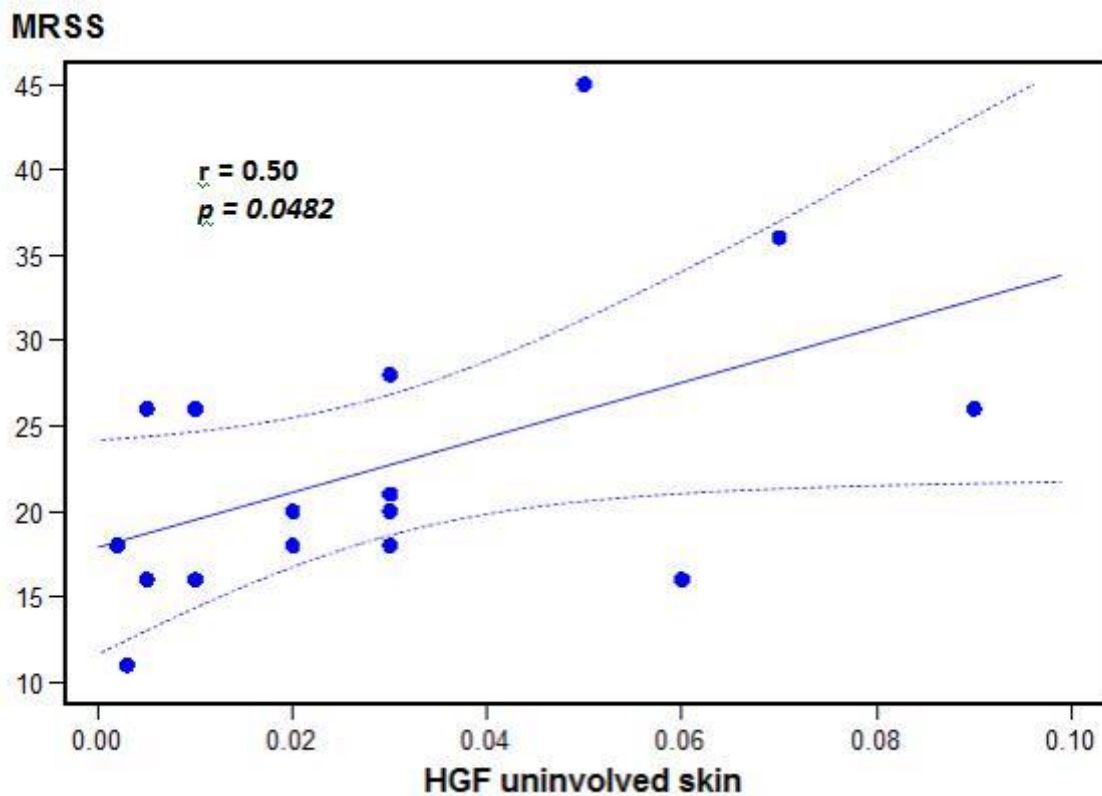
Figure 3.7. Correlation plot between the MRSS and *HGF* expression ratios in uninvolved skin
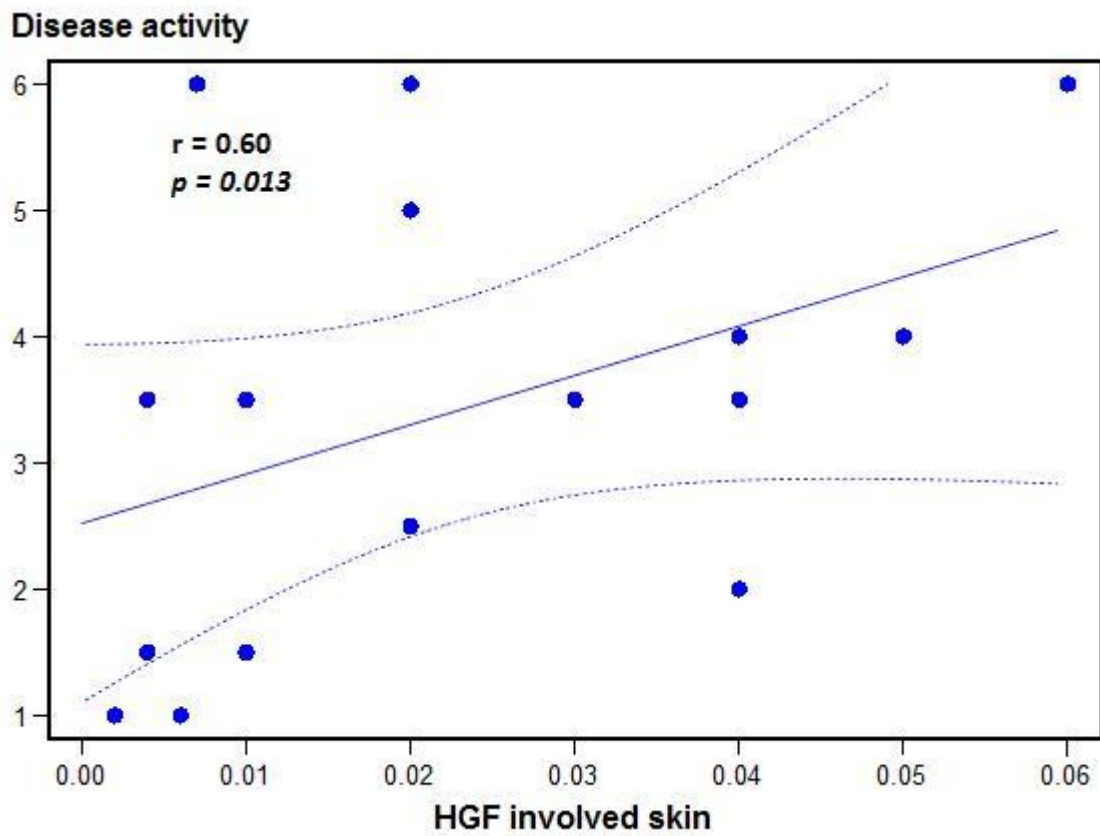
Figure 3.8. Correlation plot showing positive relationship between SSc disease activity scores and *HGF* gene expression ratios in involved skin from SSc patients

Chapter 4

Discussion

Relative quantification analysis of the genes revealed that matrix metalloproteinase 1 (*MMP1*) has decreased expression compared to the control samples, while both tissue inhibitor of metalloproteinase 1 (*TIMP1*) and hepatocyte growth factor (*HGF*) showed increased expression when compared to control samples. Due to their roles in the balance of the ECM, the results for *MMP1* and *TIMP1* were expected, whilst the results for *HGF* were surprising as they were not consistent with the hypothesised role the *HGF* plays in regulating the ECM. The clinical data that was collected from each patient was correlated to the differential expression of the genes, as the possibility that a clinical feature of the disease could be associated with the observed gene expression profiles, would be of great interest.

The discussion that follows explores the implications of the findings of this study in greater detail. Firstly, the differential expression of the genes in SSc patients compared to controls is considered. Secondly, the difference in gene expression between the two different samples obtained from each patient, that is the involved skin sample from the forearm compared to the clinically uninvolved skin sample from the back, is considered. In the last section, an assessment is done to determine whether the differential expression of these genes can explain any disease manifestations in the patients.

4.1. Clinical features

A similar number of patients (n=16) and controls (n=15) were investigated. To be included into the study as a patient, the individual had to fulfil the American College of Rheumatology (ACR) criteria for SSc and have the diffuse subtype of the disease. The duration of disease at the time of biopsy was crucial for inclusion into the study. Natural

regression of fibrosis of the skin has been reported in patients (Distler and Distler, 2009) and recruiting a patient with fibrotic regression had to be avoided. Two biopsies were required from each patient for the study; one from clinically involved skin and the other from clinically uninvolved skin, which would serve as an internal control. The area chosen for the clinically involved biopsy was on the forearm, more specifically 4cm from the ulna styloid on the left arm (Whitfield et al., 2003). The skin biopsy from the back was taken just under the scapula. The clinically involved area from which the biopsy was taken was given a skin score by one of the medical doctors who completed the clinical data sheets, for which the average skin score was 2. The biopsy that was taken from the back is not clinically affected by fibrosis and as such the skin score for this biopsy in every patient was zero.

Systemic sclerosis is considered active if a patient scores three or more on the DAS score (Valentini et al., 2001). In this patient group the mean disease activity score was 3.4, with 10 patients scoring above three. This indicates that most of the patients included in this study had active disease at the time the biopsies were taken. The MRSS takes into account the skin scores for 17 areas on the body of the SSc patient. The mean MRSS in the patient group was 22.6, ranging from 11 to 45, placing the majority of patients included in this study in the severe category for skin thickness. Muscle weakness, or myositis is quite common in SSc patients. The frequency of visceral organ involvement, including lung and renal disease was 20% and 13% respectively, which was less common than previously reported by Tager and Tikly (1999). All patients were ANA positive whilst only a quarter of them were ATA positive. This however is not surprising as all the patients had diffuse disease. This specific ATA is associated with interstitial lung disease. The large proportion of patients who had an elevated ESR is similar to previous findings in SSc patients of African decent (Tager and Tikly, 1999). Overall, there was nothing unusual about this group of patients compared to similar studies using SSc patients.

4.2. Differential gene expression

To be included as a control, the individual had to be undergoing reconstructive surgery, otherwise healthy and give informed consent to donate a skin sample. Due to the nature of the reconstructive surgeries, there are a large number of males compared to females (ratio 7.5:1). Conversely, the SSc patient group had a female to male ratio of 7:1. This is not unusual for a study based on an autoimmune disease. In a 2007 study using 3656 SSc patients there was a reported gender ratio of 6:1 in favour of females (Walker et al., 2007), while another study which included 1012 SSc patients of Italian heritage showed a female to male ratio of 7.8:1 (Ferri et al., 2002). The mean age of the patients recruited for the study was 35.6 years, 5.6 years on average more than the age of the control individuals. The mean disease duration for patients included in this study was 24.7 months.

In order to optimise the qPCR so that the assay was sensitive and reproducible, many factors were optimised. Primer concentrations started at 1µM and eventually ended at a usable 0.25µM, there were no spurious bands present and there was one clear, distinct band. The amount of cDNA added was either 500ng/µl or 1µg/µl, depending on the intensity of the resulting band. The number of cycles in the qPCR run was altered until a suitable cycle number was found. It was adjusted from 30 cycles to 45, which proved suitable for the production of sufficient product. Finally, 4µl of Betaine was added to two of the assays, this is a PCR additive which is reported to reduce the base pair composition dependence on DNA strand melting.

4.2.1. Comparison of the gene expression levels between patient and control samples

The three genes chosen for the study have previously been implicated in SSc and as mentioned previously they were chosen for their specific roles in the metabolism of the ECM. The matrix metalloproteinases (MMPs) are a group of endopeptidases that play a

pivotal role in connective tissue remodelling, especially during development, wound healing and other physiological processes. The endogenous inhibitors of MMPs are the tissue inhibitor of metalloproteinases (TIMPs), which function in controlling the activity of the MMPs (Iyer et al., 2007). MMPs degrade connective tissues and TIMPs inhibit MMPs thereby reducing MMP activity and consequently decreasing connective tissue degradation. It is the balance between these molecules that controls normal ECM degrading activity (Huang et al., 2001).

From the relative gene expression data observed, the expression of *MMP1* is decreased which is expected in fibrotic skin, as there is an accumulation of collagen. *MMP1* expression for the patient samples compared to the controls was significantly decreased. A p-value of *0.004* was calculated when comparing the difference between patient uninvolved skin and control samples. Likewise, a p value of *0.004* was calculated for differences in *MMP1* expression between patient involved skin and control samples.

The expression of *MMP1* in SSc patients, compared to controls seems to differ between studies. As far back as 1979, Uitto et al., found contrary to this study, that collagenase (*MMP1*) activity was increased in the fibroblasts of SSc patients. Conversely, other studies have reported that the activity of *MMP1* is diminished (Takeda et al., 1994; Tomimura et al., 2008). These studies all used fibroblast cell culturing and collagen biosynthesis assays to determine the levels of *MMP1*. The number of patients varied in the studies from as low as 7 (Uitto et al., 1979) to 41 (Tomimura et al., 2008). Few studies stipulate the disease duration of the patients used and this criterion seems to be quite important as a recent study argued that the expression of *MMP1* is not significantly different between late-stage disease SSc patients and controls (Zurita-Salinas et al, 2004). The rate of collagen degradation in SSc skin depends on the clinical stage and activity of the disease. Disease duration could therefore be the factor responsible for the discrepancies in the results of these studies. The reduction in *MMP1* expression that is evident in this study was not unexpected; as less collagenase is produced by *MMP1* the more collagen accumulates in the ECM resulting in fibrosis. It is evident from previous studies that cell culture does not always mimic the *in vivo* situation.

In agreement with previous studies (Ågren et al., 2001), *TIMP1* is not highly expressed in normal skin and in comparison, the expression of *TIMP1* was very much higher in the patient samples, irrespective of whether it was in clinically affected or unaffected skin compared to the controls. Statistically, the increases in expression of *TIMP1* in both the involved and uninvolved skin is significant with p-values of *<0.00001* for both sample types. Due to the role of *TIMP1* in the metabolism of the ECM, the results obtained in the relative quantification experiments were expected. The results from the present study are in agreement with the study by Zurita-Salinas et al. (2004) that found *TIMP1* synthesis was increased in the fibroblasts of SSc patients with early disease. However, they found no significant change of *TIMP1* levels in late disease (>7 years) compared to controls. Meng et al. (2008) studied the expression of TIMP1 protein in the skin lesions of diffuse SSc patients using Immunohistochemistry. Their study found that TIMP1 protein was significantly increased ($p<0.05$) in the patient samples compared to controls.

The expression of the matrix metalloproteinases and their inhibitors are transcriptionally regulated by a number of cytokines (TNFα), hormones, interleukins (IL-1 and IL-6) as well as growth factors such as TGFβ (Dhar et al., 2006). In addition, many cell-cell and cell-matrix interactions are responsible for the expression of the MMP genes (reviewed in Nagase and Woessner, 1999). Inflammatory cytokines such as TNFα trigger the ceramide-signalling pathway that drives the expression of MMP1 in human fibroblasts. Conversely TGFβ is known to repress *MMP1* expression while inducing *TIMP1* expression. This multifunctional growth factor has profound effects on ECM homeostasis via its ability to alter the balance between proteinases and their inhibitors at the level of gene expression (Hall et al., 2003). This key growth factor in ECM homeostasis signals via transmembrane receptors to intracellular mediators of the Smad family as well as through the mitogen-activated protein kinase pathway (Dhar et al., 2006). It is well established that TGFβ plays an important role in the development of fibrosis and the pathogenesis of SSc (Kubo et al., 2002; Yamane et al., 2002). It is evident that the explanation of *MMP1* regulating *TIMP1* and vice versa is very simplistic. Many different cytokines, interleukins and growth factors are involved that constitute various pathways, all of which are involved in the expression of both *MMP1* and *TIMP1*.

Although comparison of gene expression data with TIMP1 and MMP1 serum levels was not done in this study, it is still essential that some comparison be made with previous studies that have been done looking at serum levels in SSc patients. In a study by Young-Min et al. in 2001, it was found that TIMP1 serum levels were significantly raised in patients with diffuse SSc compared to normal controls ($p=0.01$), and that the increase was higher in patients with early disease of less than two years. The higher levels of TIMP1 observed supports the hypothesis that there is an accumulation of the extracellular matrix due to a decrease in matrix degradation. The variation in the levels of TIMP1 between early and late disease reflects the early progression of fibrosis that is observed clinically. The expected reduction in MMP1 serum levels was not observed as the difference between SSc patients and controls was not significant. Another study by Kikuchi et al., (1995) also showed elevated levels of TIMP1 in the serum of SSc patients. These patients had the diffuse form of the disease and showed an increased incidence of lung fibrosis and anti-topoisomerase 1. This study concluded that serum levels of TIMP1 are a useful indicator of disease activity.

Hepatocyte growth factor (HGF) is secreted by mesodermal cells and has mitogenic and morphogenic properties on epithelial and endothelial cells (Galimi et al., 1993). HGF could be important as an endocrine mediator through an epithelial-mesenchymal interaction in wound healing, tissue or organ regeneration and morphogenesis (Nakamura, 1991). The range of activity of HGF and its impact on many physiologic and pathologic processes are reflected by the expression of its receptor, c-met, in a wide variety of organs and cell types. The interaction of HGF and c-met within the immune system serves to control the functions of some immune cell types (Skibinski, 2003).

Hepatocyte growth factor has not been widely studied in the context of SSc, although some studies report on the gene studied in animal models of SSc, such as tight-skin (tsk) mouse. HGF has been shown to have anti-apoptotic activity and has a role in suppressing fibrosis in the liver (Iwasaki et al., 2006).

The study in lung fibroblasts of African American patients suggests that HGF expression might be decreased in SSc patients, but the present study shows the opposite. *HGF*

expression was increased and the highest level of expression was found in the patient uninvolved skin. The expression of HGF in the skin samples from the SSc patients was higher than the control samples. The uninvolved skin had increased expression compared to controls (p=*0.002*) and the involved skin was also significantly increased compared to the expression of HGF in the controls (p=*0.004*). Also, there was a direct correlation between disease activity and MRSS with respect to HGF expression in the patient samples. These findings suggest that HGF may play a compensatory antifibrotic role, but not sufficient to overcome the pro-fibrotic state.

4.2.2. Gene expression comparisons between the involved and uninvolved skin from the SSc patients

It was not only of interest to compare the expression of the genes between the patient and control groups, but also to assess the differences in expression of the clinically uninvolved and involved skin samples from the patients. The uninvolved skin sample from the back was used as an "internal" control for each patient and therefore it is important to note if any differences are observed in gene expression between these two skin samples from the same individual.

The difference in expression of *MMP1* between the uninvolved and involved skin was not significant (p=0.87). Conversely, *TIMP1* was found to have a marginally significant difference (p=*0.05*) in expression between the uninvolved and involved skin. This study found that the expression of *TIMP1* was greater in the clinically involved skin than in the clinically uninvolved skin. As there was still an increase in expression of *TIMP1* in seemingly unaffected skin, it would seem to indicate that *TIMP1* expression is generally up regulated in the skin of systemic sclerosis patients, but at a higher level in skin affected by fibrosis.

HGF showed no difference in expression between the uninvolved and involved skin samples (p=0.83). Interestingly though, the gene was expressed at a slightly higher level in the uninvolved skin. It is thought that this could be the result of a compensatory

mechanism. As *TIMP1* expression increases, leading to reduced degradation of collagen and the development of fibrosis, the levels of *HGF* increase in response to this to try and break down the collagen. This could account for the high expression levels of *HGF* in the uninvolved skin of the patients, which show no physical signs of fibrosis, whilst in the forearm the *HGF* levels are not as high and there is more fibrotic tissue. Studies have shown that *HGF* expression is increased in the skin fibroblasts of individuals with SSc and this is stimulated by growth factors such as epidermal growth factor (*EGF*), platelet-derived growth factor (*PDGF*), basic fibroblast growth factor (*bFGF*) and transforming growth factor alpha (*TGFα*) (Gohda et al., 1994). Similarly the protein kinase C (PKC) pathway stimulates the production of HGF in skin fibroblasts (Matsunaga et al., 1994).

A possible explanation for the increase witnessed in *HGF* expression could be that an increase of the expression of this gene in these patients could be the result of a compensatory mechanism that is induced by the decrease in *MMP1* expression and the increase in *TIMP1* expression. Perhaps the increase in *TIMP1* and the decrease in *MMP1* promote the increased expression of *HGF* in an attempt to suppress the development or proliferation of fibrosis. Many studies (Nakamura et al., 2000; Cowin et al., 2001; Yoshida et al., 2003) have found that up-regulated *HGF* expression was observed after injury of tissues such as the lung, kidney, heart, and skin, therefore the up-regulation of *HGF* might be part of the general response to tissue damage. There are cytokines that seem to be responsible for this, such as interleukin-1 or 6 which activate *HGF* transcription (Michalopoulos and DeFrances, 1997). Additionally, Chmielowiec et al. (2007) showed that c-Met and *HGF* expression is induced in the hyperproliferative epithelium of skin wounds, indicating that receptor and ligand may act in an autocrine manner during wound healing.

Serum levels of HGF are found to be significantly higher in SSc patients compared to controls (*p=0.05*), especially those with interstitial lung disease. Systemic sclerosis patients can manifest the disease with a variety of organ damage which is secondary to vasculitis. The elevated levels of HGF may be reflective of endothelial cell injury (Hashimoto et al., 2003). Contrary to this, Hummers et al., (2009) looked at the plasma levels of various pro-angiogenic factors, including HGF and found that there were lower

levels of HGF in patients with SSc compared to healthy controls although the difference was not statistically significant. The latter authors postulate that an increase of pro-angiogenic growth factors could represent an attempt at angiogenic tissue repair, and thus a decrease in the level of HGF is surprising due to its potent pro-angiogenic effects..

The "myth" of uninvolved skin in SSc

In 1991, Claman et al. did a study on skin biopsies taken from 19 SSc patients using immunohistochemistry. The aim of the study was to determine if there were any differences between clinically affected and clinically unaffected areas. They used antibodies to endothelial leukocyte-adherence molecule type 1 (ELAM1), which would detect endothelial activation, as well as antibodies to procollagen-1 (PC1) in order to detect newly formed, unprocessed collagen. It was found that there was an increase in the level of protein of ELAM1 and PC1 in SSc skin compared to the controls, there was however no difference between clinically affected and clinically unaffected SSc skin samples. They proposed that what appears to be "normal" skin in diffuse SSc is already pathologic, as shown by abnormal endothelial activation and procollagen production. From this article onwards, this phenomenon was termed the "myth of uninvolved skin in SSc". Many studies have been able to corroborate this finding, most recently studies by Kubo et al. (2003) and Sawaya et al. (2004). In the former study they concluded that uninvolved skin appears to represent an intermediate stage between normal skin and skin from an SSc patient. They studied the protein localisation of Fli1 in human SSc fibroblasts and found it to be down regulated in both the clinically affected skin samples as well as the clinically unaffected skin samples, although to a lesser extent in the latter. The Sawaya study found that clinically unaffected skin from SSc patients contained inflammatory cells (such as CD4+ T cells) although the number of these cells was lower than in affected skin. The observation that the presence of inflammatory and fibrotic alterations occur in clinically unaffected skin of SSc patients suggest that vascular and fibrotic abnormalities at the tissue level precede the clinical evidence of the disease.

The genes in this study show the same trend in altered gene expression compared to the normal in both clinically involved and uninvolved skin. For both *MMP1* and *HGF* the expression levels of these genes in the uninvolved skin samples were greater than in the involved samples. Many studies (Nakamura et al., 2000; Cowin et al., 2001; Yoshida et al., 2003) have found that up-regulated *HGF* expression was observed after injury of tissues such as the lung, kidney, heart, and skin, therefore the up-regulation of *HGF* might be part of the general response to tissue damage. There are cytokines that seem to be responsible for this, such as interleukin-1 or 6 which activate *HGF* transcription (Michalopoulos and DeFrances, 1997). Additionally, Chmielowiec et al., (2007) showed that c-Met and *HGF* expression is induced in the hyperproliferative epithelium of skin wounds, indicating that receptor and ligand may act in an autocrine manner during wound healing.

4.2.3. Correlations of clinical data to the gene expression profiles

Due to the small sample size used in this study, it is not possible to make many inferences with respect to clinical correlates. The only correlates that may have a biological significance are *HGF* gene expression in involved skin correlated with disease activity (r=0.60; p=0.013) and *HGF* gene expression in uninvolved skin correlated with the modified Rodnan skin scores with r=0.50 and p=0.048. The latter could be explained by the suggested compensatory role of *HGF* in response to the increase in *TIMP1* expression. The TIMP1 protein inhibits MMP1 activity, which reduces collagen breakdown. In SSc patients this would be indicated with higher MRSS scores, giving a measure of increased thickness of the skin. From the correlation analysis it is clear that *HGF* is positively correlated with increased MRSS scores. This could explain why *HGF* is observed as having increased expression, where it was expected to have decreased expression due to it's biological role in ECM degradation. With regards to the categorical data, two marginally significant observations were found, once again with *HGF*, which was found to be associated with gender in involved skin (p=*0.037*) and renal disease in uninvolved skin (p=*0.031*). Although, due to the small numbers involved these apparently significant associations may not be meaningful.

4.3. Strengths and weaknesses of the study

Due to the demographics of the individuals undergoing reconstructive plastic surgery at the time of control sample collection, there are a large number of males compared to females (ratio 7.5:1). This is opposite to the SSc patient gender ratio. This could have an impact on the expression profiles that were obtained for the control group. However, when looking at the expression ratios for the genes that were obtained for the female and males in the control group they are similar and this would lead me to believe that this is not a serious confounder in the study. It is preferable for the controls to be gender as well as age matched. All of the samples excluding one were collected from the arm area, and obtaining the skin samples from plastic surgery was the found to be the best option.

The strength of this study is the number of phenotypic measurements that were done at the same time the biopsy was taken. The availability of complete phenotypic data gives context to gene expression data collected in the laboratory, and allows a better understanding of how the differences in gene expression may contribute to or cause the clinical symptoms observed in patients.

Another strength of the study is the reproducibility of the relative quantification results. Achieving the same result in triplicate samples provides confidence that these are reliable and accurate results.

4.4. Significance of the study – implications for clinicians and patients

Clinical measurements for each patient were made at the time the skin biopsy was taken. This allows for accurate correlation of gene expression data to relevant clinical information. These comparisons between patients could lead towards a greater understanding about the genetic basis behind the clinical data.

The data provided here are important in that they constitute the first study elucidating the gene expression profiles of *MMP1*, *TIMP1* and *HGF* in black South African systemic

sclerosis patients. Comparing the expression of these genes in the South African patients to systemic sclerosis patients from other populations could provide insight into the pathogenesis of SSc and perhaps even treatment of the fibrosis. More importantly, these findings suggest that all three mediators of collagen metabolism are potential targets of drugs or therapies that could up- or down-regulate their expression.

4.5. Further research

Firstly the mechanism by which *MMP1* and *TIMP1* expression is regulated needs to be considered with regards to future research. As TGFβ is implicated in the regulation of both these genes it would be of interest to determine its differential expression in this same patient group. Other genes that are involved in either fibrosis or T-cell activation could be considered for expression studies in this patient group. In a study by Milano et al. (2008) they found that genes associated with fibrosis were co-expressed with markers of T lymphocytes and macrophages; these genes included *TGFβ*, *FBN1* (*Fibrillin 1*) and the collagen genes. The other members of the metalloproteinase family could be investigated, as well as their *trans*-activators including AP1, PEA3, β-catenin and NFκB. The A *PTPRC* (protein tyrosine phosphatase receptor C gene) has been found to be highly expressed in patients with SSc. This gene is expressed on the surface of T lymphocytes.

Sequencing of the exons, introns and promoter sequences of these genes would help to identify variants that could be responsible for differential gene expression observed. This method could help detect different isoforms of gene transcripts that are generated through alternative splicing.

Following from gene expression studies it is useful to perform immunohistochemistry. This technique uses antibodies, which are specific to antigens on proteins to detect the presence, localisation (cellular and sub-cellular) and the amount of the protein in the sample. The technique involves skin sample sections to be placed on slides and put through a series of washes in buffers and incubations with the antibodies. These slides are analysed to see if the antibodies have bound to any protein that is present in the sample. It is good practice to follow qPCR experiments with immunohistochemistry, as

the expression levels of mRNA within a cell are not always a true reflection of the amount of protein in a cell. This is due to posttranscriptional modifications or occurrences that lead to mRNA degradation, i.e. via RNA interference.

Methylation studies can be done on the promoter sequences of *MMP1*, *TIMP1* and *HGF* in order to assess the level of methylation at CpG sites. Pyrosequencing can be used to make a quantitative measurement of the amount of methylation at each CpG by providing the percentage of methylation at specific CpG sites in the promoter sequence. This would be done in order to investigate whether hyper- or hypomethylation of these genes promoters may in part be responsible for the gene expression patterns that have been found.

Finally SNP DNA microarray could be used to identify new candidate genes for study. Such association studies would require a larger sample size to elucidate the genes that are exerting an influential effect in systemic sclerosis within the South African population. Association studies require large sample sizes in order to detect small genetic effects in multifactorial diseases. In order to recruit the number of patients needed, a collaborative effort would have to be used to collect samples throughout the country. DNA microarray studies are quite expensive and the vast amount of data generated could prove difficult to analyse, but the results obtained from such an experiment could elucidate targets that could be used for therapy development in order to alleviate the burden of fibrosis.

Chapter 5

Conclusion

This study focused on gene expression of three mediators implicated in the metabolism of the ECM. This is an *in vivo* study to examine the expression of these genes in SSc patients; most studies have examined either the serum or cultured fibroblasts. Not many genetic studies have been done in South Africa, focusing on SSc, and as such this is new information, relevant and specific to the South African black population of Soweto.

A number of conclusions can be drawn from the work done in this study. The relative under expression of *MMP1* and the over expression of *TIMP1* reflects the pro-fibrotic state of scleroderma skin. The expression of both these genes in patient samples differs significantly from control samples. The increase in *HGF* expression within the patients was a surprising find and could play a compensatory role as a protective mechanism in response to the increased expression of *TIMP1.* However, the increase in *HGF* expression is not sufficient to overcome the pro-fibrotic state.

An interesting finding in this study was that even skin that was thought to be "clinically uninvolved" and chosen as such for the purpose of an internal control from the back, showed significant alterations in gene expression compared to the expression in the control samples. This seems to confirm and support previous studies that have indicated that all skin in an SSc patient is affected by the dysregulation of collagen synthesis and degradation, but not all skin shows the physical signs of this internal battle for homeostasis. This finding could also suggest that molecular modifications in fibroblasts precede phenotypic changes in the skin of SSc patients.

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Appendices

Appendix A — Ethics Approval Certificate

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Frost

CLEARANCE CERTIFICATE	PROTOCOL NUMBER M070820
PROJECT	Analysis of Three Genes that Contribute to Fibrosis in South African systemic Sclerosis Patients
INVESTIGATORS	Miss JM Frost
DEPARTMENT	Dept of Human Genetics
DATE CONSIDERED	07.08.31
DECISION OF THE COMMITTEE*	APPROVED UNCONDITIONALLY

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE   07.10.12   CHAIRPERSON ....
                           (Professors PE Cleaton-Jones, A Dhai, M Vorster,
                           C Feldman, A Woodiwiss)
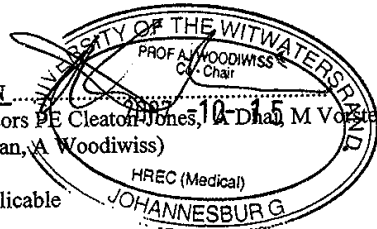
*Guidelines for written 'informed consent' attached where applicable
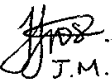
cc: Supervisor :   Prof M Tikly

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and ONE COPY returned to the Secretary at Room 10005, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. I agree to a completion of a yearly progress report.

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES   J.M. FROST

APPENDIX B – INFORMATION AND CONSENT FORMS

GENETIC STUDY OF FIBROSIS IN SYSTEMIC SCLEROSIS

(Control skin biopsy)

Information Sheet

Dear Sir/Madam

I am Jackie Frost, a research assistant and Masters student at the National Health Laboratory Service, and I would like to invite you to participate in a genetics study aimed at finding out what causes fibrosis (skin hardening) in Systemic sclerosis. If you decide to participate in the study please note that a questionnaire needs to be completed, please allow 15 minutes for the questionnaire/interview to be completed.

Participation is entirely voluntary and you will not be disadvantaged in any way if you choose not to participate.

Systemic sclerosis is a complex, multi-system disease, which is known to involve the immune system, blood vessels and the skin. The symptom, which is most commonly identified in the disease, is the gradual thickening and tightening (fibrosis) of the skin. Often ulcers are found, particularly on the fingertips, which are slow to heal due to poor circulation.

The aim of this study is to understand the genetic basis of the fibrosis of the skin. The knowledge gained could help to improve treatment for this symptom of the disease, although this will not be immediately available and could take several decades.

As in any research study I need control samples. These samples will come from individuals who do not have systemic sclerosis or keloids. To do these studies we need to take one (1) skin sample. The skin sample from non-patient participants will need to be taken while the individual is undergoing skin graft surgery. A plastic surgeon at Chris Hani Baragwanath Hospital will be responsible for the removal of the small sample of skin during the course of a prearranged surgical operation. Since this will be done under a general anaesthetic there will be no additional pain or discomfort.

All samples will be coded and any information obtained from this study will be kept confidential and used only for research purposes. Results of the study will be made available in the biomedical literature,

The test will not involve any costs to the participant and participation is completely voluntary. You will not be disadvantaged in any way if you decide not to participate.

Should you require any further information, please do not hesitate to contact:

Professor Mohammed Tikly, Division of Rheumatology

Chris Hani Baragwanath Hospital and the University of the Witwatersrand

(011) 933-9577

Or

Professor Michéle Ramsay, Division of Human Genetics

National Health Laboratory Service and the University of the Witwatersrand

(011) 489-9214

GENETIC STUDY OF FIBROSIS IN SYSTEMIC SCLEROSIS

(Patient skin biopsy)

Information Sheet

Dear Sir/Madam

I am Jackie Frost, a research assistant and Masters student at the National Health Laboratory Service, and I would like to invite you to participate in a genetics study aimed at finding out what causes fibrosis (skin hardening) in Systemic sclerosis. If you decide to participate in the study please note that a questionnaire needs to be completed, please allow 15 minutes for the questionnaire/interview to be completed.

Participation is entirely voluntary and you will not be disadvantaged in any way if you choose not to participate.

Systemic sclerosis is a complex, multi-system disease, which is known to involve the immune system, blood vessels and the skin. The symptom, which is most commonly identified in the disease, is the gradual thickening and tightening (fibrosis) of the skin. Often ulcers are found, particularly on the fingertips, which are slow to heal due to poor circulation.

The aim of this study is to understand the genetic basis of the fibrosis of the skin. The knowledge gained could help to improve treatment for this symptom of the disease, although this will not be immediately available and could take several decades.

To do these studies we need to take two (2) skin biopsies. One (1) sample will be taken from the forearm and one (1) sample from the back. This procedure might cause some discomfort and pain. It involves the removal of a small piece of skin (4mm in diameter) by punch biopsy. After the procedure a steri-strip will be placed over the biopsy region to prevent any infections and to help the wound heal better. No stitches will be needed. A qualified doctor will carry out the procedure.

All samples will be coded and any information obtained from this study will be kept confidential and used only for research purposes. Results will not be made available to you personally as they will need to be interpreted in the context of the group and their clinical value may not be apparent for several years.

The test will not involve any costs to the participant and participation is completely voluntary. You will not be disadvantaged in any way if you decide not to participate.

Should you require any further information, please do not hesitate to contact:

Professor Mohammed Tikly, Division of Rheumatology

Chris Hani Baragwanath Hospital and the University of the Witwatersrand

(011) 933-9577

Or

Professor Michéle Ramsay Division of Human Genetics

National Health Laboratory Service and the University of the Witwatersrand

(011) 489-9214

GENETIC STUDY OF FIBROSIS IN SYSTEMIC SCLEROSIS

Consent to Participate in Research Study

You have been asked to participate in a research study that will look at the genetics of systemic sclerosis.

You have been informed about the study by _____

Have you been informed about the procedures involved in the study?   **YES**   **NO**

Have you been informed that your participation in the study is entirely voluntary and you will not be penalised in any way it you wish not to participate?   **YES**   **NO**

Are you consenting to giving a skin biopsy?   **YES**   **NO**

Do you understand that results of the study will not be made available to you?

                                                                                    YES   **NO**

You may contact Professor Mohammed Tikly at (011) 933-9571 any time if you have any questions about the research.

You may contact the Research Ethics Committee at (011) 717-1234 if you have nay questions about your rights as a research participant.

The research study, including all information, has been described to me. I understand what my involvement in the study means and I voluntarily agree to participate.

Full name of participant (optional):_____

_____   _____
Signature of Participant   Date

_____   _____
Signature of witness   Date
(where applicable)

APPENDIX C – RNA EXTRACTION PROTOCOL

RNeasy Plus Micro Kit (Qiagen)

A maximum amount of 5mg fresh or frozen tissue can generally be processed with good yield. As a guide, a 1.5mm cube (3.4mm^3) of most animal tissues weighs 3.5–4.5mg. It is important to not overload the spin column with sample as this can affect the yield of purified RNA quite drastically. The average yield of RNA extracted using this kit was 2mg.

Before starting:

- *Add 10µl β-Mecaptoethanol to 1ml RLT buffer before use, can be stored at room temperature for up to a month.*
- *Make 80% ethanol by adding 4 volumes of 100% ethanol to RPE buffer.*
- *Make 70% ethanol by adding 24ml 100% ethanol to 6ml RNAse-free water.*

Grind weighed tissue in a mortar and pestle, ensuring tissue remains frozen

↓

Add tissue powder to 350µl RLT buffer in a 2ml tube

↓

Pass lysate through needle 5 -10 times until homogenised

↓

Centrifuge at 18 000rcf for 3 minutes

↓

Remove supernatant with pipette and transfer to gDNA eliminator column

↓

Centrifuge for 30 seconds at 9 000rcf, save flow through

↓

Add 1 volume 70% ethanol, mix well by pipetting

↓

Transfer to RNeasy MinElute column, centrifuge for 15 seconds at 9 000rcf
Discard flow through

↓

Add 700µl RW1 buffer, centrifuge for 15 seconds at 9 000rcf
Discard flow through

↓

Add 500µl RPE buffer, centrifuge for 15 seconds at 9 000rcf
Discard flow through

↓

Add 500µl 80% ethanol, centrifuge for 2 minutes at 9 000rcf
Discard collection tube and flow through

↓

Place column in collection tube, centrifuge for 5 minutes at 18 000rcf - lid open
Discard collection tube and flow through

↓

Place column in 1.5ml tube

↓

Add 14µl RNAse-free water to centre of column

↓

Centrifuge for 1 minute at 18 000rcf to elute RNA

↓

RNA quantification/purity/integrity assessment

↓

Store the RNA at –70˚C until needed for reverse transcription

APPENDIX D – QPCR PRIMERS

Key → **Forward primer**
Reverse primer
PCR product

MMP-1>ENST00000315274 cdna:KNOWN_protein_coding
```
GGGATATTGGAGCAGCAAGAGGCTGGGAAGCCATCACTTACCTTGCACTGAGAAAGAAGACAAAGGCCAGTAT
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GACATTCACCAAGGTCTCTGAGGGTCAAGCAGACATCATGATATCTTTT**TGTCAGGGGAGATCATCGGGACAA**C
TCTCCTTTTGATGGACCTGGAGGAAATCTTGCTCATGCTTTTCAACCAGGCCCAGGTATTGGAGGGGATGCTC
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TT
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TIMP-1 >ENST00000218388 cdna:KNOWN_protein_coding
```
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CCTTCTGCAATT**CCGACCTCGTCATCAGGGCCAAG**TTCGTGGGGACACCAGAAGTCAACCAGACCACCTTATA
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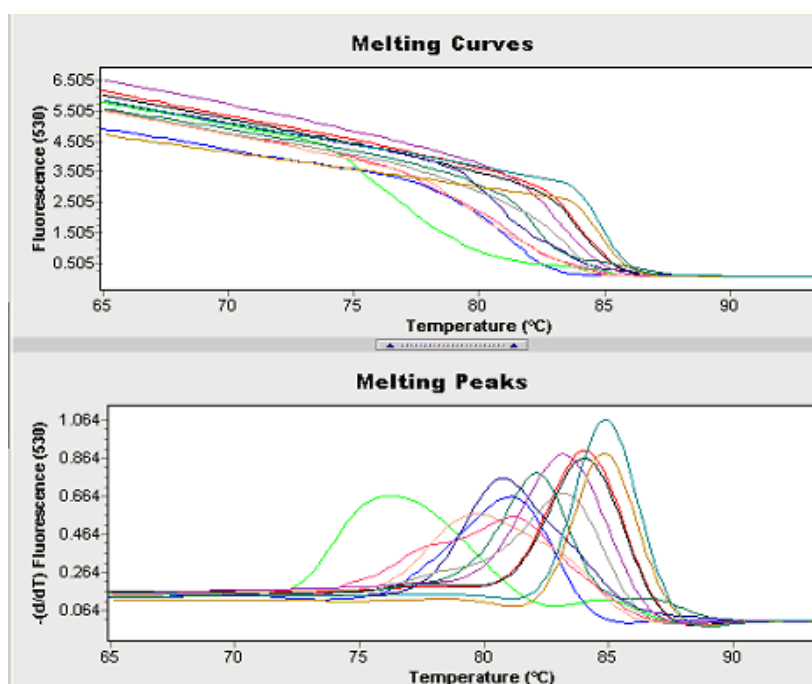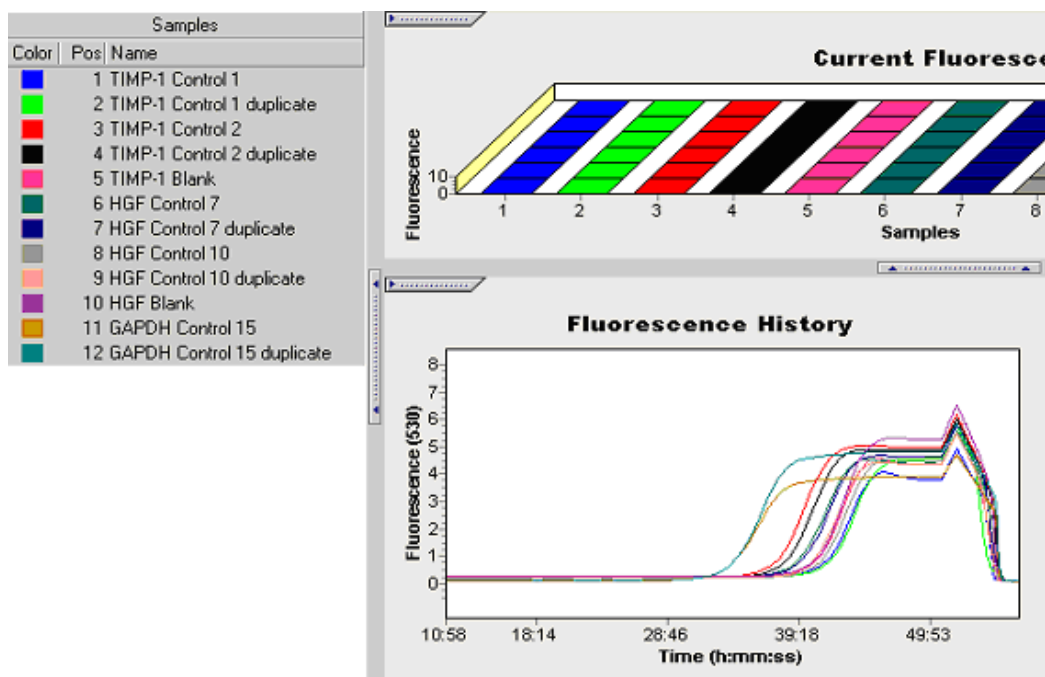
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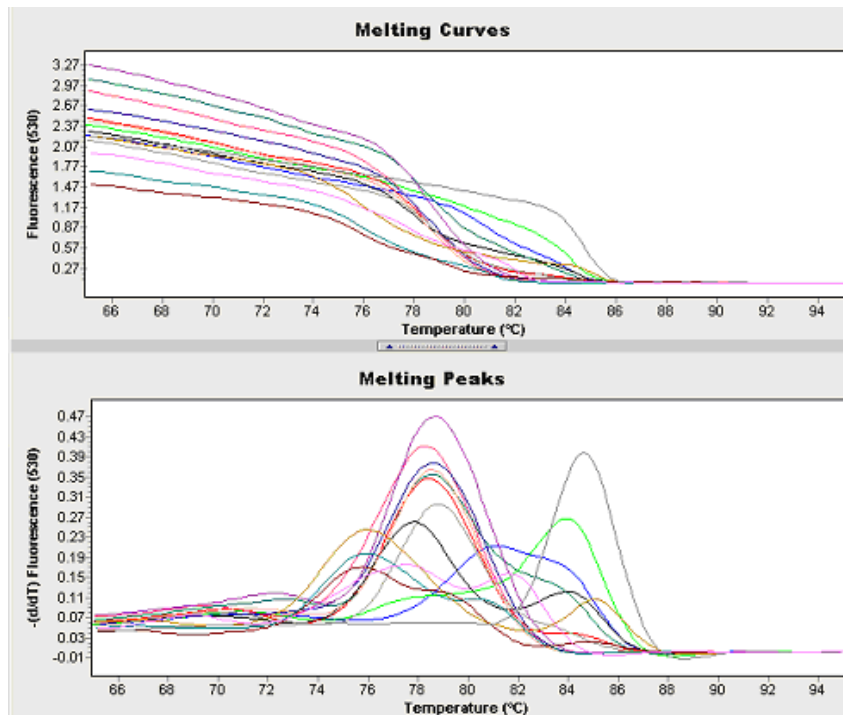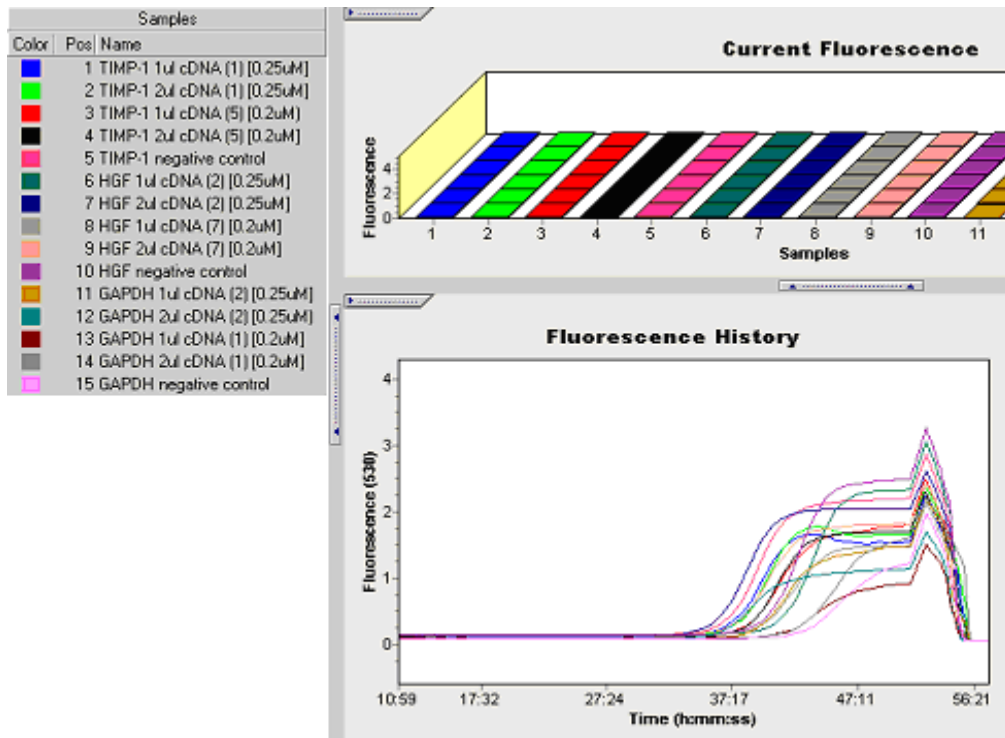
APPENDIX E – QPCR Optimisation

The series of screen shots shows the fluorescence history, melting curves and melting peaks of the optimisation steps for the qPCR assays.

First Run

Change of primer concentrations

Samples		
Color	Pos	Name
	1	TIMP-1 1ul cDNA (1) [0.25uM]
	2	TIMP-1 2ul cDNA (1) [0.25uM]
	3	TIMP-1 1ul cDNA (5) [0.2uM]
	4	TIMP-1 2ul cDNA (5) [0.2uM]
	5	TIMP-1 negative control
	6	HGF 1ul cDNA (2) [0.25uM]
	7	HGF 2ul cDNA (2) [0.25uM]
	8	HGF 1ul cDNA (7) [0.2uM]
	9	HGF 2ul cDNA (7) [0.2uM]
	10	HGF negative control
	11	GAPDH 1ul cDNA (2) [0.25uM]
	12	GAPDH 2ul cDNA (2) [0.25uM]
	13	GAPDH 1ul cDNA (1) [0.2uM]
	14	GAPDH 2ul cDNA (1) [0.2uM]
	15	GAPDH negative control

Change amount of cDNA [500ng/µl] added
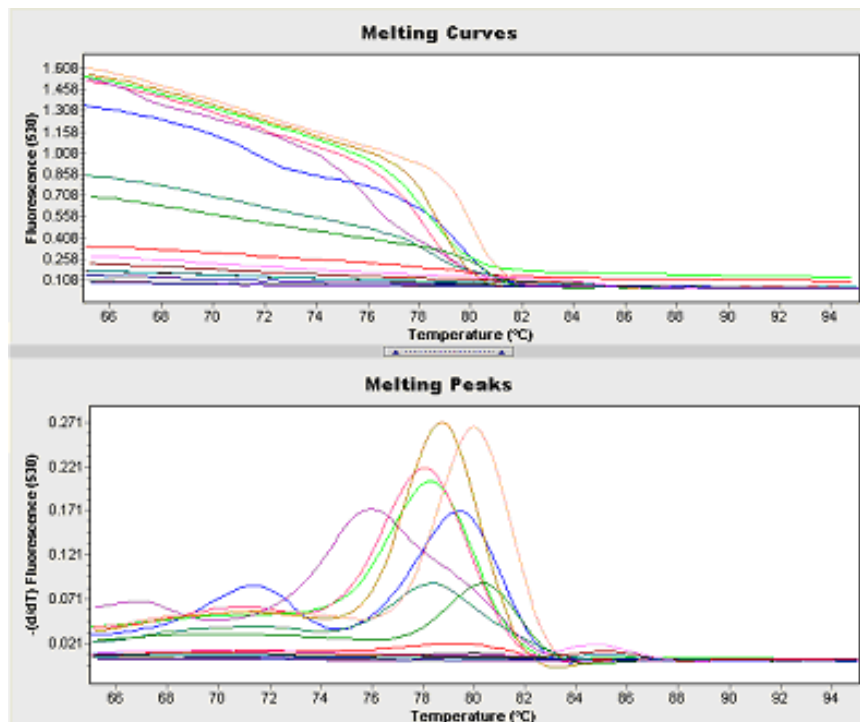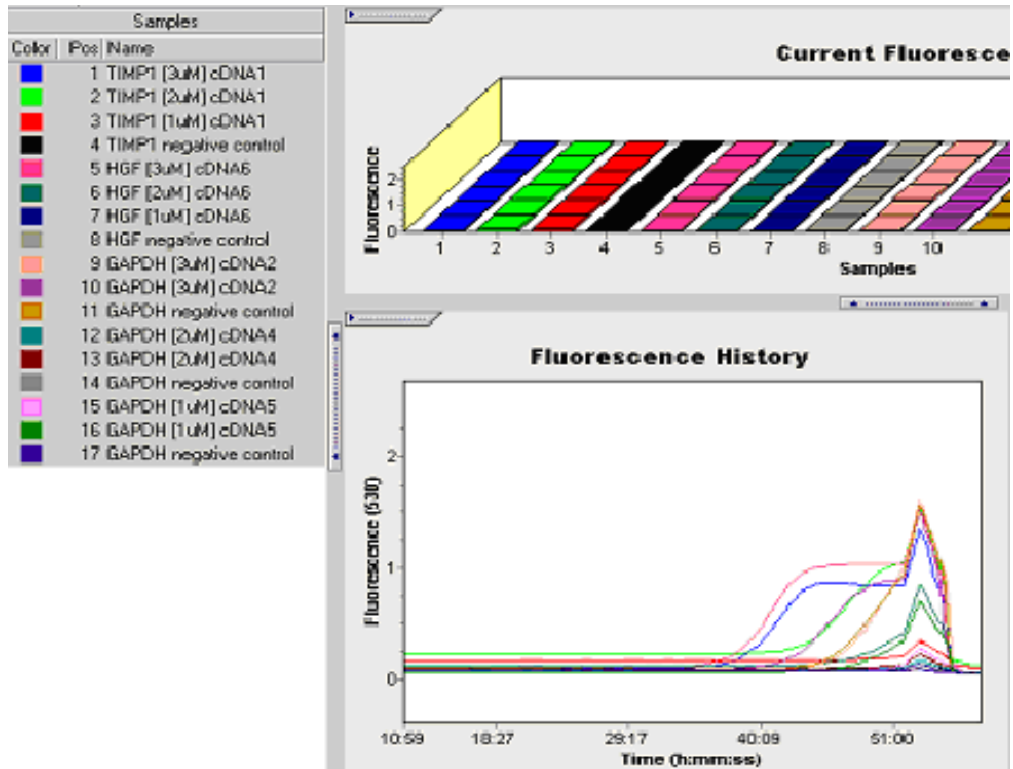
Finally optimised
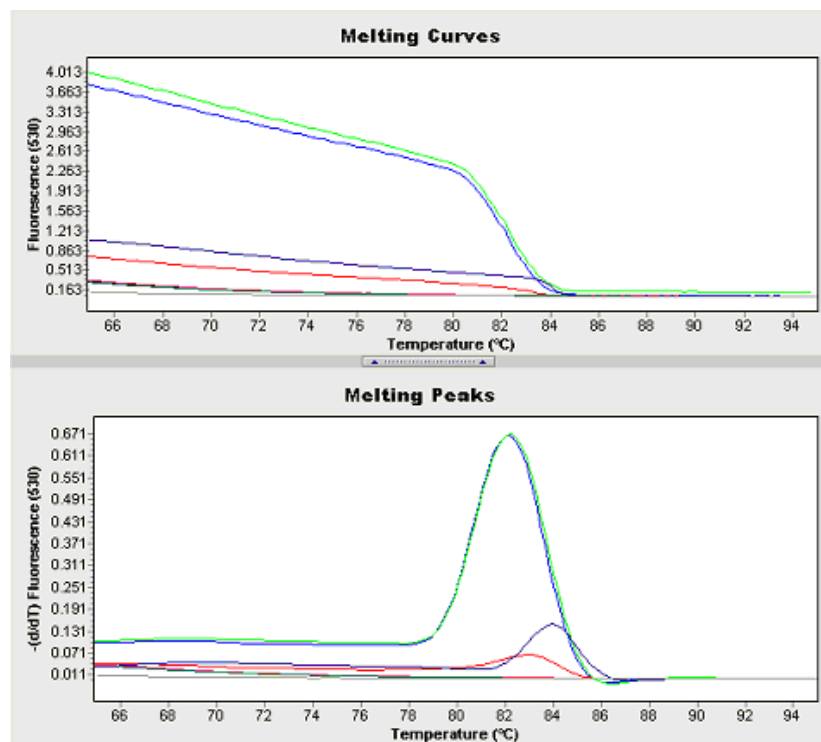
MMP1 was optimised with the addition of Betaine

APPENDIX F – DATA TABLES

Table F1. Mean qPCR ratios and CV values for MMP1 assay

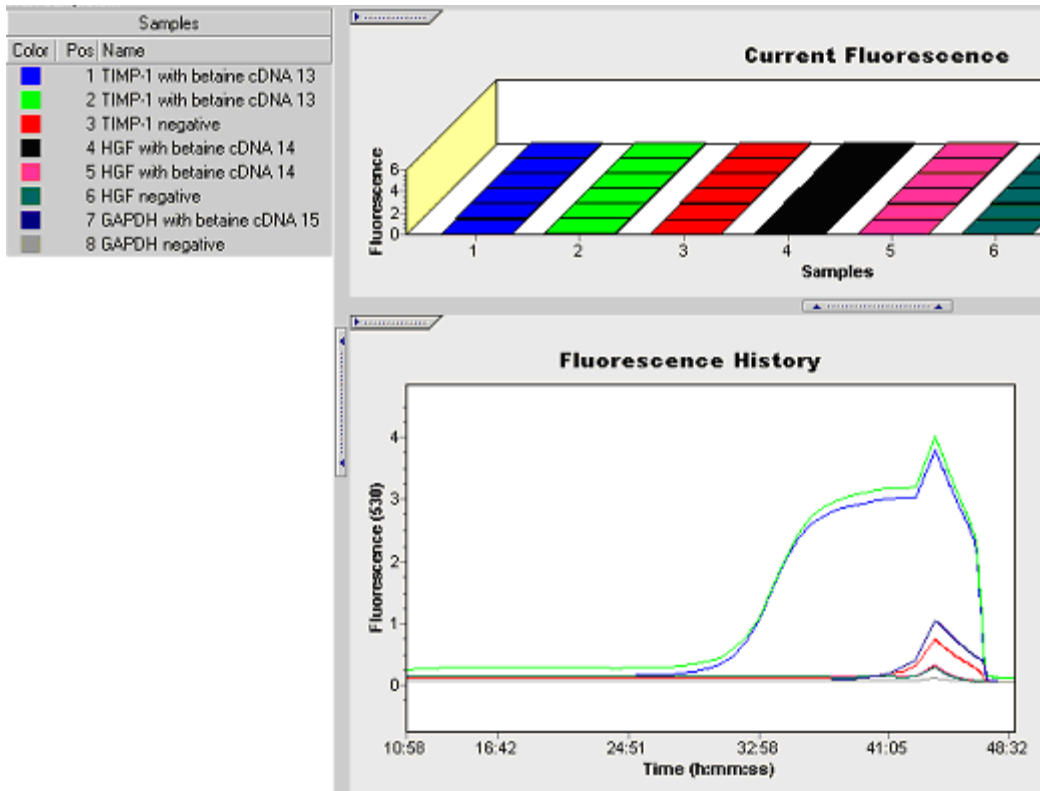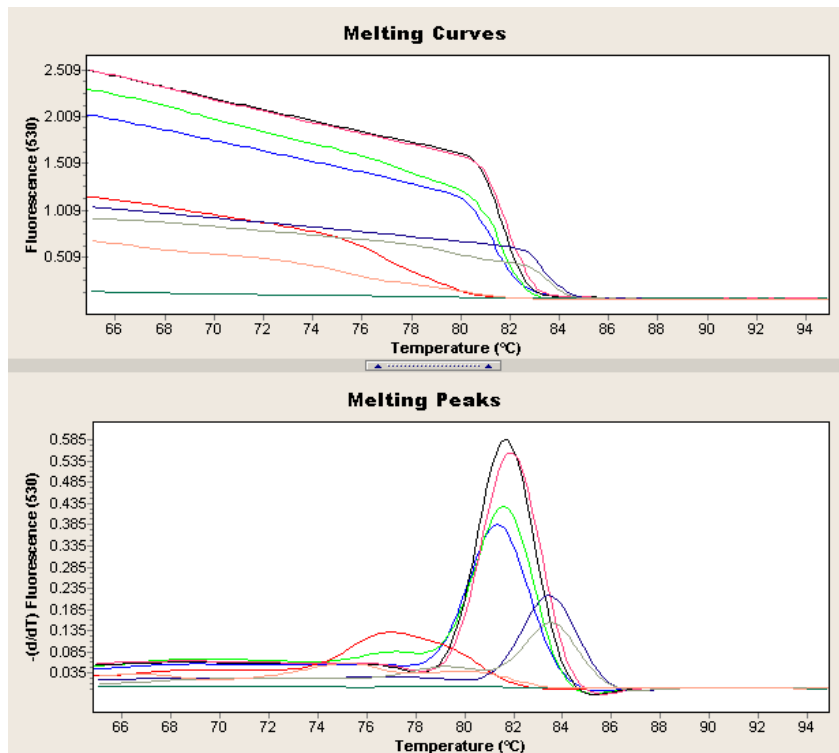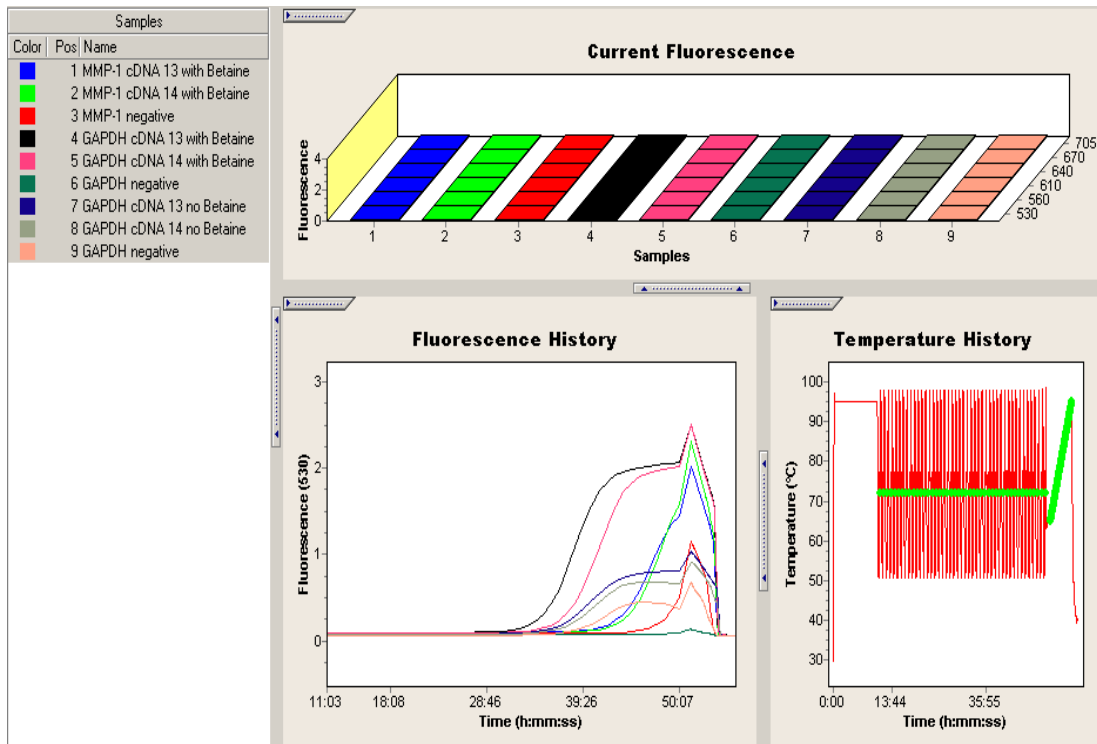| Sample	Mean Cq MMP1	Mean Cq GAPDH	Mean ratio	CV (%)	Sample (involved)	Mean Cq MMP1	Mean Cq GAPDH	Mean ratio	CV (%)	Sample (uninvolved)	Mean Cq MMP1	Mean Cq GAPDH	Mean ratio	CV (%)
Control 1	30.78	28.44	0.19	1.6	Patient 1	31.64	23.66	0.004	1.6	Patient 1	33.37	25.91	0.006	1.4
Control 2	32.07	28.52	0.09	0.2	Patient 2	30.09	19.59	0.003	2.5	Patient 2	33.32	25.06	0.003	1.2
Control 3	31.36	24.67	0.01	0.9	Patient 3	30.02	18.30	0.003	1.3	Patient 3	32.42	27.66	0.04	0.4
Control 4	28.73	24.79	0.07	0.5	Patient 4	29.07	23.19	0.02	3.0	Patient 4	31.54	25.72	0.02	1.4
Control 5	31.28	28.05	0.11	0.5	Patient 5	29.78	23.40	0.01	0.9	Patient 5	31.80	24.04	0.005	1.0
Control 6	26.09	19.19	0.008	0.3	Patient 6	31.52	21.32	0.001	1.5	Patient 6	32.52	24.88	0.005	1.3
Control 7	28.71	21.25	0.005	0.7	Patient 7	31.78	22.79	0.002	0.9	Patient 7	31.86	24.69	0.007	1.0
Control 8	19.82	15.12	0.04	0.2	Patient 8	30.27	23.05	0.006	1.8	Patient 8	31.73	23.24	0.003	0.6
Control 9	27.67	23.25	0.05	0.2	Patient 9	30.06	22.36	0.005	1.1	Patient 9	34.50	28.39	0.01	0.7
Control 10	29.31	24.84	0.05	1.2	Patient 11	31.68	23.04	0.003	1.7	Patient 11	31.28	25.26	0.01	1.3
Control 11	23.29	13.81	0.001	2.3	Patient 12	32.24	26.65	0.02	1.7	Patient 12	35.78	28.15	0.003	0.6
Control 12	30.62	22.76	0.004	1.2	Patient 13	31.58	23.02	0.003	0.8	Patient 13	35.13	26.89	0.003	2.2
Control 13	27.50	21.21	0.01	0.8	Patient 14	33.33	27.09	0.01	2.1	Patient 14	35.88	27.36	0.003	0.5
Control 14	26.87	21.11	0.02	0.3	Patient 15	25.11	27.09	0.01	3.4	Patient 15	23.17	21.31	0.02	1.2
Control 15	33.57	31.70	0.20	1.0	Patient 16	30.90	21.52	0.003	2.9	Patient 16	30.89	23.92	0.008	2.8
					Patient 17	34.38	25.28	0.002	1.3	Patient 17	32.93	29.62	0.01	2.1

Table F2. Mean qPCR ratios and CV values for TIMP1 assay

| Sample	Mean Cq TIMP1	Mean Cq GAPDH	Mean ratio	CV (%)	Sample (involved)	Mean Cq TIMP1	Mean Cq GAPDH	Mean ratio	CV (%)	Sample (uninvolved)	Mean Cq TIMP1	Mean Cq GAPDH	Mean ratio	CV (%)
Control 1	32.42	27.66	0.04	0.6	Patient 1	31.12	26.91	0.24	0.1	Patient 1	31.12	28.09	0.12	0.1
Control 2	33.06	22.45	0.0006	0.8	Patient 2	29.79	22.29	0.31	0.2	Patient 2	29.79	29.43	0.78	0.3
Control 3	34.07	22.64	0.0004	0.3	Patient 3	33.59	22.24	0.15	0.0	Patient 3	33.59	32.74	0.55	0.0
Control 4	29.85	19.20	0.0006	0.2	Patient 4	29.31	26.03	0.44	0.0	Patient 4	29.31	28.17	0.45	0.2
Control 5	32.24	26.65	0.02	2.4	Patient 5	30.05	26.34	0.12	0.0	Patient 5	30.05	27.02	0.12	0.2
Control 6	29.54	17.76	0.0003	5.1	Patient 6	31.26	26.03	0.82	0.5	Patient 6	31.26	28.46	0.14	0.1
Control 7	32.91	19.90	0.0001	0.6	Patient 7	28.86	28.93	1.56	0.3	Patient 7	28.86	28.04	0.56	0.2
Control 8	28.47	15.76	0.0001	1.3	Patient 8	27.92	27.73	1.24	0.1	Patient 8	27.92	25.54	0.19	0.1
Control 9	30.88	25.03	0.02	0.0	Patient 9	36.48	28.27	1.69	0.7	Patient 9	36.48	34.62	0.27	1.7
Control 10	33.06	21.44	0.0003	2.4	Patient 11	25.95	28.31	1.22	0.1	Patient 11	25.95	24.79	0.45	0.6
Control 11	30.53	18.95	0.0003	1.2	Patient 12	36.15	30.28	1.33	0.0	Patient 12	36.15	34.42	0.30	0.2
Control 12	34.61	23.96	0.0006	1.3	Patient 13	32.97	26.10	0.32	0.2	Patient 13	32.97	28.19	0.07	0.2
Control 13	31.40	30.10	0.04	0.1	Patient 14	32.92	31.73	0.15	0.2	Patient 14	32.92	28.62	0.05	0.1
Control 14	32.63	20.35	0.0002	1.4	Patient 15	27.72	25.15	0.03	0.3	Patient 15	27.72	21.18	0.01	0.1
Control 15	33.25	21.95	0.0004	1.5	Patient 16	36.48	29.53	0.02	0.1	Patient 16	36.48	30.64	0.02	0.1
					Patient 17	32.92	25.13	0.06	0.1	Patient 17	32.92	28.62	0.05	0.2

Table F3. Mean qPCR ratios and CV values for HGF assay

| Sample	Mean Cq HGF	Mean Cq GAPDH	Mean ratio	CV (%)	Sample (involved)	Mean Cq HGF	Mean Cq GAPDH	Mean ratio	CV (%)	Sample (uninvolved)	Mean Cq HGF	Mean Cq GAPDH	Mean ratio	CV (%)
Control 1	32.21	31.66	0.07	0.5	Patient 1	29.79	24.95	0.04	0.5	Patient 1	33.85	26.33	0.005	0.4
Control 2	34.82	25.19	0.001	0.1	Patient 2	28.72	20.64	0.004	0.9	Patient 2	33.46	25.23	0.003	2.1
Control 3	32.06	25.28	0.009	0.2	Patient 3	29.82	22.53	0.006	2.2	Patient 3	33.33	27.61	0.02	0.6
Control 4	31.59	19.54	0.0003	0.3	Patient 4	30.01	25.19	0.03	0.8	Patient 4	32.38	26.99	0.03	3.3
Control 5	35.38	27.96	0.007	0.6	Patient 5	28.25	23.97	0.05	0.7	Patient 5	30.38	24.02	0.01	1.1
Control 6	30.47	21.06	0.002	4.9	Patient 6	30.65	24.16	0.01	1	Patient 6	33.43	25.58	0.005	2.1
Control 7	31.39	23.19	0.003	1.1	Patient 7	31.13	26.19	0.03	1	Patient 7	31.82	26.22	0.02	2.6
Control 8	27.79	14.88	0.0001	4.7	Patient 8	30.88	25.03	0.02	1.3	Patient 8	32.65	23.74	0.002	1.2
Control 9	33.93	23.36	0.0007	1.3	Patient 9	29.44	26.46	0.06	1.6	Patient 9	32.60	28.71	0.07	3.8
Control 10	31.11	25.20	0.02	0.2	Patient 11	29.90	25.09	0.04	0.9	Patient 11	31.50	26.54	0.03	1.7
Control 11	30.43	15.60	0.0003	1.9	Patient 12	27.68	20.55	0.002	1	Patient 12	32.79	27.34	0.03	0.9
Control 12	31.27	21.46	0.001	1.2	Patient 13	31.76	29.15	0.004	0.6	Patient 13	30.67	27.20	0.09	0.3
Control 13	30.82	19.15	0.0003	0.7	Patient 14	28.79	26.99	0.007	0.5	Patient 14	32.82	27.57	0.03	0.7
Control 14	32.35	21.60	0.0006	0.9	Patient 15	28.15	24.68	0.02	0.5	Patient 15	28.77	22.61	0.01	0.1
Control 15	34.26	31.18	0.11	0.2	Patient 16	27.41	25.11	0.006	0.8	Patient 16	28.48	24.29	0.06	2.8
					Patient 17	33.12	27.57	0.02	0.2	Patient 17	31.40	30.10	0.04	1

APPENDIX G – REPRODUCIBILITY GRAPHS

Graphs showing reproducibility of the qPCR assays; a) control samples and b) patient clinically uninvolved and involved skin.
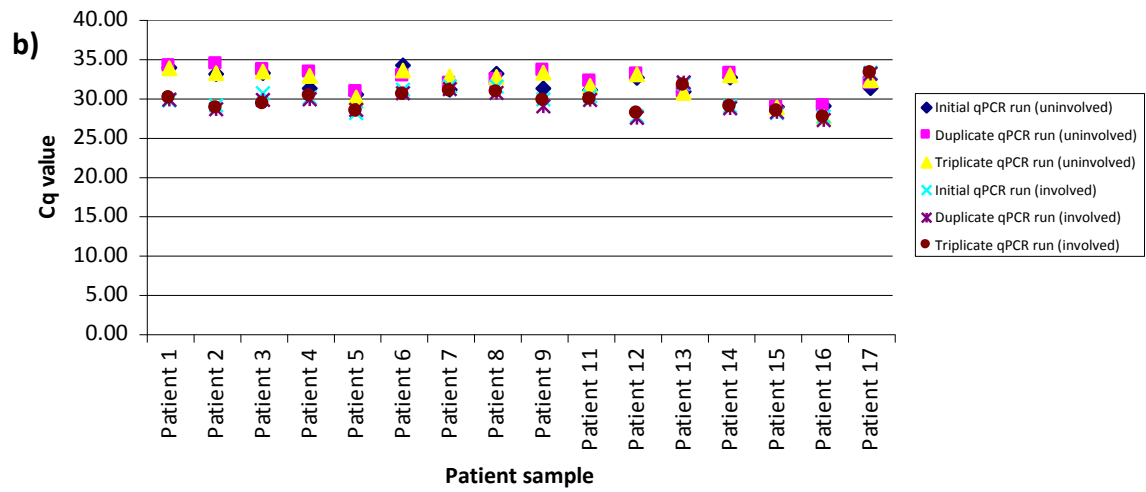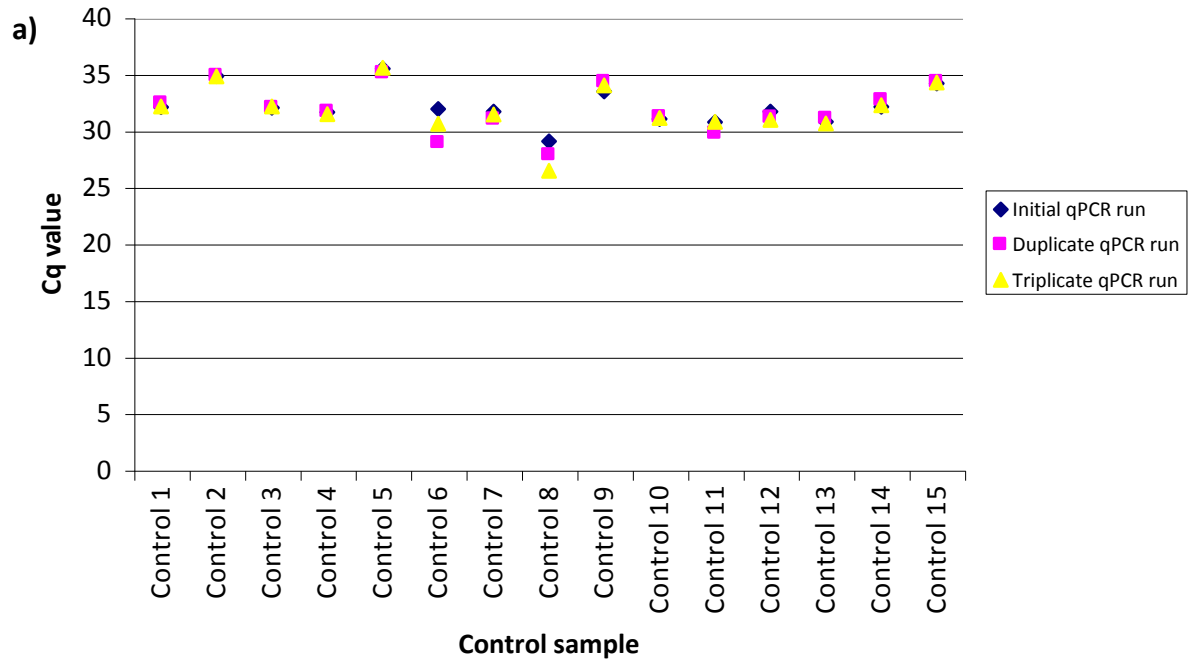
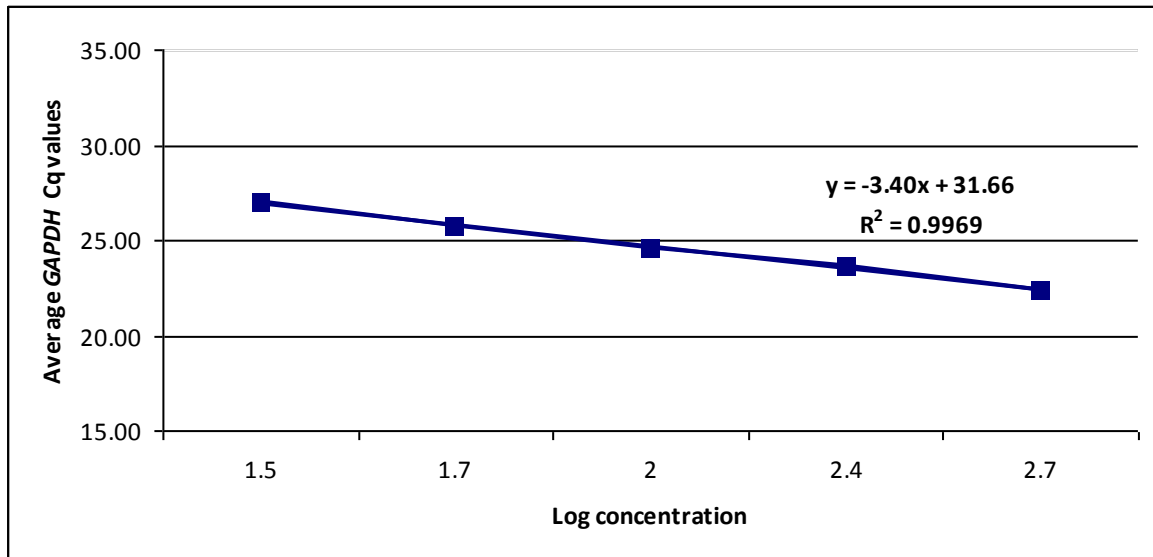## *MMP1*

a)

b)

TIMP1

a)

b)

HGF

a)

b)
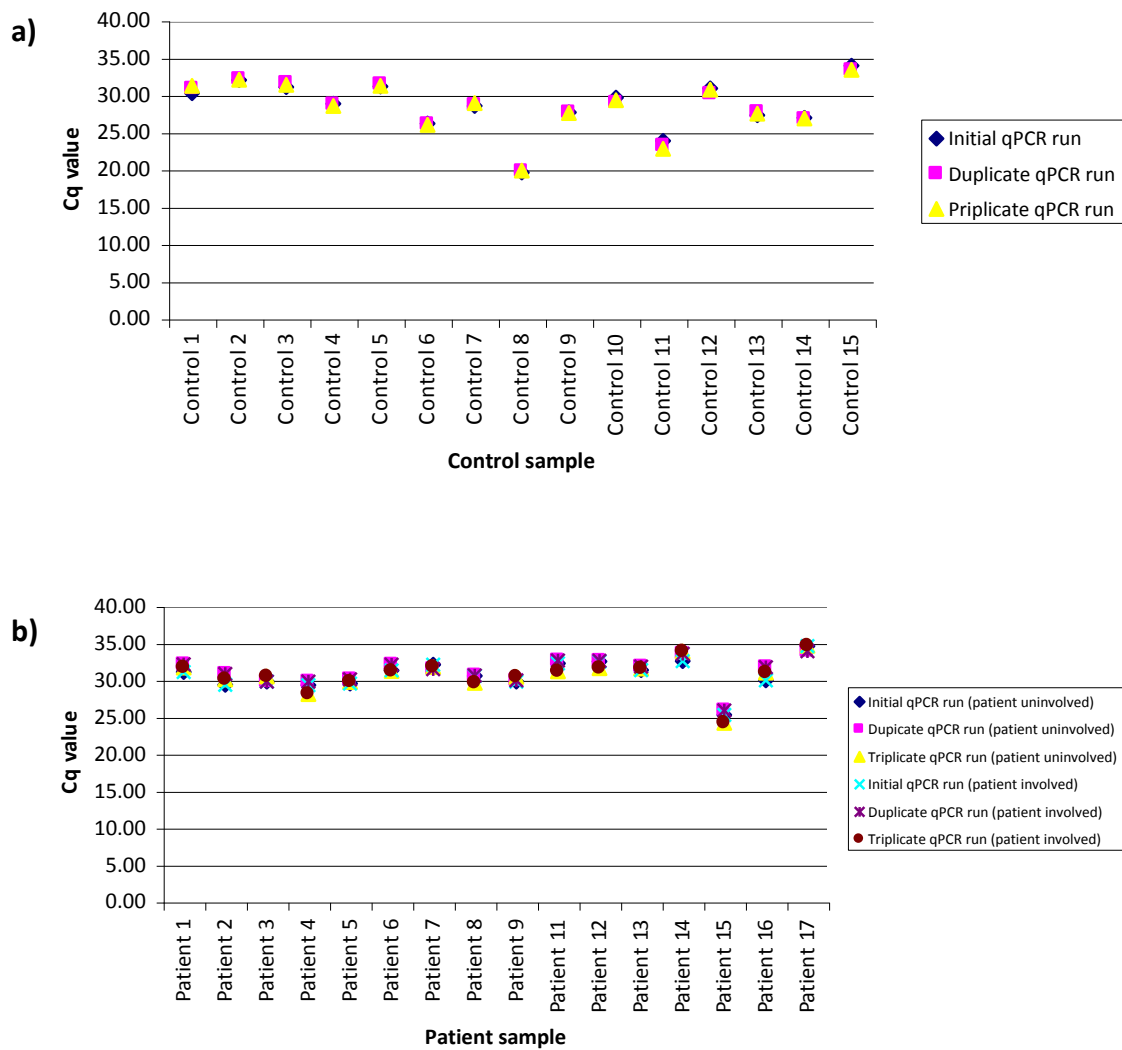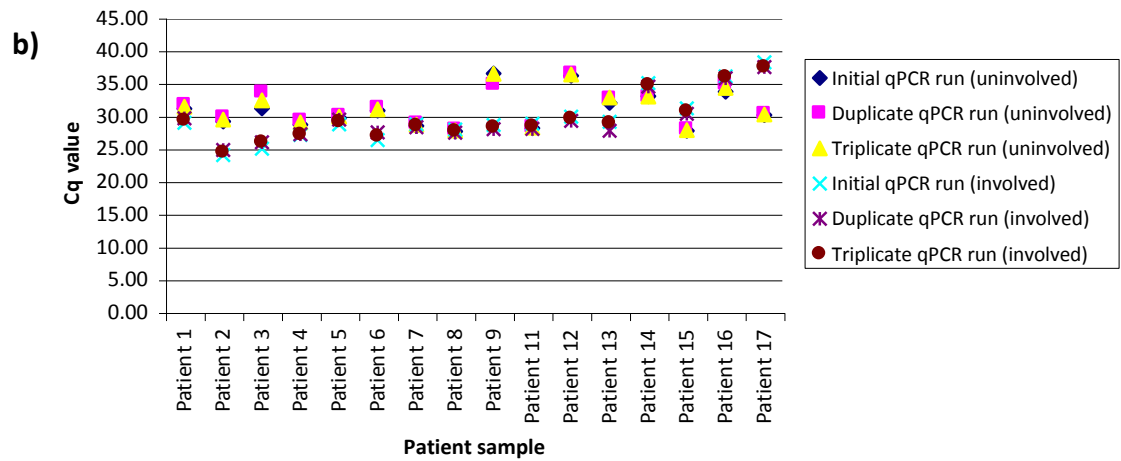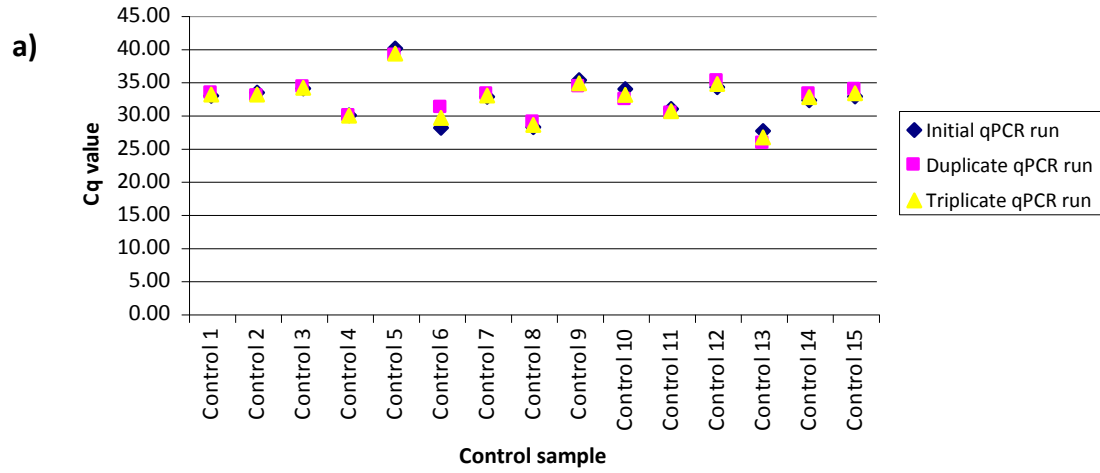
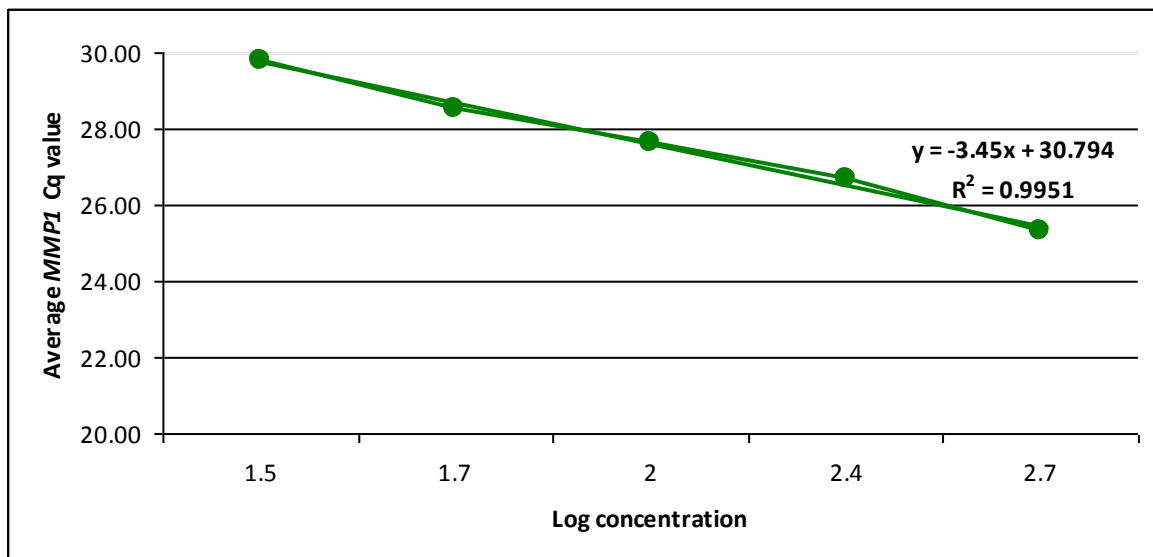# APPENDIX H – QPCR AMPLIFICATION EFFICIENCY
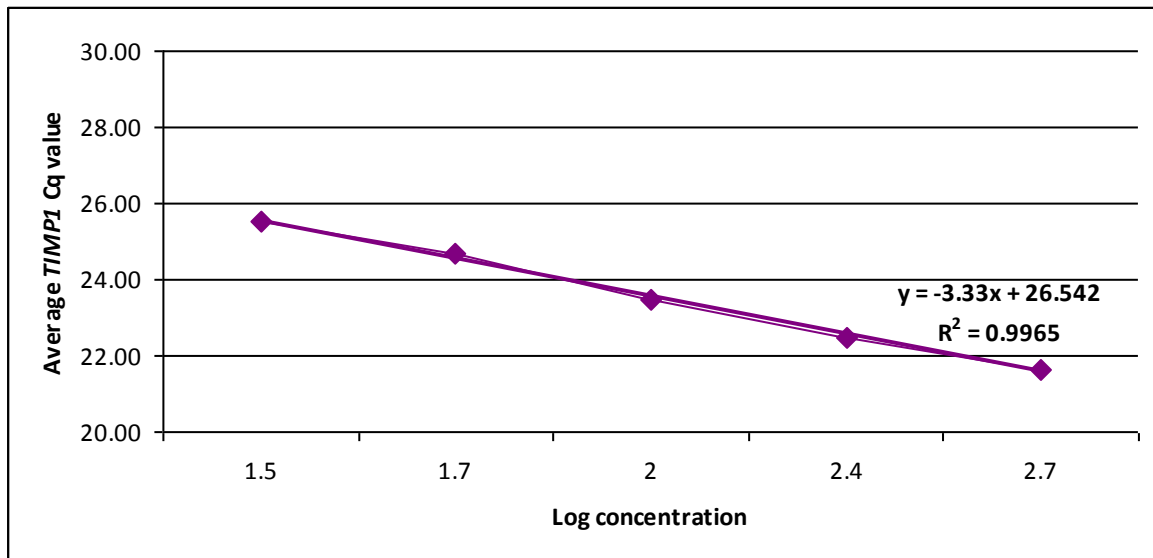

$E = 10^{(-1/\text{slope})} \times 100\%$

$E = 96.84\%$

Amplification factor = 1.97

$E = 10^{(-1/\text{slope})} \times 100\%$

$E = 94.92\%$

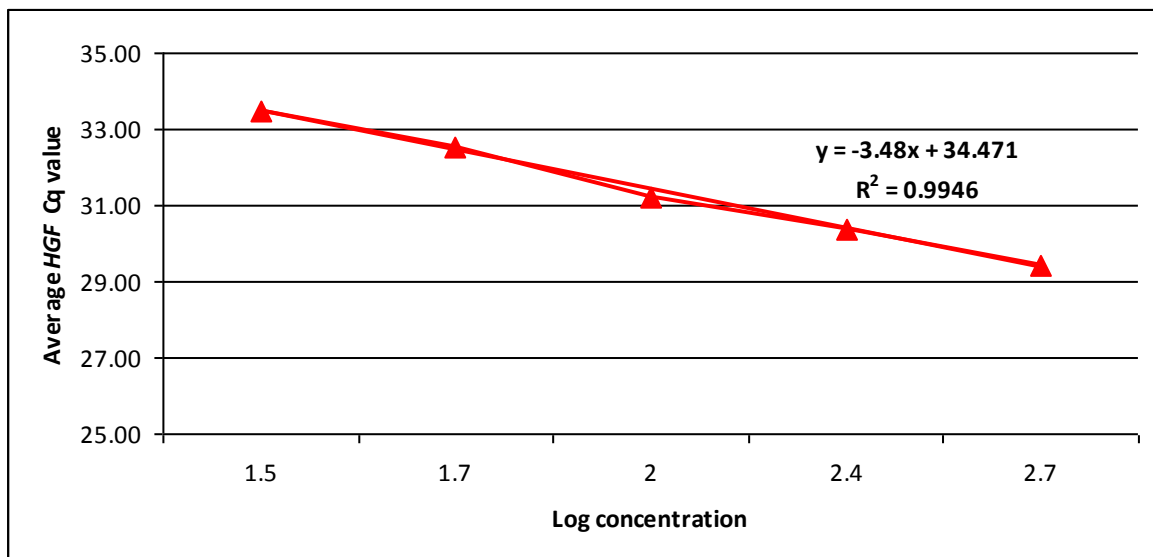Amplification factor = 1.95

E = 10$^{(-1/slope)}$ x 100%

E = 99.66%

Amplification factor = 2

E = 10$^{(-1/slope)}$ x 100%

E = 94.80%

Amplification factor = 1.94

Appendix I – Summarised Clinical Data for each patient

Table I. Clinical features of systemic sclerosis patients

Patient	Age/Sex	Duration months	ACR/diffuse disease	Skin score (0-51)	Involved/un-Involved skin score	Disease activity	ESR (>30)	CRP	Lung disease/ Renal disease	Myositis	ANA/ ATA/ Low C4
1	25/F	24	Y/Y	26	2/0	2	N	7.4	-/-	Y	+/-/-
2	34/F	23	Y/Y	11	2/0	1.5	N	14.3	-/-	N	+/-/+
3	27/F	12	Y/Y	20	2/0	1.5	N	7.2	-/-	N	+/-/-
4	50/F	22	Y/Y	21	1/0	6	Y	<1	-/-	N	+/+/-
5	26/F	2	Y/Y	16	2/0	4	Y	339.5	No data	Y	+/-/+
6	23/F	24	Y/Y	16	2/0	3.5	Y	11.1	-/-	N	+/+/-
7	48/F	25	Y/Y	18	3/0	3.5	N	7.5	+/-	N	+/+/+
8	37/F	49	Y/Y	18	1/0	2.5	Y	1.3	-/-	N	+/+/-
9	28/F	32	Y/Y	36	3/0	6	Y	9.3	-/-	Y	+/-/-
11	28/F	15	Y/Y	28	2/0	4	N	32.8	-/-	N	+/-/-
12	39/M	17	Y/Y	18	2/0	1	N	6	-/-	N	+/-/-
13	28/M	22	Y/Y	26	2/0	3.5	N	No data	+/+	N	+/-/-
14	48/F	35	Y/Y	20	2/0	6	N	8.3	+/-	Y	+/-/-
15	28/F	3	Y/Y	26	2/0	5	Y	8.9	-/-	N	+/-/-
16	50/F	74	Y/Y	16	2/0	1	N	5.9	-/-	N	+/-/-
17	52/F	14	Y/Y	45	3/0	3.5	Y	5.7	-/+	N	+/-/-