MODULATION OF SYSTEMIC SCLEROSIS PATHOBIOLOGY BY PURIFIED HUMAN FACTOR XIII - IN VITRO AND IN VIVO STUDIES

A thesis for the degree of Doctor of Medicine by Research

University College London

Dr Anna Charlotte Leslie BA (Hons) MBBS MRCP (UK)

Primary Supervisor: Professor Christopher P. Denton

Secondary Supervisor: Dr Voon Ong

Centre for Rheumatology and Connective Tissue Diseases

Royal Free Campus

University College London

United Kingdom

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Statement of contribution

I, Anna Charlotte Leslie, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Systemic sclerosis (SSc) is a multi-system autoimmune disease with significant morbidity, mortality and an unmet therapeutic need. The pathogenesis of SSc involves dysfunctional immune signalling, including thrombospondin-1 (TSP-1) signalling, fibroblast behaviour and vascular development.

Factor XIII (FXIII) is an enzyme involved in coagulation and in wound healing by promoting angiogenesis via inhibition of TSP-1. Animal research and previous small studies suggest that therapeutic administration of factor XIII could improve vascular function in SSc.

This thesis addresses the hypothesis that FXIII supplementation may affect the pathogenesis of SSc via effects on vascular and connective tissue biology. This is explored in two interlinked clinical trials: a single-dose open-label study assessing the safety and pharmacokinetics of human factor XIII treatment in SSc; followed by a phase II, double-blind, randomised, placebo-controlled study to investigate the safety and efficacy of factor XIII treatment in SSc.

The main objective of the clinical trials was met by demonstrating that FXIII is safe and well tolerated in SSc. The trial did not meet its primary endpoints, but there was a trend towards improvement in Raynaud's phenomenon in the FXIII group.

Serum from patients and controls was analysed to quantify candidate biomarkers for SSc and the proposed mechanism of action of factor XIII. Previous work confirming that cartilage oligomeric protein, TSP-1 and tumour necrosis factor alpha are raised in SSc serum was reproduced. TSP-1 levels fell significantly in the serum of participants who received FXIII, supporting the hypothesis that FXIII modulates TSP-1. Further studies with different patient populations and FXIII doses could investigate the role of this suppression further in a clinical setting.

My results support further exploration of the role of FXIII in SSc pathogenesis and suggest that therapeutic strategies to modulate links between coagulation and tissue repair using FXIII are safe and feasible.

Impact statement

Systemic sclerosis is a rare multi-system autoimmune disease whose symptoms are progressive and challenging to manage, and in some patients shortens their lifespan. Systemic sclerosis causes inappropriate scarring of the skin, lungs and other organs. Due to its rarity and incomplete understanding of why and how it occurs, clinical research has been limited and evidence-based therapeutic options are few. There is a significant unmet need for effective therapies in this condition.

In recent decades, research into autoimmune diseases has deepened our molecular-level understanding of how these diseases occur. This has led to the development of targeted therapies which block key signalling molecules in the disease process. An alternative view is to consider where positive signalling pathways may be enhanced. By hyper-stimulating molecules with a positive effect, we could theoretically modulate dysfunctional pathways and may be able to alter downstream signalling and disease activity.

My research builds on existing knowledge of the systemic sclerosis disease process. Interestingly, in health, the body's pathways involved in blood clotting and wound healing are linked. We know that wound healing is abnormal in systemic sclerosis, therefore this raises the question of whether modulating the clotting process could positively impact abnormal wound healing. Previous animal studies and small-scale clinical work have suggested a potential role for clotting factor XIII in the treatment of systemic sclerosis.

I have conducted two inter-linked clinical trials to investigate the safety, pharmacokinetics and efficacy of factor XIII treatment in systemic sclerosis. Firstly, 8 patients received a single infusion of factor XIII and were monitored for adverse effects and drug levels. Initial positive safety data led to 18 patients undertaking a six-month trial of factor XIII or placebo. Their wellbeing, drug levels and the progress of their systemic sclerosis was monitored. Conduct of these trials to modern research standards generated high quality data. Assessment of

pharmacokinetics produced detailed patient-centred information which allowed personalised drug dosing.

The results show encouraging safety data for factor XIII treatment in systemic sclerosis. A clear symptom benefit for factor XIII therapy was not shown, however these trials were exploratory in nature. The safety and pharmacokinetic data, as well as a discussion of possible effective trial design, open the door to further research. The clinical trial data will impact systemic sclerosis clinicians, researchers and patients on an international level.

I have extended the impact of this project further by conducting research into human proteins affected by systemic sclerosis and factor XIII treatment. By monitoring these proteins in clinical trial participants, I have generated new data on the disease process and the signalling pathways factor XIII might stimulate and inhibit. I also assessed how key cells in systemic sclerosis respond to factor XIII treatment in the laboratory setting, producing further information on the interaction between factor XIII and cell behaviour in systemic sclerosis. These data are impactful for scientific and clinical researchers in systemic sclerosis.

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List of abbreviations

ACA	Anti-centromere antibody
ACE	Angiotensin converting enzyme
ACR	American College of Rheumatology
AE	Adverse event
AFA	Anti-fibroblast antibody
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
ANA	Anti-nuclear antibody
APTT	Activated partial thromboplastin time
ARB	Angiotensin receptor blocker
AST	Aspartate aminotransferase
BCL-2	B-cell lymphoma-2
BSA	Bovine serum albumin
BUN	Blood urea nitrogen
CAPA	Corrective and preventative action plan

CCB	Calcium channel blocker
CD4+	Cluster of differentiation 4 positive
CD40L	Cluster of Differentiation 40 Ligand, CD154
CD8+	Cluster of differentiation 8 positive
cFXIII-A	Cellular factor XIII A
CHFS	Cochin hand function score
CI	Confidence interval
COAT platelet	Collagen and thrombin activated platelet
COMP	Cartilage oligomeric matrix protein
CRF	Case report form
CRISS	Composite response index in dcSSc
CRP	C-reactive protein
CTGF	Connective tissue growth factor
CXCL4	Chemokine ligand-4, platelet factor 4
dcSSc	Diffuse cutaneous systemic sclerosis
DFE	Diffuse fasciitis with eosinophilia

DLCO	Diffusing capacity of the lungs for carbon monoxide
DMEM	Dulbecco's Modified Eagle Medium
DPBS	Dulbecco's Phosphate Buffered Saline
DU	Digital ulcer
ECG	Electrocardiogram
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGF-5	Epidermal growth factor 5
eGFR	Estimated glomerular filtration rate
ELISA	Enzyme-linked immunosorbent assay
ERA	Endothelin receptor antagonist
ERK 1/2	Extracellular signal related kinase-1/2
E-selectin	Endothelial leukocyte adhesion molecule-1
ESR	Erythrocyte sedimentation rate
ET-1	Endothelin
EULAR	European League Against Rheumatism

FEV1	Forced Expiratory Volume in 1 second
FGF-2	Fibroblast growth factor 2
FVC	Forced Vital Capacity
FVII	Factor VII
FVIIa	Activated factor VII
FXIII-a	Activated factor XIII
FXIII-A	Factor XIII subunit A
FXIII-B	Factor XIII subunit B
GAPDH	Glyceraldehye-3-phosphate dehydrogenase
GAVE	Gastric antral vascular ectasia
GCP	Good clinical practice
GGT	Gamma glutyl transferase
GI	Gastro-intestinal
GORD	Gastro-oesophageal reflux disease
gp130	130 kilodalton glycoprotein receptor
HAQ	Health assessment questionnaire

HB-EGF	Heparin binding epidermal growth factor
HIF	Hypoxia inducible factor
HLA	Human leukocyte antigen
HRE	Hypoxia response element
HSCT	Haematopoetic stem cell transplantation
ICAM-1	Intercellular adhesion molecule-1
ICF	Informed Consent Form
ICH	International Council of Harmonisation of Technical Requirements for Pharmaceuticals for Human Use
IGFBP1	Insulin like growth factor binding protein 1
IIT	Institute of Immunity and Transplantation
IL-1	Interleukin 1
IL-18	Interleukin 18
IL-1β	Interleukin 1 beta
IL-4	Interleukin 4
IL-5	Interleukin 5
IL-6	Interleukin 6

IL-8	Interleukin 8
ILD	Interstitial Lung Disease
IMP	Investigational medical product
IQR	Inter-quartile range
IU	International units
IV	Intravenous
JRO	Joint research office
KCO	Transfer coefficient
KG (kg)	Kilograms
lcSSc	Limited cutaneous systemic sclerosis
lcSSc	Limited systemic sclerosis
MCDB	Microvascular endothelial cell basal medium
MCID	Minimal clinically important differences
MCP-1	Monocyte chemoattractant protein 1
MedDRA	Medical dictionary for regulatory activities
MMP	Matrix metalloproteinase

mRSS	Modified Rodnan Skin Score
MUGA	Multigated acquisition scan
NADH	Nicotinamide adenine dinucleotide
NHS	National Health Service
NR4A1	Nerve growth factor IB
NSAIDs	Non-steroidal anti-inflammatory drugs
NSIP	Non-specific interstitial pneumonia
OMERACT	Outcome Measures in Rheumatology
OP	Organising pneumonia
PAH	Pulmonary artery hypertension
PAI-1	Plasminogen activator inhibitor-1
PBS-T	Phosphate buffered saline with tween
PCR	Polymerase chain reaction
PDE-5	Phosphodiesterase 5
PDGF	Platelet derived growth factor
PFT	Pulmonary function test

PI	Principal Investigator
PIS	Patient Information Sheet
PK	Pharmacokinetic
PLGF	Placental growth factor, PGF
PRO	Patient Reported Outcome
PRR	Pattern recognition receptor
RA	Rheumatoid arthritis
RCS	Raynaud’s condition score
REDCap	Research electronic data capture programme
RP	Raynaud’s phenomenon, Raynaud’s syndrome
SAE	Serious adverse event
SBBO	Small bowel bacterial overgrowth
sCD40L	Soluble Cluster of Differentiation 40 Ligand, sCD154
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SF-36	36 item Short Form Questionnaire

sFASL	Serum soluble Fas ligand
SHAQ	Scleroderma Health Assessment Questionnaire
SLE	Systemic lupus erythematosus
SRC	Scleroderma renal crisis
SSc	Systemic Sclerosis, scleroderma
SSRI	Selective serotonin reuptake inhibitor
TBS	Tris buffered saline
TF	Tissue factor
TG2	Tissue transglutaminase 2
TGFα	Transforming growth factor alpha
TGFβ	Transforming growth factor beta
$TGF\beta_3$	Transforming growth factor beta 3
TIMP-1	Tissue inhibitor of metalloproteinase 1
TLC	Total Lung Capacity
TLR	Toll-like receptor
TLR-2	Toll-like receptor 2

TNFα	Tumour necrosis factor alpha, TNF, cachexin
TSP-1	Thrombospondin-1
UCL	University College London
UIP	Usual interstitial pneumonia
UK SMART	ScleroderMA cohoRT
ULN	Upper limit of normal
uPA	Urokinase-type plasminogen activator
VAS	Visual Analogue Score
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor
VEGF-A	Vascular endothelial growth factor A
VEGF-C	Vascular endothelial growth factor C
VEGF-D	Vascular endothelial growth factor D
vWF	Von Willebrand factor

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1 Introduction

1.1 Systemic sclerosis

1.1.1 Overview

Systemic sclerosis (SSc), also known as scleroderma, is a rare multi-system autoimmune connective tissue disease with significant morbidity and mortality. It leads to the accumulation of collagen and other extra-cellular matrix components in the skin and internal organs, and vascular damage. It is associated with the development of specific autoantibodies, which include anti-nuclear antibody (ANA), anti-Scl70 antibody (also known as anti-topoisomerase 1 antibody), and anti-centromere antibody (ACA). There are many signs and symptoms seen in systemic sclerosis. The eponymous manifestation is skin fibrosis, which patients may describe as tightness or puffiness, but the disease also leads to painful triphasic colour changes in the fingers and toes (Raynaud's phenomenon or syndrome (RP)), arthralgia, dysphagia, dyspepsia, digital ulceration, bowel disturbance and shortness of breath.

1.1.2 Classification

The first classification criteria for SSc were developed in 1980 by Masi et al. and formed the preliminary American College of Rheumatology (ACR) classification criteria for SSc (table 1.1). They were not designed as a diagnostic tool and had a high specificity, therefore there was a degree of under-diagnosis using these criteria, particularly affecting patients with mild or no skin involvement. The preliminary ACR criteria also did not take into account the autoantibodies which have been shown to correlate with systemic sclerosis (Reveille and Solomon, 2003).

Table 1.1. 1980 American College of Rheumatology preliminary classification criteria for systemic sclerosis (Masi et al., 1980)

Major Criterion	1. Proximal scleroderma
Minor Criteria	1. Sclerodactyly
	2. Digital pitting scars of fingertips or loss of substance of the distal finger pad
	3. Bibasilar pulmonary fibrosis
One major or two or more minor criteria were found to confer a high specificity for systemic sclerosis	

A set of diagnostic criteria were developed by LeRoy and Medsger in 2001 (table 1.2) (LeRoy and Medsger Jr., 2001). These criteria aimed to encompass systemic sclerosis-associated autoantibodies and consider the vascular effects of the disease.

Table 1.2. 2001 criteria for the diagnosis of systemic sclerosis (LeRoy and Medsger Jr., 2001)

Raynaud's phenomenon objectively documented by:	1. Direct observation of any 2 of:	A. Pallor (well demarcated whitening of acral skin)
		B. Cyanosis (dusky blueness, which disappears on rewarming)
		C. Suffusion (well demarcated redness)
OR	2. Direct measurement of response to cold by:	A. Objective evidence of delayed recovery after cold challenge
		B. Nielsen test or equivalent
PLUS	1. Abnormal wide-field nail fold capillaroscopy, consisting of dilation and/or avascular areas	
OR	SSc selective autoantibodies (anti-centromere, anti-topoisomerase I, anti-fibrillarin, anti-PM-Scl, anti-fibrillin or anti-RNA polymerase I or III in a titre of 1:100 or higher)	
If RP is subjective only, both SSc capillary pattern and SSc selective autoantibodies (in titre > 1:100) are required to define LcSSc. LcSSc can overlap with any other disease.		

The LeRoy criteria from 2001 were followed in 2013 by the ACR/European League Against Rheumatism (EULAR) classification criteria for systemic sclerosis (table

1.3) (Hoogen, 2013). These were developed by an international panel of physicians specialising in systemic sclerosis.

Table 1.3. Preliminary ACR/EULAR criteria for systemic sclerosis (Hoogen, 2013)

Items		**Weight**
Skin thickening of the fingers of both hands, extending proximally to the metacarpophalangeal (MCP) joints		9
Skin thickening of the fingers *(only count the highest score)*	Puffy fingers	2
	Whole Finger, distal to MCP	4
Fingertip lesions *(only count the highest score)*	Digital Tip Ulcers	2
	Pitting Scars	3
Telangiectasia		2
Abnormal nail fold capillaries		2
Pulmonary arterial hypertension and/or interstitial lung disease		2
Raynaud's phenomenon		3
Scleroderma related antibodies (any of anti-centromere, anti-topoisomerase I or anti-ribonucleic acid (RNA) polymerase III)		3
Patients having a total score of 9 or more are classified as having definite systemic sclerosis		

Systemic sclerosis with skin involvement is classified into two subsets: diffuse cutaneous systemic sclerosis (dcSSc) and limited cutaneous systemic sclerosis (lcSSc). These are defined by the extent of skin involvement and diagnostic criteria have also been established by LeRoy and Medsger (LeRoy and Medsger Jr., 2001).

Table 1.4. Criteria for diagnosis of subsets of SSc, adapted from LeRoy and Medsger Jr., 2001

Disease subset	Criteria
LcSSc	Criteria for SSc plus distal cutaneous changes
DcSSc	Criteria for SSc plus proximal cutaneous changes

1.1.3 Epidemiology

The incidence and prevalence of systemic sclerosis have historically been difficult to estimate due to the different methods of diagnosis and classification, as described above. Even with the 2013 ACR/EULAR classification criteria now widely accepted by the international community, estimates of incidence and prevalence vary geographically. This may be partly due to differing reporting rates related to different health care systems, reporting methods and disease definitions. The variation may also be partly due to true variation between populations due to genetic or environmental factors.

Many data used to estimate incidence and prevalence rates are from the latter half of the 20th century. Reviewing more recent data from the 21st century shows a wide variation in the published incidence and prevalence rates of systemic sclerosis. Diagnostic coding data from the USA from 2003 to 2008 produces an incidence rate of 46 cases per million per year, and a prevalence of 135-184 cases per

million (Furst et al., 2012). Diagnostic coding data gathered Canada in 2003 gives a prevalence of 443 cases per million (Bernatsky et al., 2009). Data gathered in England in 2000 generates a prevalence of 88 cases per million (Allcock et al., 2004), whereas data gathered in France in 2006 gives a prevalence of 132.2 cases per million (El Adssi et al., 2013).

It has been suggested that the incidence rate of systemic sclerosis is increasing over time. In 1971 Medsger and Masi published data reporting that the incidence rate of systemic sclerosis in Tennessee increased from 0.6 cases per million per year between 1947 and 1952; to 4.5 cases per million per year between 1953 and 1968 (Medsger and Masi, 1971). Similar increases have been reported in Pennsylvania where reported incidence of systemic sclerosis rose from 9.6 cases per million per year between 1963 and 1972, to 18.7 cases per million per year between 1973 and 1982 (Steen et al., 1997). However, during the periods that have been reviewed healthcare has improved significantly on a global level, perhaps leading to better diagnostics and increased reporting. In addition, the classification criteria discussed above have been published and used widely in clinical practice, so patients presenting now might receive a diagnosis of systemic sclerosis where physicians may previously have assigned an alternative diagnosis. In 2014, Andreasson et al compared incidence and prevalence rates in Sweden using both the 1980 ACR and 2013 ACR/EULAR classification criteria for systemic sclerosis. They found that applying the 2013 criteria to the patient population generates incidence and prevalence rates which are 30-40% higher than those determined using the 1980 criteria (Andreasson et al., 2014).

Geographic variation in the incidence of SSc leads to speculation that genetic factors may play a significant role. As discussed above, incidence rates for North America tend to be higher than those for Europe, even when different data collection methods are considered. In 1996, Arnett et al. reported a high incidence of SSc among a native American population in Oklahoma, and interestingly the patients from this population mostly displayed a similar phenotype of SSc, suggesting that a genetic factor might be contributing to the high incidence (Arnett et al., 1996). Other racial variations have been noted including a higher prevalence

among black patients compared to white, and a higher rate of diffuse SSc and interstitial lung disease (ILD) among black patients (Mayes et al., 2003, Le Guern et al., 2004).

Additionally, high incidences in geographic locations where there is significant genetic variation in the population could suggest environmental factors are present that predispose patients to developing SSc. Examples of such areas include higher than expected rates in Ontario, Canada, compared to other local areas and higher rates around London airports compared to the rest of the city (Thompson and Pope, 2002, Silman et al., 1990).

All epidemiological studies of SSc note that the disease is more common in females, and this is true across different geographical locations. Estimated female to male ratio of disease incidence varies from 1.5:1 (Medsger and Masi, 1971) to 14:1 (Tamaki et al., 1991).

1.1.4 Aetiology and pathogenesis

The aetiology of SSc remains unknown, but it is thought to be due to both genetic and environmental factors.

Due to the rare nature of the condition, only one twin study has been published. This compared 42 twin pairs where at least one twin had SSc. This study found a concordance rate of 4.7% with no variation between monozygotic and dizygotic twins (Feghali-Bostwick et al., 2003). There are also a limited number of studies that look at heritability in a large cohort. Published data suggest an increased relative risk of between 3.07 and 14.3 when a first degree relative has a diagnosis of SSc, and other data suggest that the presence of SSc in a first degree relative increases the relative risk of other autoimmune conditions (Frech et al., 2010, Chandran et al., 1995, Arnett et al., 2001, Hudson et al., 2008).

Though the epidemiological data show that SSc is not inherited in a Mendelian fashion, genetics are still believed to be relevant in the aetiology of the disease. It is most likely that multiple genes are involved in predisposing a person to the

development of SSc. Candidate genes are those involved in innate and adaptive immunity, apoptosis and fibrosis.

There is a well-established link between the presence of certain human leukocyte antigen (HLA) sub-types and the development of SSc. In particular SSc has been shown to correlate with HLA DQB*0301, DRB1*1104 and DQA1*0501 (Arnett et al., 2010) . Furthermore, the different SSc autoantibodies have been shown to associate with different HLA sub-types. For example, HLA DRB1*11 correlates with the presence of the anti-Scl-70 antibody whereas HLA-DRB1*01, DRB01*04 and DQB1*0501 correlate with the presence of ACA (Reveille et al., 2001). Other immune system genetic variants linked with the development of SSc include interferon 5 and 8 (IL-5, IL-8), and toll-like receptor 2 (TLR-2) (Dieude et al., 2009, Gorlova et al., 2011, Broen et al., 2012).

Exposure to some environmental triggers has been linked to the development of SSc. The best reported environmental triggers are solvents and silica. There are multiple reports suggesting that exposure to chemical solvents can predispose people to the development of SSc. A meta-analysis of 11 case control series by Kettaneh et al. has confirmed an increased risk in those exposed to chemical solvents. However it was impossible to determine which solvents particularly may increase risk due to the wide variety of solvent types and the limited number of reports available (Kettaneh et al., 2007). A further meta-analysis by McCormic et al. indicates that the relative risk of development of SSc is increased in men who have been exposed to silica (McCormic et al., 2010).

Infectious agents have long been considered relevant to the aetiology of autoimmune diseases. The hypothesis is that the presence of specific infectious agents causes the immune system to develop antigens against self via molecular mimicry. Various pathogens have been implicated in the development of SSc, particularly viral infections including Epstein-Barr virus, cytomegalovirus and parvovirus B19 (Farina et al., 2014, Halenius and Hengel, 2014, Magro et al., 2004). However, it is difficult to establish a causal link due to the high rates of infection with these common viral illnesses in the general population.

The pathogenesis of SSc is incompletely understood and appears to involve the interaction of multiple different factors. It is generally believed that vascular dysfunction helps to contribute to immune activation, the release of soluble mediators and a dysfunctional extracellular matrix environment. This then results in inappropriate fibroblast activation and the deposition of excess collagen (1996, Sollberg et al., 1994).

Vascular dysfunction

Vascular changes occur early in the disease process of SSc and include perivascular infiltration, capillary leakage and disorganized, abnormal capillary network structure (Cutolo et al., 2003). This manifests clinically as Raynaud's phenomenon and abnormal nail fold capillaroscopy, which often pre-date other symptoms and signs.

Multiple vascular processes have been implicated in the pathogenesis of SSc, including those involved in vasoconstriction, vasodilation, angiogenesis, vasculogenesis and vascular endothelial cell function.

Endothelin is a vasoconstrictor and fibrogenic molecule which may be involved in the pathogenesis of SSc by contributing to early vascular dysfunction (Zamora et al., 1990). Levels of endothelin are raised in patients with predominantly vascular symptoms of SSc; and in patients with a more fibrotic phenotype, suggesting that it may also contribute to fibrosis (Yamane et al., 1992, Vancheeswaran et al., 1994). This is supported by data that show a dose-dependent collagen-synthesising effect for endothelin when applied to fibroblasts (Kahaleh, 1991). Endothelin is also upregulated in the fibroblasts of SSc patients (Kawaguchi et al., 1994).

Nitric oxide is a vasodilator, which in normal vessels counterbalances the effects of endothelin. Studies have shown that nitric oxide-producing compounds are overall reduced in the plasma of patients with SSc, and also the normal production of nitric oxide in response to a cold challenge is reduced in these patients (Kahaleh and LeRoy, 1999). However the relevance of nitric oxide is not clear, because a further

study has shown elevated nitric oxide levels in the plasma of SSc patients (Andersen et al., 2000).

A study by Flavahan et al has shown that the arterioles in SSc patients are abnormally sensitive to selective alpha-2 adrenergic receptor agonists. This raises the suggestion that although the vasoconstrictor/vasodilator balance may be disrupted in SSc, the vessel response to these mediators may also be abnormal (Flavahan et al., 2000).

In the healthy population, prolonged tissue hypoxia leads to angiogenesis. Angiogenesis is the formation of new blood vessels as a branch from pre-existing vessels, whereas vasculogenesis is the formation of new vessels independent of pre-existing vessels. Vessels formed by vasculogenesis are formed from circulating endothelial progenitor cells. These two processes are necessary to provide oxygen and nutrients to growing tissues, such as during gestation and childhood. They involve multiple signalling pathways and an interplay between pro-angiogenic and anti-angiogenic molecules, the potential roles of which in SSc are discussed below.

Angiogenesis is triggered by hypoxia inducible factor (HIF), a transcription factor whose expression increases in hypoxic conditions. It binds to the hypoxia response element (HRE) located in the 3'-flanking region of the erythropoietin gene (Semenza and Wang, 1992). HIF also has binding sites in the genes for vascular endothelial growth factor-A (VEGF-A), transforming growth factor β_3 ($TGF\beta_3$) and membrane type 1 matrix metalloproteinase (Forsythe et al., 1996, Caniggia et al., 2000, Petrella et al., 2005). Overall HIF activates over 40 genes, many of which are involved in angiogenesis (Hirota and Semenza, 2006).

VEGF-A is one member of five related growth factors, the others being VEGF-B, -C, -D and placental growth factor (PLGF, PGF). VEGF-A was the first of this family to be discovered, and in much of the published literature is simply referred to as VEGF. Although angiogenesis is complex, there is comprehensive research to support a key role for VEGF-A signalling in this process (Yancopoulos et al.,

2000). VEGF-A stimulates the grown of vascular endothelial cells and prevents their death by apoptosis (Ferrara and Davis-Smyth, 1997, Gerber et al., 1998b, Gerber et al., 1998a). VEGF-A is known to be an important pro-angiogenic factor to the extent that embryos with mutations in VEGF-A are unviable (Ferrara, 2001).

It has been hypothesised that patients with SSc are deficient in part of the angiogenesis pathway, so that tissue hypoxia does not trigger appropriate angiogenesis, resulting in ischaemic damage such as digital ulceration (Distler et al., 2002, LeRoy, 1996). As VEGF-A is a key player in angiogenesis, its role in SSc has been investigated.

Patients with SSc paradoxically exhibit higher levels of VEGF-A than their healthy counterparts. In skin biopsies taken from 15 patients, the mean percentage of keratinocytes expressing VEGF-A was 14% in healthy controls and 50% in SSc patients. However, the levels of HIF were not consistent with this upregulation, suggesting that a HIF-independent mechanism may be the cause of the VEGF-A upregulation in SSc (Distler et al., 2004). It seems that the above-average VEGF-A levels might paradoxically have a negative effect on blood vessels, rather than a pro-angiogenic one. Sustained high levels of VEGF-A cause vessels to form in an uncontrolled manner and with increased permeability, which has been noted to resemble the vascular changes seen in SSc (Dor et al., 2002).

Serum VEGF-A levels correlate well with the presence of digital ulcers. The link between VEGF-A and digital ulceration was studied by Distler et al in 2002. The highest levels of VEGF-A were seen in SSc patients without digital ulcers. SSc patients with digital ulcers also had raised levels, but lower than their ulcer-free counterparts. Healthy controls had lower levels of VEGF-A than SSc patients (Distler et al., 2002). This suggests that high levels of VEGF-A are protective against digital ulceration in SSc.

Platelet derived growth factor (PDGF) is a growth factor implicated in angiogenesis and tissue remodelling. It has been found in the endothelial cells of patients with SSc (Gay et al., 1989).

Thrombospondin-1 (TSP-1) is a known anti-angiogenic modulator. It works via both direct and indirect mechanisms. Its direct effect is to inhibit the migration of endothelial cells, and to induce apoptosis in these cells, therefore preventing them from contributing to new vessel formation (Lawler, 2002). Its indirect effect is to cause the activation and chemotaxis of inflammatory cells (BenEzra et al., 1993), and the growth and migration of myofibroblasts (Nicosia and Tuszynski, 1994). It is also known that TSP-1 inhibits pro-angiogenic factors such as metalloproteinases (Rodriguez-Manzaneque et al., 2001).

TSP-1 is thought to be directly involved in the pathogenesis of systemic sclerosis, as upregulated mRNA and increased protein synthesis of TSP-1 have been demonstrated in skin and cultured fibroblasts from patients with systemic sclerosis (Macko et al., 2002, Mimura et al., 2005). Levels of TSP-1 have also been shown to correlate with the extent of severity of skin fibrosis in systemic sclerosis (Farina et al., 2010, Delbarre et al., 1981).

Vasculogenesis is impaired in SSc patients. Patients have significantly reduced numbers of circulating endothelial progenitor cells from which vasculogenesis can be initiated. The endothelial progenitor cells that do exist are also less capable of differentiating into endothelial cells *in vitro* (Kuwana et al., 2004).

Vascular endothelial cells behave abnormally in SSc. This is due to multiple factors. Approximately 25% of patients with SSc produce antibodies directed against endothelial cells. These antibodies lead to the upregulation of adhesion molecules on the surfaces of endothelial cells, and can also initiate apoptotic pathways in endothelial cells (Carvalho et al., 1996, Sgonc et al., 2000, Worda et al., 2003, Ahmed et al., 2006). Endothelial cell apoptosis may also be triggered by generation of the complement-derived membrane attack complex, which has been detected in the vasculature of SSc patients (Sprott et al., 2000). This is thought to be secondary to chronic endothelial cell activation and eventually leads to endothelial cell damage (McHugh et al., 2003).

An interaction between endothelial cells and immune cells via adhesion molecules may contribute to the recruitment of inflammatory cells in the pathogenesis of SSc. Adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and endothelial leukocyte adhesion molecule-1 (E-selectin) are usually displayed by endothelial cells following trauma. These molecules recruit inflammatory cells into the extracellular matrix for tissue repair. In SSc, endothelial cells constitutively display higher than expected levels of the adhesion markers ICAM-1 and E-selectin (Claman et al., 1991, Sollberg et al., 1992, Gruschwitz et al., 1992). The soluble forms of the markers ICAM-1, VCAM-1 and E-selectin have been isolated from the blood of SSc patients. High levels of these markers have also been linked to more aggressive disease (Andersen et al., 2000, Sfikakis et al., 1993). Furthermore subtypes of T-cells from patients with SSc have an increased ability to adhere to the endothelium compared to non-SSc T-cells (Rudnicka et al., 1992).

Immune activation

A variety of immune cells behave abnormally in SSc and may be related to its pathogenesis. The innate immune system is implicated in the pathogenesis of SSc, with potential roles for toll-like receptors (TLRs), pattern recognition receptors (PRRs) and dendritic cell activation (York, 2011, Bhattacharyya et al., 2011).

Cluster of differentiation 4 positive (CD4+) T-cells are more likely to migrate across the vascular endothelium in SSc compared to controls, leading to a higher-than-expected ratio of CD4+ to cluster of differentiation 8 positive (CD8+) cells in the SSc endothelium. The migrated CD4+ cells also display an activated phenotype including expressing adhesion proteins (Stummvoll et al., 2004). The types of mononuclear cells that are recruited into the extracellular matrix in SSc are also unusual in that they display T-cell markers and multiple integrins on their surfaces (Prescott et al., 1992).

Antibodies directed against self-antigen (autoantibodies) are frequent in SSc and form part of the 2013 classification criteria (see above). The most common

autoantibodies are anti-centromere antibody and anti-Scl-70 antibody (also known as anti-topoisomerase-I antibody). Other less common antibodies include anti-RNA polymerase antibody, anti-fibrillarin antibody (also called anti-U3 ribonucleoprotein (RNP) antibody), anti PM-Scl antibody, anti U1-RNP antibody, anti Th/To antibody, anti U11/U12 antibody and anti-Ku antibody. Autoantibodies associated with other autoimmune diseases may co-exist in SSc patients with overlap conditions, for example a positive anti-Ro antibody might be seen in a patient with SSc/myositis overlap syndrome.

The SSc autoantibodies are generally regarded as markers of disease rather than key mediators in the disease pathogenesis. However some data have shown that anti-Scl-70 antibody binds specifically to topoisomerase-1 on the fibroblast surface, which in turn stimulates monocyte adhesion and activation (Henault et al., 2006).

Other less well recognized autoantibodies may have a pathogenic role in SSc. A study by Chizzolini et al showed that 58% of serum samples from SSc patients displayed anti-fibroblast antibodies (AFA), and in contrast none of the healthy control serum samples tested were positive for AFA. The serum from AFA-positive patients also induced upregulation of interleukin-6 (IL-6) and ICAM-1, suggesting that the presence of this antibody might be involved in pathogenesis (Chizzolini et al., 2002).

Soluble mediators

Multiple soluble mediators have been implicated in the pathogenesis of SSc. Mediators are produced by inflammatory cells and fibroblasts. They are produced and persist in both autocrine and paracrine feedback cycles, which may help explain the persistence of the SSc fibroblast phenotype *in vitro* even after several cell passages (Leroy, 1972, Uitto et al., 1979).

The importance of endothelin, nitric oxide, VEGF-A, PDGF and TSP-1 are discussed above.

IL-6 has been implicated in SSc pathogenesis since levels have been shown to be elevated in the serum and skin of SSc, particularly in patients with dcSSc (Khan et al., 2012). Other interleukins of interest include interleukin-1 (IL-1) and interleukin-4 (IL-4). IL-1 is overproduced in SSc, and may be linked to excess IL-6 production (Kawaguchi et al., 1999, Kawaguchi et al., 2004). IL-4 may play a role in inducing the pro-fibrotic SSc fibroblast phenotype (Distler et al., 2006).

Chemokine ligand-4 (CXCL4) (also known as platelet factor-4) is an anti-angiogenic chemokine, elevated levels of which have been found in the plasma of SSc patients (van Bon et al., 2014) and this may therefore play a role in the pathogenesis of the disease.

Connective tissue growth factor (CTGF) is a growth factor induced by transforming growth factor beta (TGFβ) which is implicated in fibrotic conditions including SSc. Levels of the N-terminal portion of CTGF have been shown to be elevated in SSc, and the levels correlate with the extent of skin fibrosis (Dziadzio et al., 2005).

Fibroblasts

Fibroblasts are a cell type that is common in the connective tissue. Fibroblasts synthesise extracellular matrix (ECM) proteins including collagen. They therefore have a key role in maintaining the function and integrity of connective tissue. There are multiple sub-types of fibroblasts whose function is specialised to meet the needs of their location.

Abnormal fibroblast functioning is thought to play a key role in SSc and is the endpoint of the other pathogenic processes described above. Though not all the mechanisms are known, we know that the pathogenic processes underlying SSc result in an inappropriate population of myofibroblasts – fully differentiated fibroblasts which lay down collagen.

In normal wound healing mechanisms, tissue injury results in fibroblast activation. Quiescent fibroblasts express endothelin-1 (ET-1) and intracellular adhesion molecules. Once activated by mechanical stress, fibroblasts transform into myofibroblasts and produce alpha-smooth muscle actin (α-SMA) and TGFβ.

Myofibroblasts contribute to granulation tissue formation, which eventually leads to scar formation as part of normal wound healing. In SSc the myofibroblast transformation occurs inappropriately, without a mechanical trigger present. Other disease processes exist in which fibroblast functioning is abnormal, such as keloid scar formation.

SSc fibroblasts dedicate 4 times more protein synthesis to collagen production than non-SSc fibroblasts. This leads to a greater volume of collagen being produced and more quickly than normal (LeRoy, 1974, Jimenez et al., 1986). This is due to the upregulation of genes expressing type I, III, VI and VII collagen (Buckingham et al., 1978, Peltonen et al., 1990, Rudnicka et al., 1994). This fibrogenic phenotype is common to both dermal and pulmonary SSc fibroblasts, suggesting that excess collagen deposition is also part of the pathogenesis of SSc-related pulmonary fibrosis (Shi-Wen et al., 1997). However, not all populations of fibroblasts in SSc patients display the excess collagen-producing phenotype. The phenotype appears to be isolated in specific subpopulations, particularly those close to mononuclear cells and proximal to blood vessels (Kahari et al., 1988, Scharffetter et al., 1988).

There are multiple non-collagen extracellular matrix molecules produced excessively by SSc fibroblasts which are also thought to contribute to the pathogenesis of SSc. These include elevated levels of fibronectin (Xu et al., 1991), increased synthesis of glycosaminoglycans (Falanga et al., 1987), increased expression of tissue inhibitor of metalloproteinase 1 (TIMP-1) (Bou-Gharios et al., 1994, Kirk et al., 1995), increased expression of intercellular adhesion molecules (Abraham et al., 1991) and constitutive secretion of IL-6 (Feghali et al., 1994).

Much of the abnormal protein production seen in scleroderma fibroblasts mimics a normal fibroblast response to exposure to TGFβ (Massague, 1990). Dysregulated TGFβ production and signalling has been proposed as a key mechanism in the development of the fibrogenic SSc myofibroblast phenotype (Gilbane et al., 2013). TGFβ signalling pathways are dysregulated in SSc partly because there are high levels of autocrine TGFβ produced by scleroderma myofibroblasts, and partly due

to increased expression of TGFβ receptors on scleroderma fibroblasts compared to normal. Excessive TGFβ signalling via these receptors leads to downstream dysregulation of many of the genes regulated by TGFβ including collagen (Kawakami et al., 1998). Furthermore, excessive TGFβ expression seems to dysregulate its own feedback processes that would allow a return to homeostasis. Persistent activation of the TGFβ signalling pathways results in inhibition of nuclear receptor subfamily 4 group A member 1 (NR4A1), an endogenous inhibitor of TGFβ signalling (Palumbo-Zerr et al., 2015). The importance of this dysregulated TGFβ signalling can be seen in mouse models, where inhibition of the TGFβ receptor in fibroblasts reduced the amount of fibrosis occurring as a response to injury (Hoyles et al., 2011).

1.1.5 Clinical features

Clinical features are systemic and yet variable between patients. They are commonly sub-divided by body system as below.

Skin thickening is the hallmark of SSc and informs the disease classification. Patients often experience an initial phase of ‘puffy’ skin, during which time they might state that their hands became swollen or that they could no longer remove rings. After this initial phase, the skin involvement progresses to cause skin thickening. Patients commonly describe this as ‘tight’, ‘hard’ or ‘restricted’ rather than thickened. The skin involvement most commonly affects the hands and face but can spread to involve the entire dermis. Patients may develop a classic SSc facial appearance including thinning of the lips and nose, microstomia, and loss of tissue from the cheeks leading to a ‘hollowed’ appearance. In late disease the skin thickening can recede and leave patients with loss of subcutaneous fat, leading to a ‘bony’ appearance in the fingers and toes (sclerodactyly).

Skin involvement can be functionally disabling for patients as it restricts the motion of the joints and structures underlying the affected skin. This is a particular issue in the hands where thickened skin can lead to contractures and result in loss of function. Patients often describe limitations to personal care and activities of daily

living (ADLs). When affecting the mouth, restricted skin results in microstomia which can affect the patient's ability to eat and can lead to weight loss. Skin involvement can also cause neuropathic pain.

SSc can affect the entire gastrointestinal (GI) tract. In the oesophagus SSc commonly causes gastro-oesophageal reflux disease (GORD), dysphagia and regurgitation of food. In the stomach SSC causes early satiety and gastric antral vascular ectasia (GAVE), also known as watermelon stomach, which can cause chronic bleeding and anaemia.

In the lower gastrointestinal tract SSc can cause bloating, diarrhoea, abdominal pain, small bowel bacterial overgrowth (SBBO), constipation, faecal urgency, and faecal incontinence.

In common with other chronic diseases, living with systemic sclerosis can cause depression and anxiety (Thombs et al., 2007).

Systemic sclerosis causes widespread fatigue that can limit the patient's ability to work and even to perform ADLs (Thombs et al., 2008).

Arthralgia is a common complaint, seen in up to 80% of patients (Di Franco et al., 2015). Patients may also have an overlap syndrome with inflammatory arthritis or myositis. This leads to joint pain and swelling secondary to synovitis and muscle pain and weakness respectively.

Vascular manifestations are among the most common symptoms of the disease and can cause significant pain, morbidity and loss of function. Systemic sclerosis causes Raynaud's phenomenon, a vasculopathy where digits pass through a triphasic colour change when exposed to cold. Patients can also develop digital ulcers which are painful and slow to heal. In severe cases peripheral vascular damage secondary to SSc can result in auto-amputation of the digits or gangrene requiring surgical amputation.

Systemic sclerosis can cause pulmonary artery hypertension (PAH) which manifests as progressive breathlessness that limits the patient's ability to walk, speak and perform ADLs.

Patients with systemic sclerosis can develop scleroderma renal crisis (SRC). This is associated with steroid use therefore high dose steroids are not recommended (Steen and Medsger, 1998).

Systemic sclerosis can cause interstitial lung disease (ILD), which causes breathlessness, cough, and reduced exercise capacity. Typically, the ILD is of the non-specific interstitial pneumonia (NSIP) pattern, but it is also possible to find usual interstitial pneumonia (UIP) and organising pneumonia (OP) related to SSc. Additionally, patients can develop uncommon forms of SSc-related lung disease including bronchiectasis. Respiratory involvement can lead to significant morbidity and affect a patient's independence and ability to self-care.

1.1.6 Treatment

Management guidance was produced by the EULAR Scleroderma Trials and Research Group (EUSTAR) in 2009 (Kowal-Bielecka et al., 2009) and updated by the same group in 2017(Kowal-Bielecka et al., 2017). Management depends on the body systems affected.

Conservative treatments for skin thickening include emollients and hand waxing to improve skin flexibility. Neuropathic pain due to skin involvement is managed using gabapentin, pregabalin or amitriptyline.

There are many options for the treatment of Raynaud's phenomenon. Hand warmers and lined gloves are commonly used to both treat and prevent attacks. Medical therapies focus on vasodilators to improve arterial supply to the digits and reduce ischemia. Di-hydropyridine calcium channel blockers (CCBs) are recommended to treat Raynaud's phenomenon (Thompson et al., 2001). Meta-analysis data show that phosphodiesterase-5 inhibitors (PDE-5 inhibitors) such as sildenafil are also effective in treating SSc-related Raynaud's phenomenon (Roustit

et al., 2013). Additionally prostacyclin analogues such as iloprost are effective but have a higher incidence of adverse effects, therefore are recommended for severe Raynaud's phenomenon (Pope et al., 2000) or digital ulceration (Wigley et al., 1994, Tingey et al., 2013). The endothelin receptor antagonist (ERA) bosentan is effective in preventing digital ulceration but does not speed up the healing time of active ulcers (Korn et al., 2004, Matucci-Cerinic et al., 2011).

Musculoskeletal pain can be treated with simple analgesia, such as paracetamol and ibuprofen. Non-steroidal anti-inflammatory drugs (NSAIDs) can exacerbate GORD so should be used cautiously. Musculoskeletal pain in the context of an overlap syndrome may be related to active myositis or synovitis, in which case it should be treated with immune suppression.

Proton-pump inhibitors (PPIs) are widely used to treat acid reflux in SSc (Pakozdi et al., 2009). Prokinetics such as domperidone and metoclopramide are recommended for symptoms related to motility disturbance such as dysphagia, GORD, early satiety and bloating, though evidence is limited (Soudah et al., 1991, Fiorucci et al., 1994). Diarrhoea and constipation are managed using loperamide and laxatives such as movicol and senna. Antibiotics are given for SBBO, and courses are often required on a cyclical basis. Frequently used antibiotics include ciprofloxacin, co-amoxiclav and doxycycline (Grace et al., 2013). Faecal incontinence is difficult to treat but there have been some successes using posterior tibial nerve stimulation (van der Wilt et al., 2017).

It is recommended that SRC is treated early with angiotensin converting enzyme (ACE) inhibitors as this is associated with increased survival at 1 year (Steen et al., 1990, Guillevin et al., 2012, Penn et al., 2007). Intravenous iloprost infusion can be used as a renal vasodilator, although the benefits remain unclear (Scorza et al., 1997). Renal replacement may be needed; often temporarily, and therefore these patients should be under the care of a renal physician.

Treatment options for PAH include PDE-5 inhibitors such as sildenafil and ERAs such as bosentan and macitentan which have all been shown to improve exercise

tolerance, functional class and some haemodynamic measures in PAH (Ghofrani et al., 2011, Pulido et al., 2013, Taichman et al., 2014). The prostacyclin analogue epoprostenol given continuously has also been shown to be beneficial in PAH and can be considered for severe (World Health Organisation (WHO) class III/IV) PAH in SSc (Badesch et al., 2000). Other prostacyclin analogues (treprostinil and inhaled iloprost) have also been approved for use in severe SSC-associated PAH (Kowal-Bielecka et al., 2017).

Systemic immune suppression is indicated in SSc where there is progressive skin thickening in a diffuse distribution, or interstitial lung disease. This takes the form of methotrexate (van den Hoogen et al., 1996) (Pope et al., 2001) or mycophenolate mofetil (Volkmann et al., 2017, Omair et al., 2015).

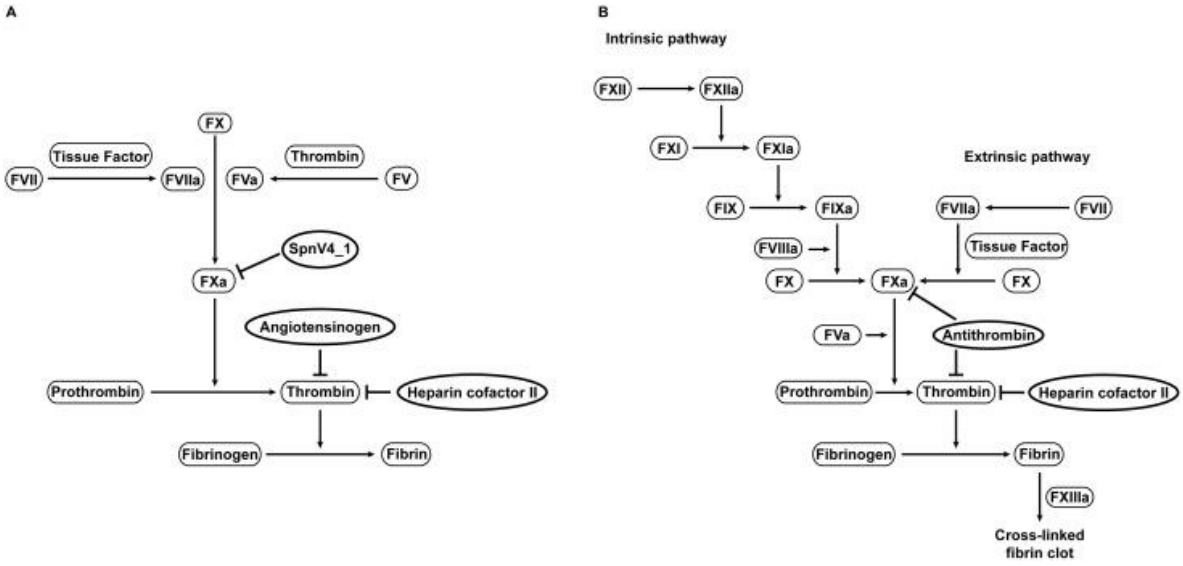
In progressive SSc-related interstitial lung disease that does not respond to mycophenolate mofetil, cyclophosphamide may be used, and is associated with improvements in lung volume, dyspnoea score and quality of life (Tashkin et al., 2006, Hoyles et al., 2006). Haematopoetic stem cell transplantation (HSCT) has been shown to improve skin involvement and stabilise declining lung function. However, it carries a significant risk of treatment-related mortality and is therefore reserved for patients with rapidly progressive systemic sclerosis at risk of organ failure (Burt et al., 2011, van Laar et al., 2014).

1.2 Factor XIII

1.2.1 Coagulation

Coagulation, also known as clotting or haemostasis, is the process by which liquid blood becomes solid blood clot or thrombus. This is a mechanism employed by the human body to reduce blood loss. It is a complex process involving multiple serine proteases known as clotting factors. The series of molecular events which lead to coagulation occur via two pathways which are known as the intrinsic and extrinsic pathways, and finally combine into a common end-point pathway (figure 1).

Figure 1 The coagulation cascade (Wang et al., 2014). Section A represents the common end-point pathway and section B represents the entire coagulation cascade.



The extrinsic pathway is also known as the tissue factor (TF) pathway. It is triggered by trauma which causes activation of TF. Activated TF forms a complex with factor VII (TF-FVIIa) which then activates factor IX and factor X. Once activated, factor X feeds into the common end-point pathway which is described below.

The intrinsic pathway is also known as the contact activation pathway. It is also triggered by trauma, which exposes vascular collagen leading to the activation of factor XII. Activated factor XII results in the downstream activation of factors XI, IX and VIII. Activated factors VIII and IX then activate factor X, which feeds into the common end-point pathway.

The common end point pathway leads to the conversion of prothrombin to thrombin by activated factor X. Thrombin has two roles in the coagulation cascade: it activates fibrinogen to form fibrin, and it activates factor XIII to form activated factor XIII (FXIIIa). Fibrin forms the basis of the blood clot and is further altered into a stable cross-linked fibrin clot by activated factor XIII.

1.2.2 Factor XIII and coagulation

Factor XIII is a plasma clotting factor and a pro-transglutaminase enzyme. It consists of 2 A subunits (FXIII-A) and 2 B subunits (FXIII-B). It was discovered by Robbins in 1944 (Robbins, 1944). The B subunit is produced in the liver (Nagy et al., 1986) whereas the A subunit is produced by haematopoetic cells (Poon et al., 1989). Activated factor XIII catalyses an acyl-transfer reaction which crosslinks fibrin chains. Specifically, this reaction results in the formation of covalent lysine cross-links between fibrin α chains (leading to α polymers) and between fibrin γ chains (leading to γ dimers) (Standeven et al., 2007, Siebenlist et al., 2001). This reaction, when amplified, results in cross-linked fibrin clots (Greenberg et al., 1991). The α and γ cross-linking processes result in increased blood clot stability and strength (Shen et al., 1974, Standeven et al., 2007).

Multiple lysine donor sites are present on the fibrin α chain, creating the potential for fibrin chains to cross-link whilst also recruiting other plasma proteins, such as thrombospondin-1 (TSP-1) (Sobel and Gawinowicz, 1996). The recruitment of TSP-1 and alpha 2-antiplasmin to the fibrin clot is catalysed by FXIII.

TSP-1 is a glycoprotein involved in platelet aggregation (Roberts et al., 2010). TSP-1 cross-links with itself in the presence of activated FXIII, forming homopolymers (Bale and Mosher, 1986, Lynch et al., 1987). Radioactive labelling has shown that these homopolymers are integrated into fibrin clots, and again this is increased in the presence of FXIIIa. The integration of TSP-1 homopolymers into the fibrin clot increases the clot's density in a dose dependent manner (Bale et al., 1985). The recruitment of TSP-1 by FXIII therefore further strengthens the fibrin clot.

Alpha 2-antiplasmin is a serine protease inhibitor that inhibits plasmin, and therefore prevents plasmin from breaking down fibrin. Alpha 2-antiplasmin is integrated into the fibrin clot during its cross-linking, a process facilitated by FXIII (Lee et al., 2004).

Factor XIII therefore helps to stabilise the fibrin clot whilst also recruiting a protein to protect it from being broken down. This is physiologically important, as clots deficient in activated FXIII or α2-antiplasmin lyse more quickly than clots containing both of these proteins (Fraser et al., 2011).

1.2.3 Other roles for factor XIII

Factor XIII facilitates cross-link formation between endothelial and platelet integrins, helping to recruit platelets to sites of trauma to contribute to wound healing (Dardik et al., 2002).

A further action of factor XIII on platelets may involve stimulating transformation to a pro-thrombotic platelet subset, known as collagen and thrombin activated (COAT) platelets. COAT platelets express fibrinogen, von-Willebrand factor (vWF), fibronectin, α2-antiplasmin and TSP-1 at their surface. These are pro-thrombotic proteins released from platelet α-granules which make COAT platelets more likely to engage in thrombosis than non-COAT platelets (Richardson et al., 2013). As discussed above, α2-antiplasmin and TSP-1 are known FXIII substrates. Inhibiting FXIII-A activity reduces the appearance of the specific COAT protein complexes on the surface of COAT platelets (Dale et al., 2002). It has been suggested that coordinated activity between the platelet α-granules, FXIII, phosphatidyl and platelet glycoprotein receptor activation must occur for the COAT platelet subset to develop (Szasz and Dale, 2002, Dale, 2005).

Factor XIII-A acts on fibronectin, which is cross-linked to the fibrin chain by FXIII (Matsuka et al., 1997). Fibronectin is a protein which binds integrins, fibrin, collagen and heparin. It has key roles in cell adhesion, migration and tissue repair (Pankov and Yamada, 2002, Brotchie and Wakefield, 1990). For example, fibronectin cross-linking to the fibrin chain appears to improve platelet recruitment into a clot (Cho and Mosher, 2006). In the cell-cell matrix setting, fibronectin cross-linking to fibrin aids the adhesion and spread of fibroblasts on fibronectin, and the spread of megakaryocytes on type-1 collagen (Corbett et al., 1997, Malara et al., 2011). Therefore, the FXIII-dependent cross-linking of fibronectin to fibrin has

important roles in the recruitment and spread of various cell types within the cell-cell matrix.

The extracellular matrix is also affected by FXIII. In addition to cross-linking fibronectin to fibrin, FXIII also cross-links type I, II, III and IV collagen to fibronectin. The formation of collagen-fibronectin connections affects extracellular matrix remodelling, cell proliferation and cell migration *in vitro* (Sevilla et al., 2013, Sottile et al., 2007) suggesting that these FXIII-dependent interactions may play a role in wound healing.

FXIII creates bridges between endothelial αvβ3 and platelet glycoprotein IIb/IIIa integrins, which are involved in the interaction between platelets and endothelial cells during wound healing (Dallabrida et al., 2000). Furthermore, activated FXIII improves endothelial tube formation, increases endothelial cell migration and decreases apoptotic cell numbers in a dose-dependent manner (Dardik et al., 2002). Further cell types affected by factor XIII are fibroblasts and vascular smooth muscle cells. Factor XIII is known to influence the migration of fibroblasts and vascular smooth muscle cells (Brown et al., 1993, Naito et al., 1998) as well as to increase fibroblast proliferation and adhesion of fibroblasts to the extracellular matrix (Barry and Mosher, 1990, Muszbek et al., 1996). Endothelial cells adhere to activated FXIII in an integrin-dependent manner (Dallabrida et al., 2000).

A key function of factor XIII in wound healing is thought to be due to downregulation of the anti-angiogenic protein TSP-1 (Dardik et al., 2003). TSP-1 is known to cause endothelial cell apoptosis via upregulation of the pro-apoptotic protein *Bax*, reduction of the anti-apoptotic protein B-cell lymphoma 2 (Bcl-2), and activation of the caspase death pathway (Mirochnik et al., 2008, Nor et al., 2000). When TSP-1 is overexpressed in mice, this is associated with poor levels of cutaneous healing, granulation tissue formation and wound-related angiogenesis, suggesting that inhibition of TSP-1 by factor XIII might be a mechanism by which FXIII assists in wound healing (Streit et al., 2000) .

The downregulation of TSP-1 by factor XIII appears to be mediated through the VEGF pathway. Factor XIII cross-links vascular endothelial growth factor receptor 2 (VEGFR-2) to αvβ3 integrin, which may trigger a downstream intracellular pathway leading to the downregulation of TSP-1, a model of which has been proposed by Dardik et al (Dardik et al., 2006).

In summary, factor XIII plays the following roles in wound healing: stabilising the fibrin clot, activating and recruiting pro-thrombotic platelets, fibroblasts and vascular smooth muscle cells into the wound, facilitating cell-cell and extracellular matrix connections, and inhibiting the anti-angiogenic TSP-1.

FXIII-A accelerates self-cross-linking of plasminogen molecules, as well as cross-linking plasminogen to fibronectin (Bendixen et al., 1993). Once activated, plasminogen becomes plasmin which is involved in clot fibrinolysis. This suggests that although factor XIII is a key player in the coagulation process where it promotes clot stability, it may also facilitate timely clot degradation, which is necessary for complete wound healing. Furthermore, plasmin activates matrix metalloproteinases (MMPs) which are essential for tissue repair, so factor XIII indirectly activates MMPs and assists in tissue repair by this mechanism (Castellino and Ploplis, 2005, Pepper, 2001).

Beyond coagulation and wound healing, FXIII is involved in bone health. Together with tissue transglutaminase 2 (TG2), factor XIII-A is one of the most abundant transglutaminases expressed in the cartilage and bone and is produced by chondrocytes and osteoblasts (Nurminskaya et al., 2002, Piercy-Kotb et al., 2012, Aeschlimann et al., 1996). FXIII-A may have a role in the maturation of both chondrocytes and osteoblasts, as well as the maturation of pre-osteoblasts into osteoblasts (Nurminskaya et al., 1998, Al-Jallad et al., 2006, Nakano et al., 2007). It also may play a role in the generation and maintenance of the extracellular matrix in cartilage and bone. We can see that FXIII-A has cross-linking activity in the cartilage and bone matrix (Nurminskaya et al., 2002, Nurminskaya et al., 1998, Sorensen et al., 1994, Kaartinen et al., 2002). Together with TG2, FXIII-A helps stabilise the osteoblast microtubule system, allowing enhanced secretion of type 1

collagen and fibronectin, which contribute to the extracellular matrix (Piercy-Kotb et al., 2012, Al-Jallad et al., 2011). By inhibiting FXIII-A we can see that its microtubule-stabilising role is important for effective deposition and mineralisation of the bone matrix (Al-Jallad et al., 2006, Al-Jallad et al., 2011) however when FXIII-A is not present its role appears to be compensated for by other transglutaminases (Nurminskaya and Kaartinen, 2006, Tarantino et al., 2009).

Furthermore, the factor XIII A subunit is found intracellularly, where it is known as cellular factor XIII A (cFXIII-A). A variety of cell types express cFXIII-A including platelets, megakaryocytes, monocytes, macrophages, fibroblasts, mast cells and dendritic cells. Factor XIII-A is involved in cytoskeletal remodelling, which contributes to platelet activation and macrophage phagocytosis (Poon et al., 1989, Adany and Bardos, 2003, Collin et al., 2013, Nemeth and Penneys, 1989, Shubin et al., 2017).

1.2.4 Factor XIII deficiency and replacement

Clinically significant factor XIII deficiency was first described in 1960 in a case report describing a boy with a severe bleeding diathesis and normal standard clotting tests. The only abnormality that could be found was that his blood clots were soluble in urea (Duckert et al., 1960). The urea clot lysis test has since been found to correlate directly with undetectable factor XIII levels on factor XIII assay (Al-Sharif et al., 2002).

In factor XIII deficiency, tests of the clotting pathways (activated partial thromboplastin time, bleeding time and prothrombin time) are normal. This is because these tests assess the formation of the clotting end point, fibrin, which is unaffected by the deficiency. It is the stability of the fibrin clot which is abnormal in FXIII deficient patients.

Inherited factor XIII deficiency is now known to be a rare, autosomal recessive disorder (Board et al., 1993b) that affects approximately 1 in every 2 to 5 million people (Lorand et al., 1980). It can be divided into deficiencies of subunit A and subunit B (Anwar and Miloszewski, 1999, Ivaskevicius et al., 2007).

In type 1 factor XIII deficiency both A and B subunits are physiologically absent (Girolami et al., 1977). Genomic studies have found that the genetic mutation in this disorder is in the gene for the B subunit alone. Subunit B is not detected in plasma of these patients. It is proposed that the B subunit is required for the stability of the A subunit in plasma (Saito et al., 1990), hence patients with type 1 deficiency have no appreciable subunit A activity, although their subunit A gene is normal (Izumi et al., 1996). This condition is rare and causes less than 5% of all reported cases of factor XIII deficiency. The classical deficiency is type 2, in which subunit A is absent.

The subtype of disease affects the clinical picture: type 1 deficiency is often asymptomatic other than during delivery where it causes post-partum haemorrhage (Saito et al., 1990) whereas type 2 deficiency is symptomatic and causes high proportions of umbilical cord bleeding and intra-cranial haemorrhage (Duckert, 1972).

Table 1.5. Common sites of bleeding in factor XIII deficiency (Karimi et al., 2009)

Site of bleeding	Percentage of patients affected (%)
Umbilical	80
Superficial bruising	60
Subcutaneous haematoma	55
Oral	30
Intracranial haemorrhage	30
Muscles	27

Laceration	26
Joints	24
Post-operative	17
Peritoneal	14
Epistaxis	10

A knockout mouse model for type 2 factor XIII deficiency has been established by targeted deletion of exon 7 of the FXIII-A gene. Mice which are homozygous for the mutant allele have no detectable FXIII-A activity and exhibit increased bleeding time, spontaneous subcutaneous, intra-thoracic and intra-peritoneal bleeding, and reduced survival. Bleeding time is restored to normal in the mouse model by administration of factor XIII concentrate (Lauer et al., 2002).

A 2016 study showed that patients who have a low endogenous plasma factor XIII level have reduced post-operative blood loss after coronary surgery when they receive peri-operative factor XIII (Godje et al., 2006).

Beyond an increased bleeding tendency, patients with factor XIII deficiency display impaired wound healing, the mechanisms of which are discussed further in section 1.2.5 below (Board et al., 1993a, Ivaskevicius et al., 2007, Seitz et al., 1996).

Fibrogammin® P is purified, pasteurised, plasma derived human FXIII that has been available for medicinal use in Europe since 1993. It is produced by CSL Behring as a white powder and reconstituted in normal saline (0.9% sodium chloride) for intravenous administration. It is supplied in vials containing 250 or 1250 international units (IU) of factor XIII.

Fibrogammin® P is used in prophylactic treatment of patients with FXIII deficiency and is known to be effective and well tolerated with a low risk of viral transmission (Nugent, 2012). Post-marketing data has shown that adverse events resulting from treatment with Fibrogammin® P are rare. The development of inhibitors to factor XIII occurs in < 1/10,000 people treated, anaphylactoid-type reactions occur in <1/1000 people treated and a rise in temperature occurs in <1/1000 people treated (US_National_Library_of_Medicine, 2021). Fibrogammin® P is known as Corifact™ in the USA.

1.2.5 Factor XIII and systemic sclerosis

1.2.5.1 Background data

The impaired wound healing noted in patients with factor XIII deficiency is likely to be at least partly due to the pro-angiogenic effect of factor XIII and related to its downregulation of the antiangiogenic TSP-1 (see section 1.2.3 above).

The link between FXIII and TSP-1 is of interest because TSP-1 has been shown to be upregulated in systemic sclerosis in several studies. Mimura et al have demonstrated that there is constitutive upregulation of TSP-1 in systemic sclerosis fibroblasts, and this appears to have autocrine effects on TGFβ (Mimura et al., 2005). Immunohistochemical staining of systemic sclerosis fibroblasts has also demonstrated increased TSP-1 expression (Macko et al., 2002, Avouac et al., 2011).

Furthermore, the upregulation of TSP-1 in systemic sclerosis may be linked to disease severity. A study in 2010 suggested that the degree of skin thickening correlates with the extent of TSP-1 upregulation (Farina et al., 2010).

Additional data of interest come from Paye et al. who have shown that activated factor XIII supplementation (1 U/ml) of SSc fibroblasts inhibits collagen deposition *in vitro,* which is another potential mechanism by which factor XIII signalling and systemic sclerosis may be linked (Paye et al., 1990).

1.2.5.2 In vivo studies

Excisional wounds in mice with congenital factor XIII deficiency take longer to heal when compared to a second group of mice with congenital factor XIII deficiency in which factor XIII has been replaced. Histology of the lesions shows delayed re-epithelialisation and necrosis in the factor XIII deficient mice, but normal healing histology in mice where factor XIII was replaced, therefore confirming that administration of factor XIII can reverse the abnormalities in wound healing (Inbal et al., 2005).

Dardik et al. have studied the effects of factor XIII on neovascularisation *in vivo*. Injection of thrombin-activated FXIII into the graft tissue in a heterotopic mouse heart allograft model produces increased numbers of new vessels and improved cardiac contractile performance. The development and infiltration of new blood vessels into a Matrigel™ graft (an artificial mixture that replicates the extracellular environment) is reduced in FXIII-knockout mice. This effect was almost entirely reversed by the addition of activated factor XIII (Dardik et al., 2003). This concept has also been applied in rats, where the development of blood vessels into a hydroxyapatite bone implant is stimulated by the addition of FXIII (Kilian et al., 2005). Administration of FXIII-A in rabbits results in corneal neo-vascularisation associated with inhibition of TSP-1 at 48 hours, an effect not seen in the placebo group (Dardik et al., 2003).

These studies demonstrate *in vivo* the effects of factor XIII on wound healing.

1.2.5.3 Clinical trials

Multiple studies have already shown promising results regarding factor XIII treatment in patients with systemic sclerosis. The first was undertaken in 1975 and was a case report series of 20 patients who received factor XIII. The study included patients with both systemic sclerosis and localized scleroderma. The study was not conducted to modern research standards and the published data are limited. The dose and frequency of FXIII administration are not stated, and the duration of treatment varied significantly (from 15 days to 6 months). Of the 17 patients with

systemic sclerosis included in the study, 12 are reported to have shown an improvement in their systemic sclerosis symptoms after treatment with factor XIII. The measures used to assess this improvement are not specified, but it seems a holistic approach was used as the report states that improvements in skin suppleness, joint mobility, vasomotor disturbances and alveolar-capillary diffusion were all found (Thivolet et al., 1975).

A double-blind, randomised, placebo-controlled, cross-over study analysing factor XIII efficacy in systemic sclerosis compared to placebo was undertaken in 1982. 25 patients with limited or diffuse systemic sclerosis received intravenous factor XIII (1 vial per dose, concentration not specified) or placebo twice each day for 3 weeks. Patients then had a 6-week wash-out period after which they were crossed-over into the other group (factor XIII or placebo) for 3 further weeks. Symptoms and signs of systemic sclerosis were assessed by patient-based assessment, physician assessment and functional assessment (grip strength and mouth opening) at the end of each 3-week treatment period. This study was more similar to modern day clinical trials in its design. These assessments showed improvement in patients following treatment with factor XIII in 20/25 cases, versus 3/20 following placebo. (Guillevin et al., 1982). In a second report on the same study the author reported that there was a high concordance between patient and physician-reported outcomes for the factor XIII treatment (Guillevin et al., 1985a).

Guillevin performed a second study in 1985 looking at the long-term efficacy and safety of factor XIII in systemic sclerosis. Eighty-six systemic sclerosis patients received different dosing regimens of intravenous factor XIII for a mean duration of 19 months. The patients were followed up for a mean period of 22.9 months. Guillevin and his team found that 44 out of the 86 patients had improvements in their skin disease, and the factor XIII was well tolerated. The greatest response was seen in patients treated more intensively, with 500 units of factor XIII per day for 21 days, cycle repeated 2-4 times. The lowest response was seen in patients who received 1 cycle of intensive treatment (500 units of factor XIII per day for 21 days) followed by a maintenance dose of 500 units of factor XIII every 10 days (Guillevin et al., 1985b).

Efficacy was also tested by Marzano et al in 1995. In this study 12 patients received 500 units of factor XIII on alternate days for 21 days, then entered a maintenance phase where they received 500 units every 10 days. This cycle was repeated every 6 months for a mean duration of 35.5 months. The study found that after a mean treatment period of 10 months, 75% of patients noticed improved skin symptoms. Smaller numbers reported improvements in Raynaud's syndrome, gastrointestinal symptoms, joint mobility and mouth opening (Marzano et al., 1995).

Once factor XIII treatment began to show benefit in systemic sclerosis, it was hypothesised that a relative factor XIII deficiency might be contributing to the aetiology of the disease. Endogenous factor XIII levels were measured in 22 patients with systemic sclerosis by Marzano and colleagues in 2000 (Marzano et al., 2000). Levels were found to be normal in 19 out of 22 patients therefore excluding a deficiency of factor XIII related to the aetiology of systemic sclerosis. It is therefore hypothesised that the positive effects of factor XIII administration are related to enhancement of factor XIII's endogenous effects on cell migration, cell-cell interactions and angiogenesis as discussed above.

Paye et al noted reductions in collagen synthesis when factor XIII (1U/ml) was administered to SSc fibroblasts *in vitro*, but this result was not replicated *in vivo* in this study. Patients received IV factor XIII treatment in two courses, 1250 U once daily for 15 days, followed by 1250 U once daily for 16 or 21 days. They had skin biopsies analysed before and after treatment. Fibroblasts extracted from these biopsies did not vary in their degree of collagen synthesis (Paye et al., 1990).

1.2.6 Further relationships between coagulation and systemic sclerosis

D-dimers are fibrin degradation products generated by plasmin during the process of fibrinolysis (the physiological degradation of cross-link fibrin clots). Plasma D-dimer level is tested clinically to exclude thrombosis. It is raised in various thrombotic conditions including deep vein thrombosis and pulmonary embolism.

In most clinical laboratories, plasma D-dimer level is measured using an enzyme-linked immunosorbent assay (ELISA). Rapid point-of-care tests have recently been developed for faster results (Riley et al., 2016). The D-dimer ELISA assay has high sensitivity but low specificity. Therefore, a normal plasma D-dimer level can essentially exclude a thrombotic condition, however a raised D-dimer level does not confirm thrombosis.

Plasma D-dimer level is also raised in physiological and pathological non-thrombotic conditions. These include pregnancy (Kovac et al., 2015, Hansen et al., 2011), cardiovascular disease (Lowe and Rumley, 2014) and autoimmune inflammatory conditions such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) (Nakashima et al., 1998, Li et al., 2010). A raised D-dimer level in these conditions is thought to be related to a pro-coagulant state, supported by evidence of higher rates of thrombosis in these conditions. Pregnancy is associated with an increased risk of thrombosis, primarily venous in nature (Ornaghi et al., 2015). It is well known that RA and SLE are associated with increased risk of thrombosis, accelerated atherosclerosis and cardiovascular disease (Roman et al., 2003, Hurlimann et al., 2004). D-dimer appears to be useful in predicting thrombotic events in SLE (Wu et al., 2008).

Several small studies have investigated a link between raised plasma D-dimer levels and SSc. In 2000, Trifiletti et al. found D-dimer levels in SSc patients to be no different from healthy controls (Trifiletti et al., 2000). However, in 1996 Falanga et al. found that SSc patients have raised D-dimer levels compared to healthy controls (Falanga et al., 1991). In 2006 Lippi et al. showed that patients with SSc have raised D-dimer levels compared to age and sex-matched controls. Upon stratifying the patients for disease subset, the team found that the raised levels were only seen in the diffuse cutaneous SSc; there was no difference in D-dimer level seen between limited cutaneous SSc patients and controls (Lippi et al., 2006).

In 2008 Marie et al. confirmed these findings in a larger study, and found that the presence of a raised D-dimer in these patients correlated with “macrovascular

impairment responsible for peripheral ischaemia" (Marie et al., 2008), suggesting that macrovascular impairment in SSc could be related to a pro-coagulant state.

Solfietti *et al* in 2016 performed plasma D-dimer levels and thrombin generation assays on groups of patients with RA and SSc, as well as healthy controls. Raised plasma D-dimer levels were confirmed in both RA and SSc groups, and additionally these groups had thrombin generation assay profiles compatible with a hypercoagulable state (Solfietti et al., 2016).

1.3 Sclero XIII clinical trials

The main experimental medicine study central to this thesis was a phase II double-blind, randomised, placebo-controlled study to investigate the safety and efficacy of intravenous factor XIII treatment in patients with diffuse or limited SSc (Sclero XIII). The trial was composed of two interlinked phases: a pharmacokinetic phase and a treatment phase. The pharmacokinetic phase involved administration of a single dose of factor XIII concentrate, followed by close monitoring of patient wellbeing and factor XIII levels. The treatment phase was a 24-week double-blind, randomized, placebo-controlled trial aiming to continue to observe the safety profile of factor XIII concentrate, and to monitor pharmacokinetics, adverse events, possible therapeutic benefit and associated biomarkers. The Sclero XIII clinical trials recruited eligible patients with a confirmed diagnosis of SSc and classification to either limited or diffuse subset (LeRoy and Medsger Jr., 2001). The investigational medicinal product (IMP) was Fibrogammin® (purified, pasteurised concentrate of human blood coagulation factor XIII) and the placebo was 0.9% sodium chloride solution.

The trial is registered on ClinicalTrials.gov (number NCT02551042) and is now closed (US_National_Library_of_Medicine, 2021).

Designing and conducting clinical trials in SSc is challenging for several reasons. As described, the disease has two different subtypes, and both have varied multi-system signs and symptoms. While patients may share some symptomatology, each patient's disease is unique. Thus, generating a primary efficacy end point for

a clinical trial is difficult. The disease is also rare, and lower patient numbers and geographic spread hamper recruitment.

Most clinical trials in SSc published in recent years use skin or lung fibrosis as the primary endpoint, measured using the modified Rodnan Skin Score (mRSS) and by radiological and functional pulmonary assessment respectively (Khanna et al., 2020b, Distler et al., 2019). Markers of less common clinical manifestations of disease, such as polymyopathy and cardiac involvement are used as secondary outcome measures (Chung et al., 2012).

The modified Rodnan Skin Score is a validated assessment of skin fibrosis shown to correlate with internal organ involvement and disease progression (Clements et al., 1993, Steen and Medsger, 2000, Steen and Medsger, 2001). It is widely used as the primary endpoint in clinical trials in SSc, including in the Sclero XIII trials. There are, however, several drawbacks with using the mRSS as an outcome measure. The mRSS score improves spontaneously over time, following the natural progression of SSc (Medsger, 2003, Shand et al., 2007). There is also intra and inter-assessor variability when performing the mRSS, though work has been done to reduce this and standardise skin scoring for use across trials (Khanna et al., 2017).

The mRSS has been used to estimate the minimal clinically important difference (MCID) in skin fibrosis in SSc. The MCID has been estimated for lcSSc as 3-4 units on the mRSS and for dcSSc as 5 units. Patients experiencing this degree of improvement in skin fibrosis show significant improvements in other symptoms as well, including shortness of breath and extent of joint contracture (Khanna et al., 2019).

When assessing digital vascular effects of SSc in a clinical trial setting, the Raynaud's condition score (RCS) is commonly used. This is a patient recorded assessment and asks the patient to record the number and duration of Raynaud's attacks, followed by rating the overall severity of their Raynaud's syndrome on a 10-point Likert scale (a 100-point Likert scale is used in some variations). The RCS

has been validated against the outcome measures in Rheumatology criteria (OMERACT) (Merkel et al., 2003). However, the results of patients' RCS results do not correlate well with objective measures of microvascular dysfunction; and concerns have also been raised that the RCS might be subject to the placebo effect (Pauling et al., 2015, Pauling et al., 2018b).

Pauling et al. have published the results of an international study investigating the patient experience of SSc. They have identified 7 'themes' that patients with SSc and associated RP rate as causing the greatest distress. This group plan to develop PROs that encompass these themes, with a view to recording the RP experience more effectively in future clinical trials (Pauling et al., 2018a).

Objective measurements of digital vascular dysfunction have been suggested for use in clinical trials. These include infra-red thermography and laser Doppler measures, but they have not been validated and are not considered feasible for use in large scale trials (Merkel et al., 2003).

The challenges faced when designing clinical trials in SSc and how to overcome them have been reviewed. Suggestions include trial design modifications including the early-escape design and the add-on design (Mendoza et al., 2012). The Sclero XIII clinical trials use an add-on design. An expert committee published guidelines in 2015 documenting 22 'points to consider' when designing clinical trials in SSc. Their intention was that this guideline will "lead to more uniform and higher-quality trials and clearly delineate areas where further research is needed" (Khanna et al., 2015).

In 2016 the American College of Rheumatology gave provisional endorsement to a new composite measure for use in randomised controlled trials in diffuse cutaneous systemic sclerosis, known as the Composite Response Index in DcSSc (CRISS). This has been shown to be both sensitive and specific in detecting changes in key organ systems in systemic sclerosis over time, and has been shown to differentiate the effects of methotrexate on disease progression when compared to placebo over a 1-year period in a randomised controlled trial (RCT)

(Khanna et al., 2016a). The benefits of the CRISS and other patient reported outcomes have been noted, specifically their ability to provide breadth of information to clinical trial endpoints, which is highly relevant in a multi-system disease such as systemic sclerosis (Pauling, 2019).

Composite measures such as the CRISS may be utilised in trials in SSc in the future and will hopefully generate high quality data for analysis. They have already been used successfully in clinical trials in systemic lupus erythematosus, a connective tissue disease with similar clinical heterogeneity which also presents challenges in clinical trial design (Furie et al., 2011). Composite measures are currently being validated for use in SSc clinical trials (Khanna et al., 2017, Khanna et al., 2020a).

Another future possibility for clinical trial design in SSc is to gather data by patient self-assessment. A recent publication suggests skin disease can be monitored remotely using the Patient self-Assessment of Skin Thickness in Upper Limb (PASTUL), and that this correlates well with validated SSc outcomes measures (Spierings et al., 2021).

Clinical trials in systemic sclerosis have mostly focused on diffuse SSc. As noted by Allanore et al, in his 2016 paper, lcSSc represents the majority of SSc diagnoses (between 56 and 73% depending on geographic population) and yet most clinical trials in SSc recruit diffuse SSc patients only (Allanore, 2016). At the time of writing (June 2021), a review of the ClinicalTrials.Gov website shows 351 trials registered for diffuse SSc and only 9 for limited SSc (US_National_Library_of_Medicine, 2021). Given that lcSSc patients comprise most patients seen in clinical practice, this exclusion could negatively affect many patients in the clinical setting. Some therapies may never be licensed for lcSSc patients as they have not been trialled in this cohort. Others may have varying efficacy in the lcSSc group compared to dcSSc, but the published data would not reflect this. Part of the issue with performing clinical trials in lcSSc relates to finding workable endpoints. As discussed, these are primarily focused on skin thickening and lung fibrosis, both of which predominantly affect the dcSSc population.

The Sclero XIII clinical trials were open to both limited and diffuse SSc patients. This allowed the IMP to reach a broader patient population, therefore generating data which represents the SSc patient population more completely.

1.4 Study objectives

The primary objective of the pharmacokinetic phase of the Sclero XIII clinical trial was to assess the safety and pharmacokinetics of a single dose of intravenous factor XIII therapy in systemic sclerosis. The main objective of the treatment phase was to assess the safety and tolerability of intravenous factor XIII therapy in systemic sclerosis over time. The study aimed to gather safety data based on clinical findings, number of treatment failures and safety-related organ assessments. Secondary objectives involved assessment of possible therapeutic effect; specifically considering effects on skin fibrosis, digital ischaemia, hand function, pulmonary function and quality of life.

1.5 In vitro work objectives

The *in vitro* work presented below provides extended breadth to the clinical trial work. The collection of serum from clinical trial participants, non-clinical trial SSc patients and healthy controls provides 3 comparator groups in which to review biomarkers relevant to the pathogenesis of SSc, and the effect of treatment with FXIII on these. Furthermore, the studies of fibroblast protein synthesis and behaviour provide data on the effects on FXIII administration *in vitro.*

1.6 Summary of background

Systemic sclerosis is a multi-system disease whose aetiology and pathogenesis are only partly understood. The disease leads to fibrosis of the skin and lungs, as well as vascular damage to the kidneys and peripheral blood vessels. It carries significant morbidity and mortality. The disease pathogenesis is mediated via a series of complex interactions between soluble mediators, the endothelium, vascular dysfunction, immune dysregulation, and fibroblast dysfunction. There are

no curative treatments available, and despite recent advances in clinical research there remain limited treatment options. Most clinical trials focus on the treatment of skin thickening or lung fibrosis in the dcSSc patient population. There remains a high unmet need for effective treatment for all manifestations of systemic sclerosis.

Factor XIII is a multi-functional endogenous human protein with roles in coagulation, angiogenesis and wound healing. This is likely mediated via its interactions with TSP-1, which is known to be upregulated in systemic sclerosis. Cellular and animal data provide support for a role for factor XIII in wound healing, and as a potential therapeutic treatment in systemic sclerosis.

Human factor XIII is available commercially as a pasteurised concentrate. It has been used for decades in the treatment of factor XIII deficiency and is therefore known to have an excellent safety profile.

Clinical trials involving the administration of factor XIII to systemic sclerosis patients took place in the 1980s and 90s and showed promising results. However, these were not conducted to modern GCP standards, the doses used were variable and in most studies subjects' factor XIII levels were not monitored.

The clinical trial at the centre of this thesis aims to address questions which are currently unanswered: namely to assess the safety, tolerability and efficacy of factor XIII therapy in systemic sclerosis by conducting a placebo-controlled randomised controlled trial. Close monitoring of factor XIII levels during the administration periods will generate data regarding the pharmacokinetics of exogenous factor XIII in the SSc patient population and will inform safe and efficacious dosing.

Given the many unknowns in the role of factor XIII in systemic sclerosis, and the pathogenesis of the disease itself, this work will also involve testing for candidate biomarkers alongside the clinical trial. By linking the biomarker work to the clinical trial, I hope to maximise data generation and provide added scientific value. Changes in inflammatory proteins during the trial may provide insight into signalling pathways affected by factor XIII, and by monitoring TSP-1 levels I hope to reaffirm

the link between systemic sclerosis, factor XIII and TSP-1. I will perform cellular studies using systemic sclerosis fibroblasts and factor XIII, to gain better insights into the effects of factor XIII on the disease processes. Additionally, since coagulation is a multi-step process of which FXIII plays only one part, I will also look more closely at the links between other proteins involved in coagulation and systemic sclerosis.

1.7 Hypotheses

My primary hypothesis is that exogenous administration of factor XIII is safe in systemic sclerosis.

My secondary hypotheses are:

- That factor XIII levels rise following administration of exogenous factor XIII and TSP-1 falls accordingly.
- That factor XIII leads to clinical improvements in skin thickening and digital ischaemia.
- That inflammatory proteins known to be involved in the pathobiology of systemic sclerosis are modulated by factor XIII administration.
- That abnormal behaviour seen in systemic sclerosis fibroblasts is modulated by the addition of factor XIII *in vitro*
- That other parameters in the coagulation pathways are also abnormal in systemic sclerosis, whether as active proteins involved in disease pathogenesis or as biomarkers.

2 Methods

2.1 Sponsorship, funding and ethics

The clinical trials were sponsored by University College London. The Sponsor representative was the UCL Joint Research Office (JRO), 1st Floor Maple House, Suite A, 149 Tottenham Court Road, London, W1T 7DN.

The clinical trials were funded by an academic research grant form CSL Behring to UCL through the Joint Research Office. The study drug was funded and supplied by CSL Behring. The study was approved by the National Health Service (NHS) National Research and Ethics Service, Hampstead Local Ethics Committee.

The trials were conducted in compliance with the approved protocol, European Union GCP and United Kingdom Regulations for Clinical Trials of an Investigational Medicinal Product (SI 2004/1031), the UK Data Protection Act (1998), the Royal Free Hospital Trust Information Governance Policy, the Research Governance Framework (2005' 2nd Edition), the Sponsor's Standard Operating Procedures, and other regulatory requirements.

2.2 Study recruitment

Participants were recruited from NHS outpatient clinics and the planned investigation and treatment unit at the Royal Free Hospital. All participants provided their free and informed written consent.

2.3 Eligibility

All patients met the eligibility criteria and no changes to the eligibility criteria were made once the trial had commenced.

Table 2.1 Clinical trials eligibility criteria

Inclusion criteria
Subject selection ≥ 18 years
Male and female adults
Subjects with a diagnosis of SSc according to the 2013 ACR EULAR classification criteria. They will be classified according to LeRoy criteria as limited or diffuse subset.
Subjects will have serological status for hepatitis A and B assessed at screening.
Patients who have given their free and informed consent
Patients willing to use an effective method of contraception (hormonal or barrier method of birth control; abstinence) from the time consent is signed until 6 weeks after treatment discontinuation (females of childbearing potential and males).
Patients who have a negative pregnancy test within 7 days prior to being registered for trial treatment (females of childbearing potential). Note: Subjects are considered not of child-bearing potential if they are surgically sterile (i.e., they have undergone a hysterectomy, bilateral tubal ligation, or bilateral oophorectomy) or they are postmenopausal.
Exclusion Criteria
Females must not be breastfeeding.

Must not have allergies to excipients of IMP and placebo
Must not have uncontrolled systemic hypertension as evidenced by sitting systolic blood pressure > 160 mmHg or sitting diastolic blood pressure > 100 mmHg.
Must not have portal hypertension or chronic liver disease defined as mild to severe hepatic impairment (Child-Pugh Class A-C). Subjects positive for hepatitis C with evidence of active viral replication on sensitive polymerase chain reaction (PCR) testing are also excluded.
Must not have hepatic dysfunction, defined as aspartate aminotransferase and/or alanine aminotransferase more than 3 times the upper limit of the normal range (ULN) at the screening visit.
Must not have chronic renal insufficiency as defined by a serum creatinine > 221 µmol/L or requires dialysis.
Must not have a haemoglobin concentration < 100 g/L at the screening visit.
Must not have a history of left-sided heart disease and/or clinically significant cardiac disease, including but not limited to any of the following: • Aortic or mitral valve disease (stenosis or regurgitation) defined as more than minimum aortic insufficiency and more than moderate mitral regurgitation; (stenosis or regurgitation > grade 1). • Pericardial constriction. • Restrictive or congestive cardiomyopathy. • Left ventricular ejection fraction < 40 % by multigated acquisition scan (MUGA), angiography, or echocardiography. • Left ventricular shortening fraction < 22 % by echocardiography.

• Symptomatic coronary disease with demonstrable ischaemia.
Must not have a history of malignancies within 5 years of screening visit, with the exception of localised skin or cervical carcinomas.
Must not have psychiatric, addictive, or other disorder that compromises the ability to give informed consent for participating in this study. This includes subjects with a recent history of abusing alcohol or illicit drugs.
Must not be receiving ongoing treatment with hyperbaric oxygen.
Must not have pulmonary arterial hypertension.
Must not have received IV iloprost within the last 2 months.
Must not have been treated with sympathectomy or toxin botulinum A within the last 3 months.
Must not have had a thrombosis, stroke, pulmonary embolism or myocardial infarction in the last 6 months.
Must not have a diagnosis of diabetes mellitus requiring dietary restriction of carbohydrate.
Must not have a low sodium diet on medical advice.
Must not be participating in another clinical trial involving an investigational medicinal product.
Must not be taking bosentan or have taken bosentan within the last 4 weeks

2.4 Concomitant medications

Patients were permitted to commence or continue most common medications used in the treatment of systemic sclerosis for the duration of the trial. Of note background immunosuppressive therapy, for example mycophenolate mofetil, was permitted. Additionally, most medications that are commonly used for the treatment of secondary Raynaud's syndrome and digital ulceration in systemic sclerosis were permitted. This included vasodilators, selective serotonin reuptake inhibitors, phosphodiesterase type 5 inhibitors, and IV prostacyclin analogues.

The only medication of note which was prohibited was the endothelin receptor antagonist bosentan.

2.5 Endpoints

No changes were made to trial endpoints after trial commencement.

The clinical trial analysis was descriptive, and the study was not powered for efficacy. The analysis primarily looked for numerical differences between the endpoints at week 12 and week 24, compared to baseline. The exploratory nature of the studies allowed the generation of data which may help to guide future studies with adequate statistical power. With the small patient numbers present in these studies, any numerical differences seen should be interpreted cautiously.

Table 2.2 Primary endpoints

Description of Endpoint	**Method of Assessing Endpoint**	**Timing of Assessments**
Change in skin involvement	Comparison of modified Rodnan skin score (mRSS) at week	Screening, baseline and week 4 during PK phase

	24 compared with baseline	Screening, baseline, week 4, 8, 12, 16, 20, 24 and 28 during treatment phase
Severity of Raynaud's phenomenon	Raynaud's condition score at week 24 compared to baseline	Baseline and week 4 during PK phase Baseline, week 4, 8, 12, 16, 20, 24 and 28 during treatment phase

Table 2.3 Secondary endpoints

Description of Endpoint	**Method of Assessing Endpoint**	**Timing of Assessments**
Pulmonary function	Pulmonary function testing measured by percentage of forced vital capacity (FVC) predicted, and percentage of diffusing capacity of the lung for carbon monoxide (DLCO) predicted compared with baseline	Baseline during PK phase Screening and week 24 during treatment phase
Healing of digital ulcers	Change from baseline in digital ulcer count measured at 12 and 24 weeks	Baseline and week 4 during PK phase Baseline, week 4, 8, 12, 16, 20, 24 and 28 during treatment phase

Number of new digital ulcers developed during treatment period	Change from baseline in digital ulcer count measured at 12 and 24 weeks	Baseline and week 4 during PK phase Baseline, week 4, 8, 12, 16, 20, 24 and 28 during treatment phase
Hand function	Cochin hand function score, score at week 12 and 24 compared to baseline	Baseline and week 4 during PK phase Baseline, week 4, 8, 12, 16, 20, 24 and 28 during treatment phase
Digital ulcer pain assessment	Visual analogue scale for pain (VAS) and Raynaud's condition score, score at week 12 and week 24 compared to baseline	Baseline and week 4 during PK phase Baseline, week 4, 8, 12, 16, 20, 24 and 28 during treatment phase
Digital ulcer worsening	Positive findings on the following digital ulcer count parameters: • Overnight hospitalisation for digital ulcers • Additional surgical treatment for digital ulcer	Baseline and week 4 during PK phase Baseline, week 4, 8, 12, 16, 20, 24 and 28 during treatment phase

	• Gangrene and/or amputation • Need of local sympathectomy • Need of botulinum toxin A • Need for systemic antibiotic • Need of unplanned IV Iloprost Number of positive findings meeting the definition of 'worsening' compared between groups at week 12 and week 24	
Treatment failure	• Positive result on any of the following parameters: • Rapid progression of skin involvement defined as more than 8 skin score units and 40% increase.	Week 1, 2, 3, 4, 5 and week 7 during PK phase Week 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24 and 28 during treatment phase

	• Rapid progression of lung involvement defined as 15% fall in FVC or 20% fall in DLCO • DU evolution to gangrene • Autoamputation or surgery for digital ulcer management (amputation) or hospitalisation	

Table 2.4 Safety endpoints

Safety and tolerability of study medication	Adverse event recording Serious adverse event recording Physical examination Electrocardiogram (ECG) Vital signs Clinical laboratory parameters Pregnancy Adverse events of special interest (thromboembolic events)	Baseline, week 1, 2, 3, 4, 5 and week 7 Baseline, week 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24 and 28 during treatment phase

2.6 Assessment and management of risk

There are limited published data reviewing the possible adverse effects of the administration of factor XIII concentrate in SSc. One publication discusses a cohort of 86 cases who were treated without signs of significant toxicity (Guillevin et al, 1985). Factor XIII concentrate is licensed and used routinely for the treatment of factor XIII deficiency. The major adverse events reported with the use of factor XIII for factor XIII deficiency are documented in the summary of product characteristics (SmPC) (Electronic_Medicines_Consortium, 2022).

Allergic reactions are potentially the most serious adverse effects. A further potential adverse effect is the extension of any existing blood clots, via the fibrin

stablising effects of factor XIII. The table below discusses the potential adverse effects and the mitigation strategies in place for this trial.

Table 2.5 Potential adverse events associated with the administration of intravenous factor XIII concentrate (Electronic_Medicines_Consortium, 2022) and their risk mitigation plan in the Sclero XIII trials

The table presented below is according to the medical dictionary for regulatory activities (MedDRA) system organ classification. Frequencies have been evaluated according to the following convention: very common (≥1/10); common (≥1/100 to <1/10); uncommon (≥1/1,000 to <1/100); rare (≥1/10,000 to <1/1,000); very rare (<1/10,000).

MedDRA Standard System Organ Class	Adverse Reaction	Frequency	Risk mitigation plan
Immune System Disorders	Allergoid-anaphylactoid reactions (e.g. generalised urticaria, rash, fall in blood pressure, dyspnoea)	Rare	Exclude from trial if allergies to IMP or any of the excipients are known. Any participants experiencing an allergoid-anaphylactoid reactions will have the have administration of Fibrogammin discontinued

			immediately and appropriate treatment initiated. Such subjects will not receive further doses of Fibrogammin. All Fibrogammin administration will occur under the supervision of medical staff in an appropriately equipped environment. All participants will be observed, and their vital signs monitored for 1-hour post-infusion.
	Development of inhibitors to FXIII	Very rare	The presence of inhibitory antibodies may manifest as an inadequate response to treatment. If expected, plasma FXIII activity levels are not attained, this

			diagnosis can be considered. Given the placebo-controlled nature of the trial, FXIII levels will be compared to the randomisation list during post-trial analysis. If discrepancies are present, then this diagnosis will be considered.
General Disorders and Administration Site Conditions	Rise in temperature	Rare	Review, consider study drug discontinuation and treat as appropriate
Thrombo-embolic events	Development of new (or extension of pre-existing) thrombosis due to the fibrin-stablising effects of FXIII	Effect not seen in post-marketing data for Fibrogammin	Patients with recent thromboses will be excluded from the trial Patients who develop thrombo-embolic events during the trial will not receive further study drug.

2.7 Sclero XIII clinical trial – pharmacokinetic phase

The pharmacokinetic (PK) phase involved administration of a single dose of IMP in 8 subjects followed by close review to assess the safety and tolerability of the IMP, and its pharmacokinetics. The data generated by this trial were used to generate a dosing algorithm for the treatment phase of the study.

Patient visits took place in the Institute of Immunity and Transplantation, an Outpatient Clinic at the Royal Free Hospital, Pond Street, London. All data relating to patient visits were gathered at this location. Capillaroscopy, pulmonary function test (PFT) and echocardiography results were gathered from patient records at the Royal Free Hospital. All tests performed as part of the trial took place at the Royal Free Hospital.

Table 2.6 PK phase participant flow

Week	-4	-3	-2	-1	0	1	2	3	4	5	6	7
N=8	Screening				IMP	Follow-up						

8 patients were recruited: 4 with limited cutaneous systemic sclerosis and 4 with diffuse cutaneous systemic sclerosis.

The 8 subjects received a single dose of IMP: Fibrogammin®. The treatment allocation was therefore unblinded to participants and investigators. The dose of IMP was calculated based on the patient's weight and their endogenous factor XIII level, both of which were assessed at the screening visit.

Participants with endogenous factor XIII level greater than or equal to 90 IU/dL received 20 IU per kilogram of patient bodyweight (IU/kg). Participants with endogenous levels less than 90 IU/dL received 40 IU/kg. The dosing protocol aimed to maintain factor XIII levels below 220 IU/dL.

The dosing protocol was based on the experience gained by CSL Behring in the use of Fibrogammin in factor XIII deficiency, for which Fibrogammin has been in use since 1993. Routine dosing of Fibrogammin as a prophylactic therapy in factor XIII deficiency is 40 IU/kg. The SmPC for Fibrogammin for this indication recommends titrating the dose against the trough factor XIII level and the patient's clinical condition and adjusting as needed. The Berichrom assay is recommended (Electronic_Medicines_Consortium, 2022). Although the approach of titrating based on clinical condition was not possible in the study, the study did use the approach of measuring trough levels and titrating based on these. Berichrom assays were performed throughout. To prioritise patient safety, only patients with low endogenous factor XIII levels received 40 IU/kg of Fibrogammin, others received a dose of 20 IU/kg. In animal studies, doses of 350 IU/kg have been administered without evidence of significant toxicity (Beyerle et al., 2016).

Dosing frequency was selected based on the half-life of factor XIII which is 9-10 days (Fear et al., 1983).

Infusions were administered at a maximum rate of 4 ml/minute by unblinded investigators, as recommended in the product SmPC (Electronic_Medicines_Consortium, 2022).

Bloods for factor XIII level were drawn at screening, at baseline prior to IMP infusion, and again 1 hour after the infusion commenced. Patients were then reviewed weekly for 6 weeks. Blood draws were performed at each visit, therefore generating results for factor XIII levels 1 hour, and 7, 14, 21, and 28 days after infusion.

The baseline visit took place within 28 days of the screening visit. Other visits occurred at the specified timepoint +/- 3 days.

A tabulated view of the assessments performed during the PK phase can be viewed in the schedule of assessments (table 2.7).

2.7.1 Screening visit

The patients received the patient information sheet (PIS) in advance of the screening visit. During the visit, the study was explained to the patients, and they were given the opportunity to ask any questions. Once the patient was happy, they were asked to sign the informed consent form (ICF). This was signed prior to starting any study procedures.

The rest of the screening visit aimed to establish a complete medical history and examination, to determine a patient's eligibility to participate in the trial; as well as drawing blood to establish their endogenous factor XIII level and routine blood sampling results.

2.7.2 Baseline visit

The purpose of the baseline visit was to review the patient's history, examination, and eligibility for entry into the trial and to establish baseline findings for the trial endpoints; including mRSS, patient reported outcomes (PROs) and physician-led assessments. Once this was completed, suitable patients received the IMP infusion.

2.7.3 Visits 2, 3 and 5

These were intermediary visits used to check safety and tolerability of IMP, and to draw blood for factor XIII level.

2.7.4 Visit 4

Visit 4 was performed at week 4 and information was gathered regarding tolerability, safety and trial endpoints. Patients were examined and physician assessments and PROs were completed. Blood was drawn for factor XIII level.

2.7.5 Follow-up visit

This visit reviewed patient wellbeing following trial completion. Patients also had a physical examination and ECG.

2.7.6 Schedule of assessments

Table 2.7 PK phase schedule of assessments

Assess-ments	**Screen-ing**	**Visit 1 base-line**	**Visit 2**	**Visit 3**	**Visit 4**	**Visit 5**	**Follow up**
	Week 0	Week1	Week 2	Week 3	Week 4	Week 5	Week 7
Visit windows (days)	-7	0 ± 7d	7 ± 3d	14 ± 3d	21 ± 3d	28 ± 3d	42 ± 3d
Written Informed Consent	x						
Subject Demography	x						
Medical History	x						
Inclusion and Exclusion Criteria	x	x					
Therapy-specific assessments							
Capillaro-scopy[9]		x					
Physician VAS		x			x		

Raynaud's condition score		x			x		
mRSS	x	x			x		
SHAQ		x			x		
Digital ulcer count		x			x		
SF-36		x			x		
Cochin hand function		x			x		
Safety assessments							
Concomitant medication	x	x			x		x
Physical examination	x	x			x		x
Vital Signs [1]	x	x	x	x	x	x	x
PFT[8]		x					
12-lead ECG		x					x
AE/SAE		x	x	x	x	x	x
Laboratory assessments							
Haematology[5]		x					
Chemistry[4]		x					
Urinalysis[6]		x					
Pregnancy Test [2]	x	x	x	x	x	x	x
Factor XIII[3]	x	x	x	x	x	x	
TSP-1		x					

Investigational product							
IMP administration [7]		x					

[1.] Vital signs will include heart rate, blood pressure, oxygen saturation, temperature, body weight and height. Height will be collected only at baseline.

[2.] Urine pregnancy tests at all visits, except baseline when a serum pregnancy test will be performed

[3.] Factor XIII blood sampling for PK at: pre-infusion, 1 hour after infusion and 7-, 14-, 21- and 28-days post-infusion

[4.] Serum alanine aminotransferase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), total bilirubin, creatinine, estimated glomerular filtration rate (eGFR), amylase, blood urea nitrogen (BUN), sodium, potassium, chloride, bicarbonate, calcium, total cholesterol, urate, glucose, total protein and albumin.

[5.] Haemoglobin, haematocrit, red cell count, red cell indices, white blood cell count (total and differential) and platelet count

[6.] Including a microscopic examination of the urine sediment if indicated

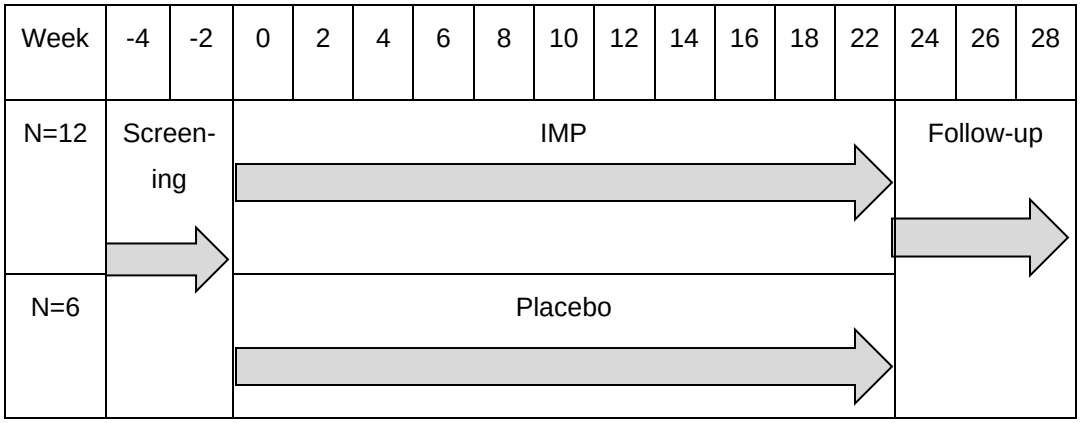
[7.] Patients to be observed for a minimum of 1-hour post-dose

[8.] If not performed in the 12 months preceding baseline visit

[9.] If never performed previously

2.8 Sclero XII clinical trial – treatment phase

Table 2.8 Treatment phase participant flow



The treatment phase was a multiple dose, double-blinded, randomised, controlled trial in 18 subjects. Subjects were randomised to the factor XIII or placebo groups using a 2:1 allocation ratio. Randomisation was stratified by disease subset (limited or diffuse SSc). Factor XIII or placebo was administered intravenously every 2 weeks for 24 weeks with all infusions taking place at the trial unit under supervision of trial investigators.

In the treatment phase of the study the intervention was IMP (Fibrogammin®) or placebo (0.9% sodium chloride in solution). Both interventions took the form of an IV infusion. The baseline visit took place within 28 days of the screening visit. Other visits occurred at the specified timepoint +/- 3 days. Blood samples were taken by the investigator and processed by the haemophilia laboratory at the Royal Free Hospital.

A tabulated view of the assessments performed during the treatment phase can be viewed in the schedule of assessments (table 2.10). An example of the prescription is shown in table 2.11.

2.8.1 Dosing

As before, dose of IMP or placebo was calculated based on patient weight and endogenous factor XIII level, which was assessed at the screening visit. The dosing algorithm was based on the pharmacokinetic phase results (section 3.1.3) and pre-existing data on the pharmacokinetics of Fibrogammin (section 2.7). The dosing protocol aimed to maintain factor XIII levels below 220 IU/dL.

Patients whose endogenous factor XIII level was ≤150 IU/dL at the screening visit received 40 IU/kg as their first dose at baseline (visit 1). Patients whose screening visit factor XIII level was 151-170 IU/dL received 30 IU/kg at the baseline visit and patients whose screening visit level was >170 IU/dL received 20 IU/kg at the baseline visit.

For the second dose (Visit 2), patients whose screening visit factor XIII level was ≤120 IU/dL received 40 IU/kg, and patients whose screening visit factor XIII level was >120 IU/dL received 20 IU/kg.

For the third dose (Visit 3) and subsequent doses, the dose was determined by the pre-dose factor XIII level from 14 days prior to the visit. If the pre-dose level 14 days prior to the visit was ≤150 IU/dL patients received 40 IU/kg. Patients whose pre-dose level from 14 days previously was 151-170 IU/dL received 30 IU/kg. Patients whose pre-dose level from 14 days previously was >170 IU/dL received 20 IU/kg. In this way the pre-dose level from Visit 2 was used to determine the dose for Visit 3, and the pre-dose level from Visit 3 was used to determine the dose for Visit 4, and so on.

If the level from 14 days prior to the visit was unavailable for any reason, the following structure was followed: patients whose screening factor XIII level was <100 IU/dL received 30 IU/kg, and all others received 20 IU/kg. To ensure patient safety, dosing was postponed for one week if the pre-dose factor XIII level from the previous visit was ≥200 IU/dL.

Table 2.9 Treatment phase dosing algorithm

Randomisation (Visit 1) and subsequent doses except Visit 2	**Dose**
Factor XIII level 14 days prior to visit ≤150 IU/dL	40 IU/kg
Factor XIII level 14 days prior to visit 151-170 IU/dL	30 IU/kg
Factor XIII level 14 days prior to visit 171-199 IU/dL	20 IU/kg
Factor XIII level 14 days prior to visit ≥200 IU/dL	No dose
Visit 2	
Factor XIII level at screening ≤120 IU/dL	40 IU/kg
Factor XIII level at screening 121-199 IU/dL	20 IU/kg
Factor XIII level at screening ≥200 IU/dL	No dose
If the factor XIII level from 14 days prior to visit was unavailable	
Factor XIII level at screening ≤100 IU/dL	30 IU/kg
Factor XIII level at screening 101-199 IU/dL	20 IU/kg
Factor XIII level at screening ≥200 IU/dL	No dose

2.8.2 Maintaining blinding

Patients and trial investigators were not aware of treatment group allocation. Pharmacy staff and study nurses were unblinded.

Factor XIII levels were reviewed only by the unblinded study nurse and were held separately to the patient's records, to maintain the investigator blind. The unblinded team members then communicated to the blinded investigator which range of the dosing algorithm included the current factor XIII level (table 2.9). The prescription was filled and signed by the Investigator using the dosing algorithm. By communicating to the investigator only a broad range which encompassed the patient's factor XIII level, rather than the precise level, the investigator was not able to deduce which treatment arm each patient had been allocated to.

The intervention was IMP (Fibrogammin®) or placebo (0.9% sodium chloride in solution). Both interventions took the form of an IV infusion. The IMP in solution was clear or slightly opalescent, and the placebo was clear. Fibrogammin® was supplied as white powder in vials and this was reconstituted in normal saline (0.9% sodium chloride solution) for intravenous administration. Vials contained 1250 IU of factor XIII.

The IMP/placebo was dispensed by the trial pharmacy to the unblinded study nurse. Only unblinded nurses were permitted to collect dispensed IMP/placebo. An opaque box was used to collect the IMP/placebo and move it around the department. IMP/placebo was prepared for administration by the unblinded nurse in a windowless preparation room which the investigators did not have access to. The correct volume of IMP/placebo was reconstituted and drawn into a syringe. The syringe was then covered in opaque material and passed to the study investigator.

Once intravenous access had been gained by the investigator, the opacified syringe was connected and IMP/placebo administered via an opacified giving set at the prescribed rate. The opacification process allowed both investigator and patient to remain blinded during the infusion.

2.8.3 Screening and baseline visits

These visits were identical to the corresponding visits performed during the PK phase (see sections 2.4.1 and 2.4.2 above) other than the additional steps of randomisation, and IMP/placebo preparation and administration as described above.

2.8.4 Visits 2, 4, 6, 8, 10, and 12

The main purpose of these visits was to check patient safety and tolerability, and to administer IMP/placebo doses.

2.8.5 Visits 3, 7, 9 and 11

Safety and tolerability were checked, and IMP/placebo administered as before. These visits also involved data collection for study end points, and thus PROs and physician assessments were completed, as well as clinical examination.
IMP/placebo administration

2.8.6 Visit 5

Visit 5 was similar to visit 3, involving data collection for study endpoints, IMP/placebo administration, safety and tolerability check and clinical examination. Additionally, routine blood sampling and thrombospondin-1 levels were performed.

2.8.7 Visit 13

Visit 13 took place at week 24. This was the end of trial visit. No IMP/placebo was administered at this visit. Patients underwent safety and tolerability check as well as blood draw, clinical examination and data collection for endpoint purposes. 12-lead ECG and pulmonary function tests were repeated.

2.8.8 Follow-up visit

The follow-up visit was completed 4 weeks after visit 13. Data were gathered for safety monitoring including routine blood tests, and for study endpoints.

2.8.9 Unscheduled and early withdrawal visits

At any unscheduled visit the patient completed study procedures as listed for Visits 3, 7, 9 and 11, plus a blood draw for haematology and biochemistry, and a urinalysis.

Patient undergoing early withdrawal completed study procedures as for the unscheduled visit, in addition to a capillaroscopy if one had not been performed previously.

2.8.10 Schedule of assessments

Table 2.10 Treatment phase schedule of assessments

Visit	Screen-ing	Base-line Visit 1	2	3	4	5	6	7	8	9	10	11	12	Unsch-edul-ed visit	Early With-drawal	End of Thera-py (visit 13)	14
Assessments	Week 0	Week 1	Wk 2	Wk 4	Wk 6	Wk 8	Wk 10	Wk 12	Wk 14	Wk 16	Wk 18	Wk 20	Wk 22			Wk 24	Wk 28
Visit windows (day)	-7	0 ± 1d	14 ± 3d	28 ± 3d	42 ± 3d	56 ± 3d	70 ± 3d	84 ± 3d	98 ± 3d	112 ± 3d	126 ± 3d	140 ± 3d	154 ± 3d			168 ± 3d	196 ± 3d
Written Informed Consent	x																
Subject Demography	x																
Medical History	x																

Inclusion/ Exclusion Criteria	x	x														
Capillaroscopy		x												x	x	
VAS		x		x		x		x		x		x		x	x	x
RCS		x		x		x		x		x		x		x	x	x
mRSS	x	x		x		x		x		x		x		x	x	x
SHAQ		x		x		x		x		x				x	x	x
Digital ulcer count		x		x		x		x		x		x		x	x	x
SF-36		x		x		x		x		x		x		x	x	
Cochin hand function		x		x		x		x		x		x		x	x	x
Concomitant Medication	x	x		x		x		x		x		x		x	x	
Physical Examination	x	x		x		x		x		x		x		x	x	x
Vital Signs [1]	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
PFT[5]	x														x	x
12-lead ECG		x													x	x
AE/SAE		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x

Haematology[6]		x				x								x	x	x	x
Chemistry[7]		x				x								x	x	x	x
Urinalysis[8]		x														x	
Pregnancy Test [2]	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Factor XIII activity [3]	x	x	x	x	x	x	x	x	x	x	x	x	x				
TSP-1 level		x				x										x	
Randomisation		x															
IMP administration .[4]		x	x	x	x	x	x	x	x	x	x	x	x				

[1.] Vital signs will include heart rate, blood pressure, pulse, body weight and height. Height will be collected only at baseline

[2.] Urine pregnancy tests for women of childbearing potential at all visits, except baseline when a serum pregnancy test will be performed

[3.] Patients who have not participated in the PK phase will have baseline factor XIII level drawn during the screening period, to guide first dose of treatment phase. They will then have sampling every 14 days 1 hour pre-dose and 1 hour post dose.

[4.] IMP dose adjustments as per established dosing algorithm. Patients to be observed for a minimum of 1-hour post-dose

[5.] Routine clinical PFT of up to 12 months prior to study visit to be used

[6.] Serum ALT, AST, alkaline phosphatase, GGT, total bilirubin, creatinine, eGFR, amylase, blood urea nitrogen (BUN), sodium, potassium, chloride, bicarbonate, calcium, total cholesterol, uric acid, glucose, total protein and albumin.

[7.] Haemoglobin, haematocrit, red cell count, red cell indices, white blood cell count (total and differential) and platelet count

[8.] Including a microscopic examination of the urine sediment

[9.] If never performed previously

Table 2.11 Treatment phase prescription

The Royal Free London NHS Foundation Trust					RHEUMATOLOGY Unit
Patient Name:		Trial Title:	SCLERO XIII ***DOUBLE-BLIND TREATMENT PHASE PRESCRIPTION***	Patient Demographics Height (m)	**FOR CLINICAL TRIAL USE ONLY**
Date of Birth:					
Hospital No.:		R&D No.:	9337	Weight (kg) (40 – 100kg)	
Gender:		EudraCT No.:	2014-001101-10		
Subject No.:		Protocol No.:	13/0417	Date weight measured	
Allergies:		Sponsor:	UNIVERSITY COLLEGE LONDON (UCL)	Page 1 of 1	

Investigator: Professor Chris Denton Sub-Investigator: Dr Anna Gill Research Nurse: Rachel Ochiel **Version: 2 Date: 16.10.17**

****DOUBLE-BLIND STUDY – PHARMACY DO NOT INFORM RESEARCH TEAM OR PATIENT OF TREATMENT ALLOCATION****
TREATMENT TO BE COLLECTED BY UNBLINDED RESEARCH NURSE ONLY

Dose Information

Factor XIII level	FIBROGAMMIN® or PLACEBO	Unblinded nurse (initial)	Pharmacist check (initial)
First dose at Randomisation (Visit 1) and **subsequent doses (apart from second dose)**			
Level at screening or pre-dose **≤150%**	Dose = 40 IU/kg		
Level at screening or pre-dose **151-170%**	Dose = 30 IU/kg		
Level at screening or pre-dose **>170% to <200%**	Dose = 20 IU/kg		
Level at screening or pre-dose **≥200 %**	**DO NOT DOSE + DELAY 1 WEEK**		
Second dose (Visit 2):			
Level at screening **≤120%**	Dose = 40 IU/kg		
Level at screening **>120% to < 200%**	Dose = 20 IU/kg		
Level at screening **≥ 200%**	**DO NOT DOSE + DELAY 1 WEEK**		

If the level from 14 days prior to the visit is unavailable:

- patients will receive 30 IU/kg if screening Factor XIII level <100%;
- patients will receive 20 IU/kg if screening Factor XIII level ≥ 100% to <200%
- **IF FACTOR XIII LEVEL IS ≥200%: DO NOT DOSE + DELAY 1 WEEK**

Treatment Duration: 24 weeks (baseline visit (day 0), weeks 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 and 22)

Please attach the following documents:

1) List of concomitant medications including dose & frequency (at Baseline)
2) Blood Results (copy of lab results for FBC, Biochem, **pre-dose Factor XIII levels**)

Disease Classification (please select): ☐ **Diffuse SSc** ☐ **Limited SSc**

Changes in concomitant medication since previous visit: ☐ Yes ☐ No; If Yes, specify here:_____

Week Number & date	Drug	Dose (IU/kg) (Please select)	Dose (IU) (Please complete)	Volume (ml) (Please complete)	Route	Rate of Administration	Admin date and time*	Nurse admin signature*	Pharmacy use: dispense	Pharmacy use: check
Date: _____ Visit No: _____ Week No.: ____	**PLACEBO (0.9% SODIUM CHLORIDE) OR FIBROGAMMIN® (FACTOR XIII CONCENTRATE)** (Trial Supply)	0 / 20 IU/kg ☐ 0 / 30 IU/kg ☐ 0 / 40 IU/kg ☐	0 IUOR _____ IU (dose = dose (IU/kg) x weight (kg))	_____ (ml) (volume (ml) = dose (IU) / 62.5 IU)	IV	Max. rate = 4ml/min: give over at least _____ minutes (Minimum duration (mins) = Volume (ml) / 4)				
		MONITOR PATIENT FOR AT LEAST ONE HOUR FOLLOWING DOSE ADMINISTRATION ***DO NOT ENDORSE THE PRESCRIPTION WITH THE TREATMENT RANDOMISATION***								

Prescribed by: I confirm that I have read and understood the clinical trial protocol for this study and I have been delegated the responsibility for prescribing by the Principal/Chief Investigator.

Prescriber Name _____ Prescriber signature _____ Ext/Blp _____ Date ____/____/____

Screened by: **Ensure documents requested attached** ☐

Pharmacist name _____ Pharmacist signature _____ Bleep /ext _____ Date ____/____/____

2.9 Randomisation

There was no randomisation for the PK phase as all patients received factor XIII.

For the treatment phase, allocation to active treatment vs. placebo was via a pre-prepared randomisation schedule generated by the sponsor University College London (UCL). This schedule was held by the trial pharmacist who had no contact with the patients. An emergency copy was held in a sealed envelope in the trial office for the purposes of emergency unblinding. This was not opened. When patients completed the screening process and were deemed eligible, they were allocated a trial number. Each trial number corresponded with the numbered allocation list held by pharmacy. There were 18 trial numbers: 9 for limited SSc patients and 9 for diffuse. The numbers were distributed in series in order of randomisation and according to the patient's subgroup.

Once a prescription was provided to pharmacy with the trial number completed, pharmacy staff compared this to the randomisation schedule which specified whether that trial number was allocated to receive active treatment or placebo. The allocated treatment was then dispensed to unblinded study nurses and administered in a blinded fashion (see section 2.8.2 above). The investigators, patients and study nurses did not view the allocation sequence as this was always held in pharmacy.

The randomisation schedule was generated using block randomisation of limited or diffuse SSc patients to active treatment or placebo with an allocation ratio of 2:1. The randomisation was performed using STATA software (version 14).

Patient enrolment was performed by the sub-investigator Dr Anna Leslie with oversight from principal investigator (PI) Professor Denton.

2.10 Study database

The database for the PK phase was created in Microsoft Access. This was chosen because entry forms could be created that matched the case report forms (CRFs). The database was created under supervision of the UCL Joint Research Office by the clinical trials team, including local statistical and data management expertise at the Royal Free Hospital.

The Treatment phase database was created in the Research Electronic Data Capture programme (REDcap) hosted by UCL. REDcap is an international secure web-based software platform designed to collect and manage data in research studies. The dataset for the Treatment phase of the trial was large and not felt to be suitable for Microsoft Access or Excel. The REDcap system allowed the design of data entry pages that matched the CRFs, making this more user-friendly and reducing data entry error.

All data were collected and reported in line with the JRO and UCL standard practice, as required by relevant authorities. Annual reports were submitted to the local ethics committee. There were no protocol violations. The majority of protocol deviations were minor, and no action was taken. One protocol deviation required a review of trial processes, and a corrective and preventative action (CAPA) plan was generated.

2.11 Safety and follow-up

Where patients were felt to require additional investigations and follow up due to clinical need, this was arranged and performed by the sub-investigator and discussed with the principal investigator. Following the trial, the patients returned to the routine clinic and their survival status was monitored until trial completion.

2.12 Adverse event reporting

Patients were monitored by study investigators for the duration of the trial and follow up period. Following each intravenous injection, patients were monitored

in hospital for one hour, and their vital signs were repeated twice during this period.

At each study visit the patients were questioned regarding adverse events that had taken place since the preceding study visit, and ongoing adverse events were followed up. Patients were also provided with telephone contact information and encouraged to telephone with any adverse events that they wished to report during the study period.

Adverse events were defined according to the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) Good Clinical Practice (GCP) guidelines (International_Council_for_Harmonisation_of_Technical_Requirements_for_Pharmaceuticals_for_Human_Use, 2016). All adverse events occurring following informed consent were recorded. Adverse events occurring before informed consent was taken were recorded in the patient's medical history. Changes to events recorded in the patient's medical history during the trial period were recorded as adverse events.

Serious adverse events (SAEs) were defined as per the ICH GCP guideline and were reported to the Sponsor in an expedited fashion, within 24 hours of the investigator being made aware of the SAE. SAEs were defined as those adverse events leading to:

- Death
- A life-threatening adverse event
- Inpatient hospitalisation or prolongation of existing hospitalisation
- Persistent or significant disability or incapacity
- A congenital anomaly or birth defect
- Other adverse events were considered serious at the discretion of the investigators if they led to action which might reasonably have prevented an SAE from occurring, as defined above.

2.13 Statistical methods

The sample size was 18 participants. This was an early phase exploratory study and so no formal sample size calculation was possible. All patients were included in the final analysis. The statistical analysis of the trial was performed once all data were collected, and the database locked. Little missing data was expected given the small sample size and therefore sensitivity analyses used to measure the robustness of the trial results were not necessary.

The continuous variables were described using the mean, standard deviation, median, minimum and maximum. Binary and categorical variables were described using frequencies and proportions. All analyses were descriptive because the study was not powered for statistical testing. Descriptive estimates were presented with 95% confidence intervals.

Patient flow was documented using a consort diagram. Demographic and baseline variable were displayed in tables.

2.13.1 Primary analyses

The primary endpoints were skin fibrosis, measured using the modified Rodnan Skin Score (mRSS); and the severity of Raynaud's phenomenon, measured using the Raynaud's Condition Score. The primary efficacy analysis therefore assessed the change in mRSS and Raynaud's Condition Score from week 0 to week 24 and compared these changes between the factor XIII and placebo groups. No statistical tests were performed. The effect size was assessed by comparing baseline with 24-week values. The following regression model (adjusted for baseline) was used to assess the treatment effect where appropriate:

$$S\_24\ week_i = \beta_0 + \beta_1\, Baseline_i + \beta_2\, Group_i$$

where:

- $S\_24\ week_i$ is the score at 24 weeks for ith patient
- $Baseline_i$ is the baseline score for ith patient

- $Group_i$ is an indicator for the treatment allocated for ith patient (0 = placebo, 1 = FXIII)
- β_0 is the regression intercept
- β_1 is the parameter of interest which quantifies the effect of baseline score
- β_2 is the parameter of interest which quantifies the effect of treatment

The changes from baseline were compared with the recognised minimally clinically important difference (MCID) which is generally taken as 20% change and at least 4 integer units change in mRSS (Khanna et al., 2019, Khanna et al., 2006).

2.13.2 Secondary analyses

For the secondary endpoints hypothesis tests were not appropriate as these analyses were considered as hypothesis generating rather than providing firm conclusions.

The binary outcomes were summarised using proportions at the relevant time points, and the information plotted to show the mean change in proportions over time. The continuous outcomes were summarised at the relevant time points and this information was also plotted to show the trend over time. Profile plots were presented for each outcome to show how each individual patient's outcome changes over time.

2.13.3 Subgroup analyses

The data were assessed for the 2 disease subgroups: limited and diffuse cutaneous systemic sclerosis.

2.14 Dates and timeframe

Recruitment for the PK phase of the trial commenced in January 2016. The first patient was randomised on 1st February 2016. The PK phase was fully recruited by 17th March 2016. The PK phase last patient last visit was 19th May 2016.

Following completion of the PK phase, the data was analysed, and a protocol amendment made based on these findings. Recruitment for the treatment phase commenced in August 2016 and continued until January 2018. The first patient was randomised into the treatment phase on 5th September 2016. The last patient was randomised on 19th February 2018. The last patient last visit was performed on 23rd August 2018. The trial was completed as planned.

2.15 Changes to the studies or planned analyses

The PK data analysis was used to generate the dosing algorithm used in the treatment phase. The protocol underwent an amendment to incorporate this dosing algorithm, and information regarding the blinding procedure was also clarified in the protocol at this time.

2.16 Clinical assessments

2.16.1 Demographics

The demographic data were recorded at the screening and baseline visits. This included, age, ethnicity, gender, height, weight, date of onset of Raynaud's, date of onset of first non-Raynaud's symptom, and history of digital ulceration.

2.16.2 History and examination

Comprehensive clinical history was taken at the screening visit. This included demographic data, review of previous and current symptoms of SSc, past medical history, medication history, allergies, social history, family history and systems review. This was followed by more tailored clinical history taking throughout the duration of the trial, as needed based on symptoms and adverse events.

History at screening was followed by full clinical examination including cardiovascular, respiratory, abdominal and neurological systems, plus ears, nose and throat and extremities. Skin was assessed for digital ulcers and SSc-

related skin thickening. Skin thickening was assessed using the mRSS (see section 2.16.4).

2.16.3 Patient-reported outcomes

The patient-reported outcomes (PROs) used in these trials were: the Raynaud's Condition Score (RCS), the Scleroderma Health Assessment Questionnaire, the 36-item Short Form Questionnaire and the Cochin Hand Function Score (CHFS).

The RCS is a validated outcome measure for Raynaud's phenomenon (Khanna and Merkel, 2007, Merkel et al., 2002). It assesses the degree of difficulty that Raynaud's phenomenon causes the patient on each day that the test is taken. This generates data on the patient's experience of their Raynaud's phenomenon. Patients self-assess the number and duration of their Raynaud's phenomenon attacks, as well as rating the degree of difficulty their Raynaud's phenomenon caused them on that day using a 10-point Likert scale.

The Scleroderma Health Assessment Questionnaire (SHAQ) is a patient questionnaire used to assess quality of life in SSc. It is adapted from the Stanford Health Assessment Questionnaire (HAQ) which is used to assess quality of life in rheumatological conditions. The original HAQ was developed by James Fries in 1980 and was modified by the author in 1982 and 2003 (Bruce and Fries, 2003). The modern HAQ assesses function over 8 domains of daily activities, such as eating and washing. The patient selects a response based on the amount of difficult they experience attempting to complete each task. There are also 4 domains in which the patient selects any devices or other people they need for assistance in their activities of daily living, and one visual analogue score relating to pain symptoms.

The SHAQ uses the same initial domains as the HAQ, but also incorporates 5 further visual analogue scores referring to symptoms specific to SSc. These are: respiratory symptoms, gastrointestinal symptoms, Raynaud's phenomenon and digital ulcers. Use of the SHAQ has been validated across several trials, and its language has been shown to correlate with patient's own descriptions of

the disease, thereby making the SHAQ applicable and personal to the patient (Johnson et al., 2005, Steen and Medsger, 1997).

The Short Form-36 Questionnaire (SF-36) is a quality-of-life assessment tool applicable to a variety of diseases. The SF-36 was developed by Ware *et al* as part of the Medical Outcomes Study (Ware and Sherbourne, 1992). It was later modified to the SF-36v2 which has been used in clinical trials and provides useful data on patient wellbeing, but is not specific to systemic sclerosis (Stewart et al., 1988, Del Rosso et al., 2004).

The Cochin Hand Function Score is an 18 question self-assessment which asks patients to score the level of difficulty they have performing various common tasks on a six-point scale from “impossible to do” to “no difficulty”. It was initially called the Duruöz Hand Index and was developed to evaluate hand function in French patients with rheumatoid arthritis (Duruoz et al., 1996). It has been validated in rheumatoid and osteoarthritis (Poiraudeau et al., 2000, Poiraudeau et al., 2001) as well as in systemic sclerosis (Brower and Poole, 2004, Rannou et al., 2007).

The PROs used in these trials can be reviewed in the Appendix, section 6.1.

2.16.4 Physician assessments

The physician assessments for these studies were the modified Rodnan Skin Score, the physician global visual analogue scale (VAS), and the digital ulcer count.

The mRSS is an internationally recognized and well validated clinical tool for the assessment of skin thickness in SSc. The body is divided into 17 sites which are commonly affected by SSc. Each of these sites is assessed clinically and the skin is given a score of 0-3. A score of 0 represents normal skin, 1 means that skin is mildly thickened, 2 moderately thickened and 3 severely thickened. The 17 sites for assessment are: face, chest, abdomen (each assessed as one area), the upper and lower arms bilaterally, the upper and lower legs bilaterally, and the dorsal surfaces of the hands, fingers and feet bilaterally. The scores

from the 17 areas are added together, giving a total score out of 51. When the test is performed by an experienced observer, the clinical score correlates with dermal weight on biopsy (Perera et al., 2007). The test has significant intra-observer variability, but this can be reduced with training. It is therefore ideal to have the same observer for all mRSS assessments (Clements et al., 1993, Czirjak et al., 2007).

It is useful to have a tool to assess skin thickness because the degree of skin thickening correlates with other organ involvement (Farmer et al., 1960). Baseline skin score correlates with increased mortality, and improvements correlate with increased survival (Clements et al., 1993, Clements et al., 2000, Steen and Medsger, 2001).

The mRSS was developed by Dr Gerald Rodnan in 1979 and has since undergone various modifications (Rodnan et al., 1979, Steen et al., 1982) (Kahaleh et al., 1986, Clements et al., 1990, Clements et al., 1993). In in its current form, the mRSS is the gold standard for the assessment of skin thickness in SSc (Domsic et al., 2011, Clements, 1995). The validity of the mRSS has been widely proven (Tashkin et al., 2006, van Laar et al., 2014).

The physician global VAS is a 10 cm analogue scale on which the physician marks their opinion on the degree of difficulty caused to the patient by their disease, with a score of 100 % representing the greatest difficulty. It is not specific to SSc. The mark on the analogue scale is then measured and translated into a percentage which is the physician VAS score entered into the trial database.

The digital ulcer count is a simple scoring tool allowing the physician to document how many digital ulcers are present on the patients' hands as well as recording any interventions that might have taken place for the ulcers.

Both the global VAS and the digital ulcer count have been recommended for use in clinical trials by the Scleroderma Clinical Trials Consortium (Khanna et al., 2008).

The physician assessments used in these studies can be found in Appendix 1, section 6.2.

2.16.5 Physiological assessments

Physiological assessments performed during these trials were: vital signs (blood pressure, respiratory rate, heart rate and temperature), 12-lead electrocardiogram, pulmonary function testing, and capillaroscopy.

2.17 Laboratory assessments

2.17.1 Routine laboratory assessments

Routine blood analyses were performed at baseline (week 0) during the PK phase and at baseline, week 8, end of therapy (week 24) and follow-up (week 28) during the treatment phase, as well as at any unscheduled visits. Blood samples were taken for full blood count and biochemistry. Blood samples were analysed by the haematology and biochemistry laboratories at the Royal Free Hospital.

Urine samples were taken at baseline and week 24. Samples were tested in the clinical trials department using urine dipstick. Urine samples with a dipstick abnormality were sent to the Royal Free Hospital biochemistry laboratory for further assessment.

Pregnancy tests were performed at all visits for women of childbearing potential. A serum pregnancy test was performed at baseline and a urine pregnancy test at all other visits.

2.17.2 Factor XIII levels

Patients participating in the clinical trials had samples for factor XIII level taken at every visit during the trial. Where a factor XIII/placebo infusion was given, the factor XIII level was drawn pre-infusion and one-hour post-infusion.

For sample collection, two 4 ml Beckton Dickinson Vacutainer tubes with 3.2% buffered sodium citrate were obtained. Samples were transferred at ambient temperature to the haemophilia laboratory at the Royal Free Hospital, where

they were processed within 4 hours of being drawn. Where samples were taken as part of the clinical trial and where a factor XIII or placebo infusion was given, the factor XIII level was drawn immediately pre-infusion and one-hour post-infusion.

The samples were analysed using the Berichrom FXIII chromogenic ammonia release assay (Siemens Healthcare Diagnostics, Marburg, Germany) on the CS-2000i coagulation analyser. This assay converts the factor XIII in the sample to factor XIIIa using thrombin. The fibrin generated by thrombin amplifies the process. A polymerase inhibitor is present to prevent the free fibrin produced in this reaction forming a fibrin clot. However, the cross-linking action of FXIIIa on fibrin continues to occur. FXIIIa cross-links glycine ethyl ester to its peptide substrate, a reaction catalysed by glutamate dehydrogenase and dependent on nicotinamide adenine dinucleotide (NADH). NADH is therefore consumed during the reaction, and this can be measured as decreased absorbance spectrophotometrically at 340 nm. The concentration change in NADH is directly proportional to the factor XIII activity in the sample.

There have been some concerns raised that the consumption of NADH in other reactions may lead to an overestimation of factor XIII activity, but this only affects measurements using this technique in severe factor XIII deficiencies (Karimi et al., 2009).

The Berichrom FXIII chromogenic ammonia release assay was developed in the early 2000s as a quantitative alternative to the clot solubility test, and it has since been shown to be more sensitive than the clot solubility test in detecting mild to moderate factor XIII deficiency (Karpati et al., 2000, Fickenscher et al., 1991).

In the older clot solubility test the patient's plasma is incubated with thrombin to trigger the final stages of the coagulation cascade (figure 1) and generate clot formation. The clot is then transferred to an acidic or urea solution for 24 hours. If the clot has dissolved at 24 hours this is indicative of factor XIII deficiency (Jennings et al., 2003).

To gain further insight into the potential role of factor XIII in systemic sclerosis, further factor XIII levels were taken outside of the clinical trials. A series of samples were analysed from systemic sclerosis patients not participating in the clinical trial, and from healthy volunteers (table 2.10). These samples were taken as part of the following study:

ScleroderMA cohoRT (UK SMART) data and tissue bank – version 7 17/02/2020.
REC reference: 20/LO/0404 (previously 15/LO/0275).
IRAS project ID: 279682.
Approved (rolling renewal) by London - Fulham Research Ethics Committee, Barlow House, 3rd Floor, 4 Minshull Street, Manchester, M1 3DZ, 24 March 2020).

All participants provided written informed consent to donate blood samples for these analyses.

A total of 6 SSc patients provided samples at week 0, 8 and 24. Together with the placebo group from the clinical trial, these patients formed a comparator group of 12 treatment-naïve systemic sclerosis patients.

12 healthy people provided samples at the same timepoints, therefore creating a third group of longitudinal factor XIII levels across a 24-week period.

Overall, therefore I gathered factor XIII levels over 24 weeks from 12 SSc patients receiving factor XIII, 12 SSc patients not receiving factor XIII and 12 healthy controls.

A further 60 patients provided a single sample, generating a larger database of non-longitudinal data on factor XIII levels in systemic sclerosis.

Table 2.12 Additional factor XIII levels analysed outside of the clinical trials

Group	n	Sample Type	Test performed		
			Week 0	Week 8	Week 24
Systemic sclerosis patients	6	Blood (citrate tube)	Factor XIII	Factor XIII	Factor XIII
	60	Blood (citrate tube)	Factor XIII		
Healthy controls	12	Blood (citrate tube)	Factor XIII	Factor XIII	Factor XIII

2.17.3 Candidate biomarker studies

Linking the candidate biomarker studies to the clinical trials ensured the availability of high-quality longitudinal samples and added scientific value to the trial. Following review of the likely mechanism of action of factor XIII in systemic sclerosis, various possible candidate biomarkers were selected for analysis. Analyses were performed using two types of immunoassays: enzyme-linked immunosorbent assays and multiplex assays.

All patients participating in the clinical trial consented to donate additional blood samples at 3 time points for research. Systemic sclerosis patients not involved in the clinical trial and healthy volunteers were recruited to provided research blood samples at the same 3 timepoints (table 2.13). Systemic sclerosis patients were recruited from outpatient clinical and PITU at the Royal Free Hospital. Healthy volunteers were friends and family members of patients, recruited during outpatient visits, or were members of staff. All participants gave their free and informed written consent.

This process produced longitudinal samples in parallel to the clinical trial data and overall generated 3 equal-sized groups for comparison: 12 SSc patients treated with factor XIII, 12 SSc patients not receiving factor XIII, and 12 healthy controls.

Table 2.13 Summary of samples obtained for candidate biomarker studies

Group		**n**	**Sample Type**	**Test performed**		
				Week 0	**Week 8**	**Week 24**
Systemic sclerosis patients participating in clinical trial	FXIII	12	Serum	ELISAs Multiplex Assays	ELISAs Multiplex Assays	ELISAs Multiplex Assays
	Placebo	6	Serum	ELISAs Multiplex Assays	ELISAs Multiplex Assays	ELISAs Multiplex Assays
Systemic sclerosis patients not participating in clinical trial		6	Serum	ELISAs Multiplex Assays	ELISAs Multiplex Assays	ELISAs Multiplex Assays
Healthy controls		12	Serum	ELISAs Multiplex Assays	ELISAs Multiplex Assays	ELISAs Multiplex Assays

Serum was chosen as it lacks fibrinogen and clotting factors, levels of which could have been affected by the clinical trial. Using serum instead of plasma

removed the risk of endogenous coagulation factors in the plasma continuing to act on other proteins within the sample after the blood had been centrifuged and separated.

Serum samples were obtained using the following validated method: two 10 ml Becton Dickinson Vacutainer spray-coated silica clot activator tubes were obtained and incubated at room temperature for 30 minutes to allow the serum sample to clot. The serum samples were then centrifuged at a rate of 3000 rotations per minute for ten minutes at 4°C. Following centrifugation, the serum samples were immediately aliquoted into 0.6 ml storage tubes and frozen at - 80°C (Ammerlaan et al., 2014). Frozen serum samples were thawed on ice and diluted 1:100.

Serum samples were obtained as part of the UK SMART data and tissue bank (see section 2.17.2 above).

Candidate biomarkers for analysis were selected because they were either known to be involved in the pathogenesis of SSc or were considered relevant to the proposed mechanism of action of FXIII on SSc. Some candidate biomarkers fell into both categories. The use of a multiplex assay for the analysis of some candidate biomarkers meant that proteins not considered relevant were also analysed. The multiplex assays are most cost-effective when purchased as a panel assay, and during the laboratory research I was conscious to conserve funds kindly provided by charity funding. The panel referenced in section 2.17.3.2 was chosen because it included the greatest number of relevant proteins compared to other panels available.

Table 2.14 Choice of candidate biomarkers

Candidate biomarkers known to be relevant to the pathogenesis of SSc	Candidate biomarkers relevant to the proposed mechanism of action of FXIII on SSc
COMP-1	TSP-1
Fibronectin	VEGF-A
TSP-1	VEGF-C
sCD40L	VEGF-D
TNFα	
IL-6	

2.17.3.1 Enzyme linked immunosorbent assays

A standard dilution series was prepared, and samples and controls were diluted according to the protocol. Standards, samples, and controls were added to a 96-well microplate coated with antibody against the biomarker of choice (capture antibody) and incubated at ambient temperature with agitation for 2 hours. The microplate was then washed and an antibody specific for the chosen biomarkers conjugated to horseradish peroxidase was added (detection antibody). The microplate was incubated at ambient temperature with agitation for 2 hours. The microplate was washed again and a solution of hydrogen peroxide and tetramethylbenzidine was added. The microplate was protected from light and incubated at ambient temperature without agitation for 30 minutes. The reaction was terminated with the addition of N-sulfuric acid. The optical density (absorbance) of each well was measured within 30 minutes using a microplate reader at 450 nm and 560 nm.

A standard curve was generated by plotting the optical density of standard wells against their known biomarker concentrations. This was used to determine the optical density of the unknown samples. Results from samples that had been diluted were multiplied by the dilution factor.

The biomarkers analysed and kits used for these assays were:

- Cluster of Differentiation 40 Ligand (CD40L) (also known as Soluble Cluster of Differentiation 40 Ligand (sCD40L): Human CD40 Ligand/TNFSF5 Immunoassay Quantikine® ELISA, RND Systems (Catalogue Number DCDL40, SCDL40, PDCDL40)
- Cartilage Oligomeric Matrix Protein (COMP): Human COMP Immunoassay Quantikine® ELISA, RND Systems (Catalogue number DCMP0)
- Fibronectin: Human Fibronectin Immunoassay Quantikine® ELISA, RND Systems (Catalogue Number DFBN10)
- Thrombospondin-1: Human TSP-1 Immunoassay Quantikine® ELISA, RND Systems (Catalogue number DTSP10)
- Tumour Necrosis Factor Alpha (TNF-α): Human TNF-α Immunoassay Quantikine® ELISA, RND Systems (Catalogue number DTA00D, STA00D, PDTA00D)
- Vascular Endothelial Growth Factor-C (VEGF-C): Human VEGF-C Immunoassay Quantikine® ELISA (Catalogue Number DVEC00)
- Vascular Endothelial Growth Factor-D (VEGF-D): Human VEGF-D Immunoassay Quantikine® ELISA (Catalogue Number DVED00)

2.17.3.2 Multiplex assays

A standard dilution series was prepared according to the protocol. The magnetic beads plus samples, standards and controls were added to the 96-well assay plate and incubated at ambient temperature in darkness for 1 hour with agitation. The plate was washed using the Bio-Plex Handheld Magnetic Washer and the detection antibodies were added. The plate was incubated at ambient temperature in darkness for 30 minutes with agitation. The plate was washed

using the Bio-Plex Handheld Magnetic Washer, streptavidin PE was added, and the plate was incubated at ambient temperature in darkness for 10 minutes with agitation.

The plate was washed using the Bio-Plex Handheld Magnetic Washer and the magnetic beads re-suspended in assay buffer. The microplate was analysed using the Bio-Plex using the Bio-Plex MAGIPIX system.

The assay used was the Bio-Plex Pro Human Cancer Biomarker Panel 2 18-Plex #171AC600M which analysed the following proteins:

- Angiopoietin-2
- sCD40L
- Epidermal growth factor (EGF)
- Endoglin
- Soluble FAS ligand (sFASL)
- Heparin-like EGF (HB-EGF)
- Insulin-like growth factor binding protein (IGFBP-1)
- Interleukin-6 (IL-6)
- Interleukin-8 (IL-8)
- Interleukin-18 (IL-18)
- Plasminogen activator inhibitor 1 (PAI-1)
- Placental growth factor (PLGF)
- TGF-α
- TNF-α
- Urokinase-type plasminogen activator (uPA)
- Vascular endothelial growth factor A (VEGF-A)
- VEGF-C
- VEGF-D

2.17.3.3 Statistical analysis

Data were analysed using SPSS statistical software and using non-parametric techniques. Independent 2-sample comparisons were assessed using the Mann Whitney U test. Dependent 2-sample comparisons were assessed using the Wilcoxon Signed Rank test. P-values less than 0.05 were considered significant. Simple descriptive statistics were generated in Microsoft Excel.

Where candidate biomarkers were assessed using both ELISA and multiplex assays, the data were converted into the same units and then analysed as a single data set, as has been performed previously (Quillinan et al., 2017).

2.17.4 Explanted dermal fibroblast studies

The explanted dermal fibroblast work described below was performed at the Centre for Rheumatology and Connective Tissues Diseases, Royal Free Campus, University College London. This work was performed in conjunction with my colleague Dr Xu Shi-Wen, who has a great deal of expertise in these techniques, and to whom I am grateful for his skill and teaching.

Skin samples were obtained as part of the following study:

Elucidating the pathogenesis of systemic sclerosis by studying skin, tissue and blood samples from scleroderma patients and healthy volunteers.
London Hampstead National Research Ethics Service Committee, Multi Research Ethics Committee reference 6398.
Lead investigator Prof. C P Denton.
Last substantial amendment approved November 2012.

All participants provided written informed consent to donate skin samples for these analyses.

2.17.4.1 Fibroblast cultures

Skin samples were taken from 6 diffuse cutaneous systemic sclerosis patients and 6 health controls by punch biopsy under local anaesthetic. Dermal fibroblasts were cultured using previously validated methods in Dulbecco's Modified Eagle Medium (DMEM) containing 10% foetal bovine serum (Invitrogen, Paisley, UK) and cell lines were used between passages 2 and 5 (Abraham et al., 2000, Chen et al., 2005, Khan et al., 2012).

2.17.4.2 Western blot analysis

Fibroblasts were starved of serum overnight. They were then incubated with or without factor XIII at a concentration of 1.0 IU/ml for 48 hours. Fibroblasts were lysed in 2% sodium dodecyl sulphate (SDS) and the proteins separated by molecular mass using SDS polyacrylamide gel electrophoresis (SDS-PAGE) on tris-glycine gels (Novex). Proteins were transferred onto nitrocellulose membrane by electroblotting (Hybond-C Extra, GE Healthcare Amersham, UK). Non-specific blinding was blocked by incubating membranes for 2 hours at room temperature in 5% (w/v) non-fat dry milk in 0.1% tris buffered saline (TBS).

Membranes were then incubated overnight at 4 ºC with primary antibodies: anti-glyceraldehye-3-phosphate dehydrogenase (GAPDH) (1:5000, Sigma) (a housekeeping protein used as a reference antibody), anti-α-SMA (1:1000, Sigma) and anti-type I collagen (1:1000, Meridian, Abingdon, UK). Membranes were then washed with phosphate-buffered saline with 0.1% Tween-20 (PBS-T) (Sigma-Aldrich) before being incubated with secondary antibodies (dilution 1:1000, Zymed) in 5% (w/v) non-fat dry milk in 0.1% TBS at room temperature for 1 hour (Shi-Wen et al., 2004, Chen et al., 2005, Denton et al., 2018). Protein antigens were detected by enhanced chemiluminescence (Amersham, Buckinghamshire, UK) and quantified using a camera imager (Biospectrum AC imaging; UVP, Cambridge, UK). Arbitrary integrated density values were recorded.

2.17.4.3 Collagen gel contraction assays

Collagen gel contraction assays were performed in 24-well tissue culture plates which were pre-coated with 0.22 µm sterile filtered 2.5% bovine serum albumin (BSA). Plates were washed with Dulbecco's Phosphate Buffered Saline (DPBS) before use. Trypsinised human fibroblasts were suspended in microvascular endothelial cell basal medium (MCDB) (Sigma-Aldrich), mixed with collagen solution [one part 0.2 M N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid, pH 8.0; four parts collagen (Nutragen, 3 mg/ml) and five parts MCDB × 2] to yield a final concentration of 80 000 cells/ml and 1.2 mg/ml collagen. The collagen/fibroblast suspension (volume 1 ml) was added to each well and allowed to polymerise by incubating at body temperature for one hour in a humidified incubator in a 5% CO_2 atmosphere. Gels were detached from the plates by adding 1 ml of MCDB medium with or without factor XIII at a concentration of 1.0 IU/ml. Contraction of the gel was quantified by loss of gel weight over a 48-hour period. Data were analysed using Student's T-test (Knowles et al., 2012, Grinnell et al., 1999, Shi-Wen et al., 2004, Shi-wen et al., 2010).

2.17.4.4 Fibroblast migration assays

Cultured fibroblasts were plated on 12-well plates in DMEM containing 10% foetal bovine serum as before. The cells naturally proliferate and adhere to the plates. Once the cell layer was approximately 70% confluent, the DMEM was removed, and the cells were rinsed with MCDB medium + 0.1% BSA and cultured for an additional 24 hours in serum-free medium + 0.1% BSA. The monolayer was artificially injured by scratching across the plate with a sterile polypropylene 200-1000 µl pipette tip (approximately 1.3 mm width) (Thermo Fisher). The wells were washed with DPBS (Thermo-Fisher) to remove detached cells. The cell monolayer was then cultured in MCDB with and without factor XIII at a concentration of 1.0 IU/ml, in the presence of 10 µg/ml mitomycin C (Merck-Millipore, UK) to prevent cell proliferation. After 48 hours, wells were imaged using an Olympus CDK2 microscope. Five representative images of the scratched areas under each condition were photographed. Scratch diameter

was measured and compared to baseline, allowing a semi-quantitative measurement of cell migration, expressed as percentage change compared to the 0-hour scratch diameter (Knowles et al., 2012, Ranzato et al., 2009, Liang et al., 2007, Cory, 2011, Xu et al., 2009).

2.17.5 Coagulation studies

To further investigate the links between systemic sclerosis and coagulation, the 60 patients who consented to a single time point blood draw for factor XIII analysis in the outpatient clinic also had a Beckton Dickinson 4 ml Vacutainer tube with 3.2% buffered sodium citrate taken for analysis of D-dimer (ng/ml), fibrinogen (g/L) levels; and activated partial thromboplastin time (APTT) (seconds). These analyses were performed in the coagulation laboratory at the Royal Free Hospital.

Clinical data were gathered from the patient record, including age, sex, SSc subtype (limited or diffuse) and overlapping conditions, autoantibody, duration of SSc and complications of SSc. Complications of scleroderma recorded were pulmonary fibrosis, pulmonary arterial hypertension, cardiac scleroderma, scleroderma renal crisis, atypical scleroderma gastro-intestinal involvement (primary biliary cirrhosis, gastric antral vascular ectasia), active digital ulceration at the time of test, and history of previous digital ulceration.

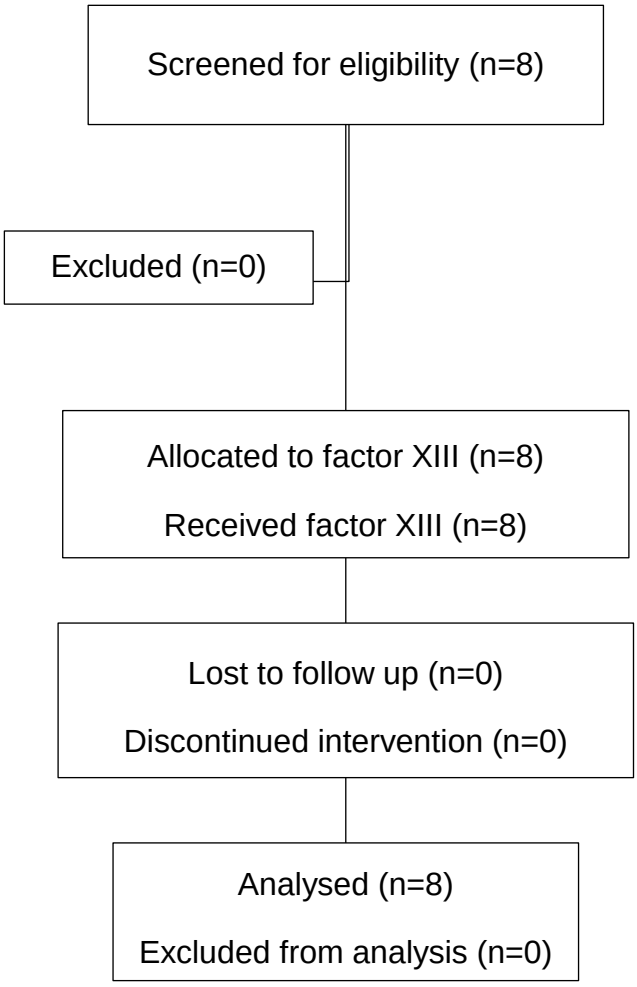
Associations of demographic and clinical characteristics with FXIII were assessed using linear regression, while associations with D-dimer were analysed using Fisher’s exact test and logistic regression.

3 Results

3.1 Sclero XIII clinical trial – pharmacokinetic phase

Eight patients were screened and randomised into the pharmacokinetic phase. No patients were excluded after being assessed for eligibility. All patients received the full dose of study medication. All patients completed the study, and none were lost to follow up.

Figure 2 Pharmacokinetic phase schematic



3.1.1 Demographics and disease characteristics

Table 3.1 Pharmacokinetic phase demographics and disease characteristics

Baseline criteria (n = 8)	
Mean age, years (SD)	59 (8)
Gender, No. (% female)	7 (87.5)
Race, No. (% Caucasian)	6 (75)
Disease characteristics	
Digital ulcers at baseline, No. (%)	2 (25)
Pulmonary arterial hypertension, No. (%)	0 (0)
Secondary Sjogren's syndrome, No. (%)	1 (12.5)
History of renal crisis, No. (%)	1 (12.5)
GAVE, No. (%)	1 (12.5)
Overlap condition, No. (%) • Polymyositis • SLE	2 (25) 1 1

Early phase of SSc, defined as disease duration < 2 years from the first non-Raynaud's symptom, No. (%)	0 (0)

3.1.2 Safety assessments

Thirteen adverse events were reported during the PK phase. There were no serious adverse events.

Table 3.2 Adverse events – pharmacokinetic phase

	Factor XIII
Adverse events	13
Serious adverse events	0

3.1.3 Factor XIII levels

Figure 3 displays the sharp increase in factor XIII seen during the first hour after administration of factor XIII during the PK phase of the study. The adjusted mean change during the first hour was + 44.31 IU/dL which, as expected, demonstrated a significant change from the baseline level (p=0.01).

Figure 3 Factor XIII levels on Day 0

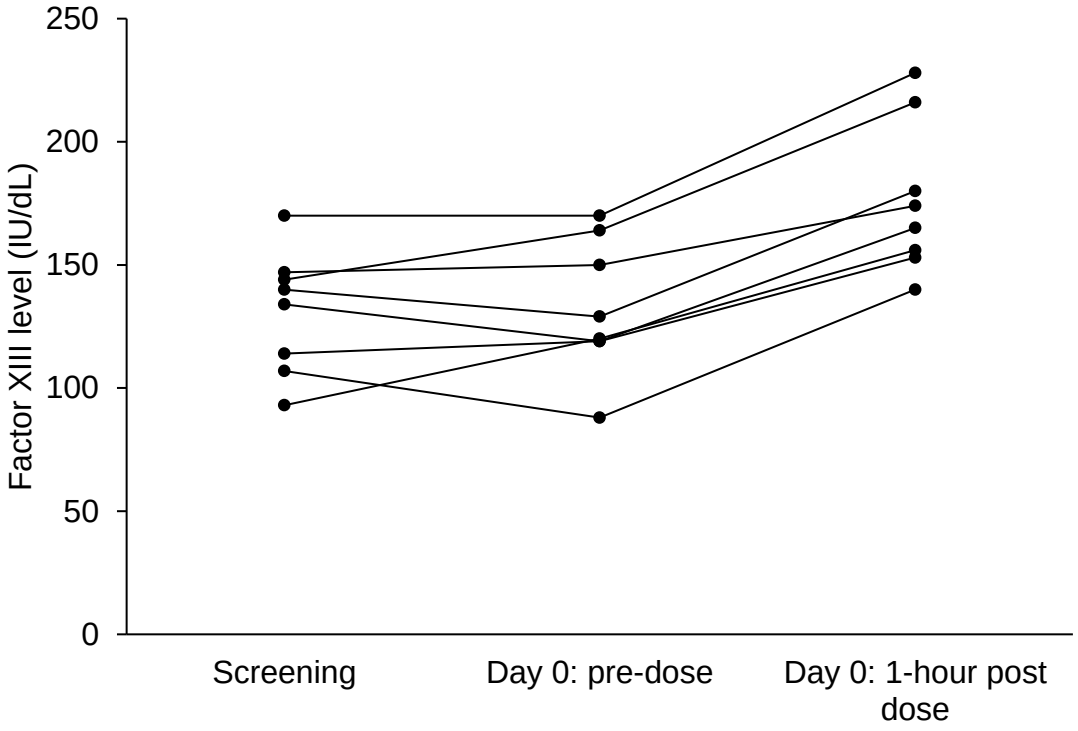


Table 3.3 Descriptive statistics for Day 0 PK phase factor XIII levels (IU)

	Screening	**Day 0 pre-dose baseline**	**Day 0 post-dose**	**p-value**
n	8	8	8	
Mean	131.13	132.38	176.50	
Min	93.00	88.00	140.00	
Max	170.00	170.00	228.00	
SD	23.30	25.54	28.87	
Adjusted mean change from baseline (95% CI)		44.13 (36.61 to 51.64)		**0.01**

Over the next 28 days (+/- 8 days) the factor XIII levels gradually declined (figure 4).

Figure 4 PK phase factor XIII levels in the 28 days post-dose

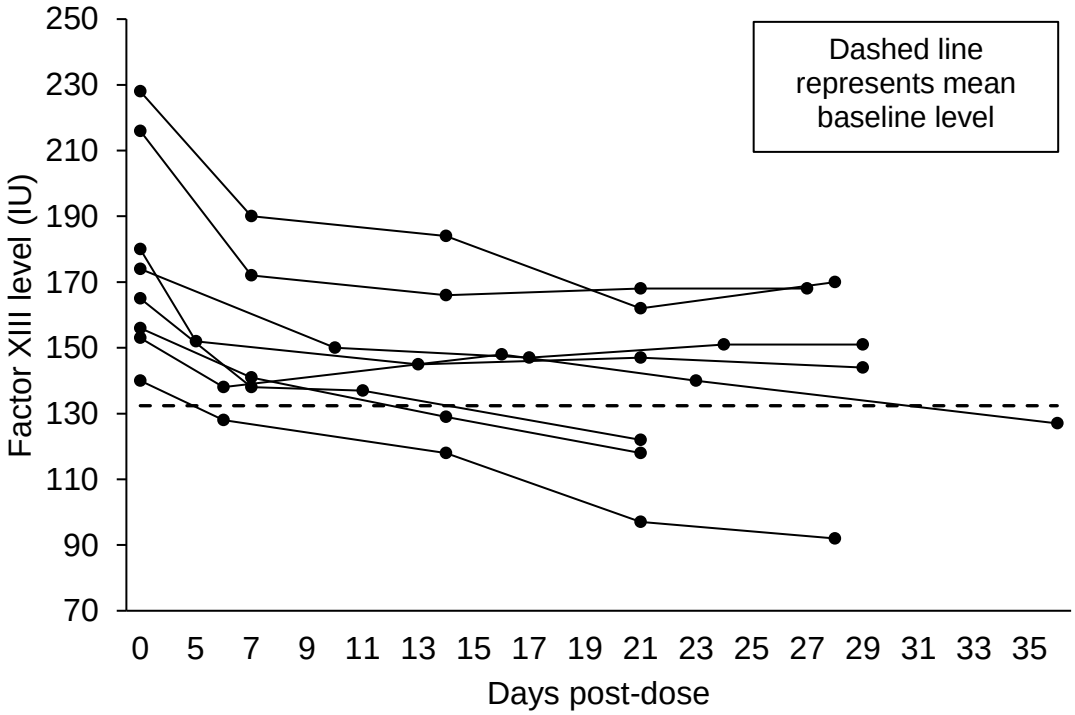
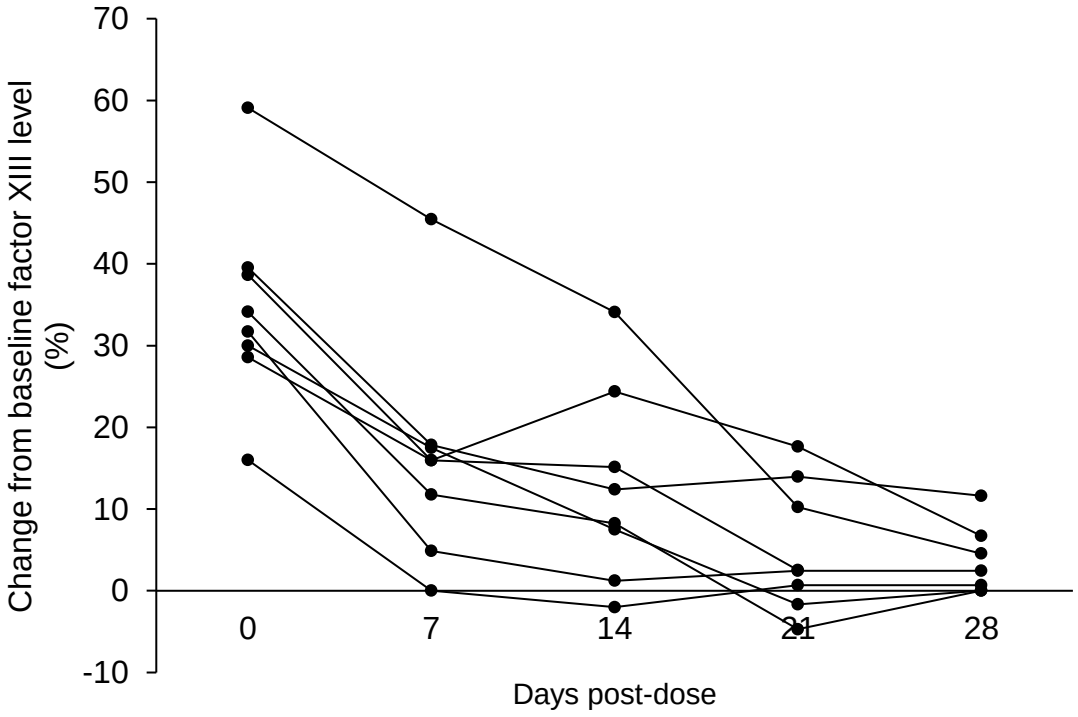


Table 3.4 Descriptive statistics for PK phase post-dose factor XIII levels (IU)

Day	**0 pre-dose baseline**	**7**	**14**	**21**	**28**
n	8	8	8	8	6
Mean	132.38	151.13	146.75	138.13	142
Min	88	128	118	97	92
Max	170	190	184	168	170

SD	25.54	19.14	19.36	22.55	26.68
Adjusted mean change from baseline (95% CI)		+ 20.4 (12.87 to 27.93)	+ 14.38 (6.79 to 21.96)	+ 5.75 (-0.62 to 6.37)	+ 3.4 (-0.08 to 6.88)

Figure 5 Percentage change from post-dose factor XIII level in the 28-days post-dose



The mean time taken for the factor XIII level to return to within 10 IU/dL of the highest endogenous level (screening or baseline) was 16.6 days (SD 8.7).

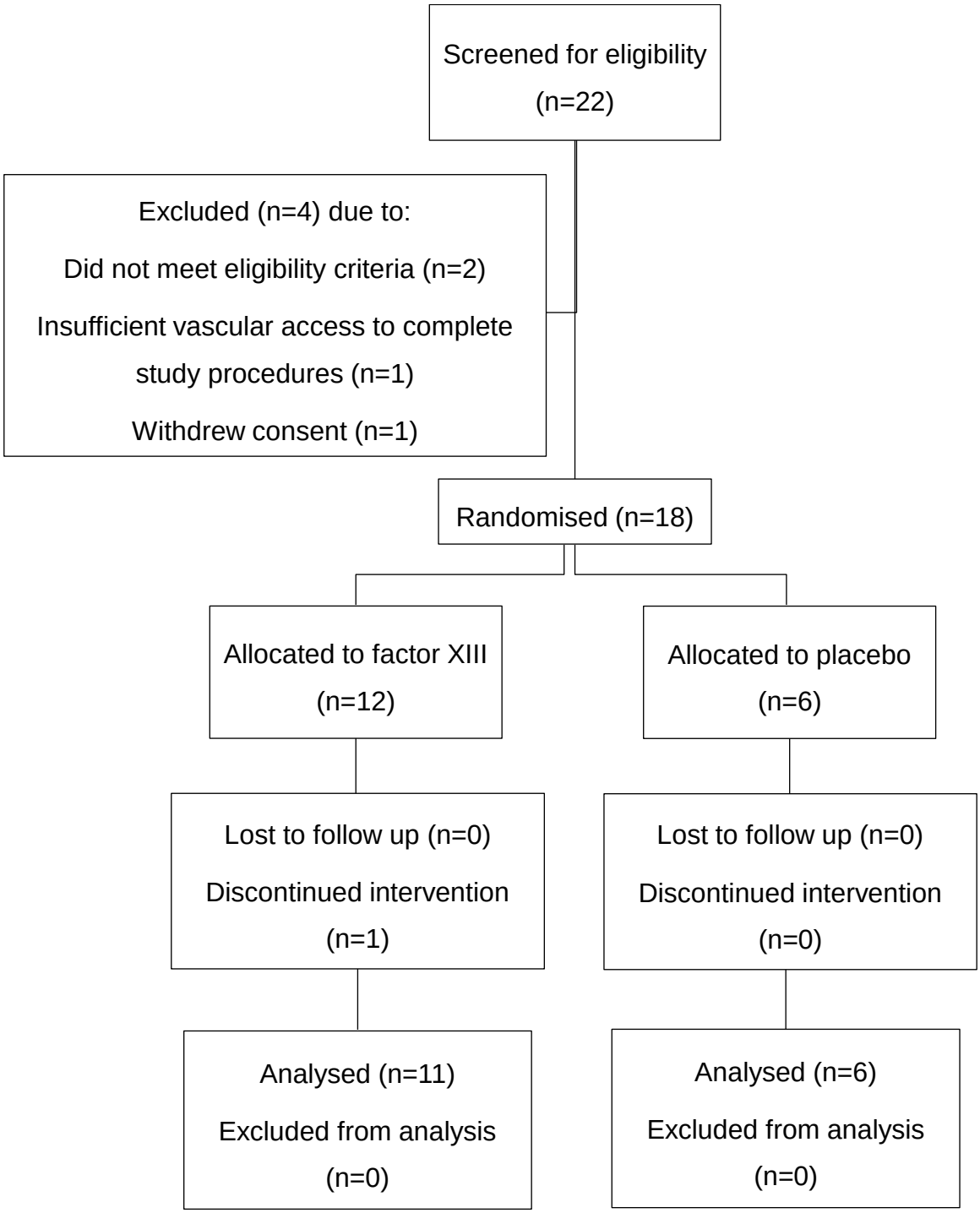
These PK data were analysed by CSL Behring who produce purified factor XIII and have experience of calculating appropriate doses in non-SSc patient

populations. The dosing algorithm for the treatment phase was then established and incorporated into the protocol (see section 2.8.1).

3.2 Sclero XIII clinical trial – treatment phase

Twenty-two patients were screened for the treatment phase, eighteen were randomised and there were four screen failures. All eighteen patients randomised received at least one dose of study medication or placebo. Seventeen patients completed the study. One patient allocated to active treatment withdrew consent to receive the study intervention but participated in the week 24 visit to generate primary endpoint data. No patients were lost to follow up.

Figure 6 Treatment phase schematic



3.2.1 Demographics and disease characteristics

Table 3.5 Treatment phase demographics and disease characteristics

The results are presented as mean (SD) or absolute number (%)

Baseline criteria	**Factor XIII (n=12)**	**Placebo (n=6)**
Age, years	59.8 (11)	57.5 (11.6)
Gender, No. (%) female	12 (100)	6 (100)
Race, No. (%) Caucasian	9 (75)	6 (100)
Disease characteristics		
Digital ulcers at baseline, No. (%)	3 (25)	0 (0)
Pulmonary arterial hypertension, No. (%)	0 (0)	0 (0)
SSc-related lung disease, No. (%)	3 (25)	0 (0)
Pulmonary hypertension secondary to SSc-related lung disease, No. (%)	1 (8.3)	0 (0)
Secondary Sjogren's syndrome, No. (%)	1 (8.3)	0 (0)
GAVE, No. (%)	0 (0)	1 (8.3)
History of renal crisis, No. (%)	2 (16.7)	1 (8.3)
Overlap condition, No. (%)	2 (16.7)	0 (0)

• Dermatomyositis • Rheumatoid arthritis	1 (8.3) 1 (8.3)	
Early phase of SSc, defined as disease duration < 2 years from the first non-Raynaud's symptom, No. (%)	0 (0)	0 (0)
Vital signs		
Heart rate, bpm	69.6 (10)	66.7 (5.5)
Systolic blood pressure, mmHg	123.5 (14.8)	129.6 (9)
Diastolic blood pressure, mmHg	70.5 (7.8)	78.3 (13.9)
Temperature, °C	36.6 (0.1)	36.6 (0.2)
Weight, kg	68.3 (12.9)	58.7 (10.2)
Height, cm	167 (7.3)	164.3 (4.9)

3.2.2 Safety assessments

During the treatment phase there were 100 reported adverse events (75 in the FXIII arm and 25 in the placebo arm). No patients withdrew from the trial due to adverse events. Two events were reported as serious adverse events. These were a traumatic metatarsal fracture which occurred before the randomisation visit, therefore before any study drug had been administered. This has therefore been omitted from analysis by the Sponsor. The second was considered serious based on investigator judgement, although it did not meet the protocol-specified definition of an SAE. This was because the patient telephoned an ambulance for her symptoms, though she was not admitted to hospital. The IMP/placebo was temporarily interrupted while she was investigated for her

symptoms and significantly deranged liver function tests, and then restarted with patient consent. She made a good recovery. The patient was later found to be receiving placebo.

The most common adverse events which were likely to be related to the study medication were headaches and diarrhoea. These events were self-resolving, and patients reported that they were tolerable.

Table 3.6 Adverse events – treatment phase

	Factor XIII	**Placebo**
Total adverse events	75	25
Adverse events that occurred more than once		
	Factor XIII	**Placebo**
Diarrhoea	9	2
Digital ulcer	12	0
Dizziness	3	0
Headache	6	0
Leg pain	0	4
Lower respiratory tract infection	4	0
Tooth infection	2	0

Upper respiratory tract infection	5	3
Serious adverse events	0	1

3.2.3 Secondary endpoints

Only descriptive results are reported for the secondary outcomes. Table 3.7 presents the means at baseline and 24 weeks, and mean change from baseline to 24 weeks, in pulmonary function, Cochin hand function, SF-36 and digital ulcer counts by treatment group.

Figure 7 shows the trend for Cochin hand function score over the 24 weeks. It suggests that the Cochin hand function is relatively stable for the FXIII group compared with the placebo group where we see a large decrease in both the first 4 weeks and week 8 to 16 in contrast to a rapid increase from week 4 to 8 and week 16 to the end of the trial. Figure 8 is profile plot for trend in Cochin hand function by treatment group. One patient in placebo group reported a high score (above 40) throughout the study period.

Table 3.7 Secondary endpoint results

	Sample size at baseline		Baseline Mean (SD)		Sample size at 24 weeks		24 weeks Mean (SD)		Sample size for change of mean calculation		Change at 24 weeks Mean (95% CI)	
	FXIII	Placebo	FXIII	Placebo	FXIII	Placebo	FXIII	Placebo	FXIII	Placebo	FXIII	Placebo
Pulmonary function												
Percentage of FVC predicted	12	6	99.98 (23.72)	116.4 (12.47)	11	6	100 (26.26)	113.95 (16.67)	11	6	-1.25 (-7.34, 4.83)	-2.45 (-10.52, 5.62)
Percentage of DLCO predicted	11	6	63.72 (16.02)	65.47 (21.36)	11	6	64 (21.21)	59.5 (18.66)	10	6	-4.05 (-9.17, 1.07)	-1.47 (-9.05, 6.11)

Cochin hand function	12	6	19.33 (13.34)	16.83 (22.1)	11	5	16.73 (11.87)	23.2 (23.53)	11	5	-3.64 (-6.03, -1.25)	1.26 (-0.51, 6.51)
Digital ulcer count	11	6	0.55 (1.04)	0	11	6	0.55 (1.21)	0	10	6	-0.1 (-0.51, 0.31)	0
Physician global VAS			35.2 (17.1)	34.1 (11)			25.8 (14.9)	21.2 (14.3)				

Figure 7 Mean values for Cochin hand function score over 24 weeks

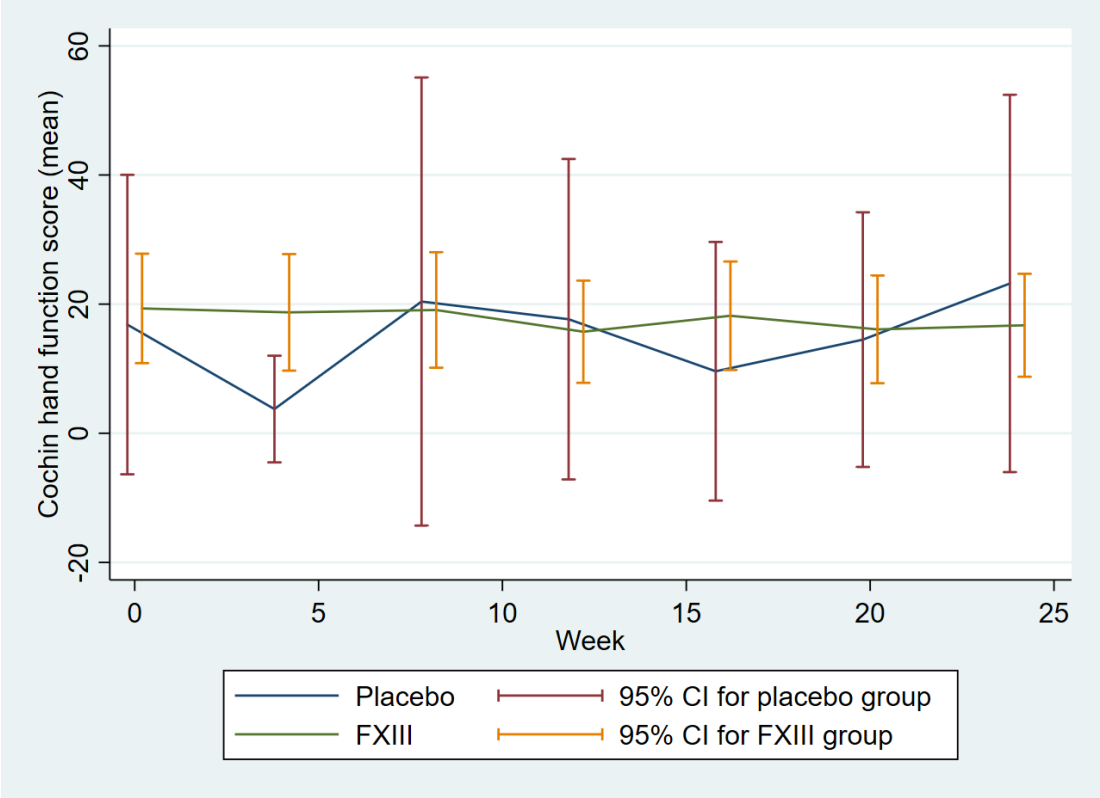


Figure 8 Cochin hand function for individual patients over 24 weeks

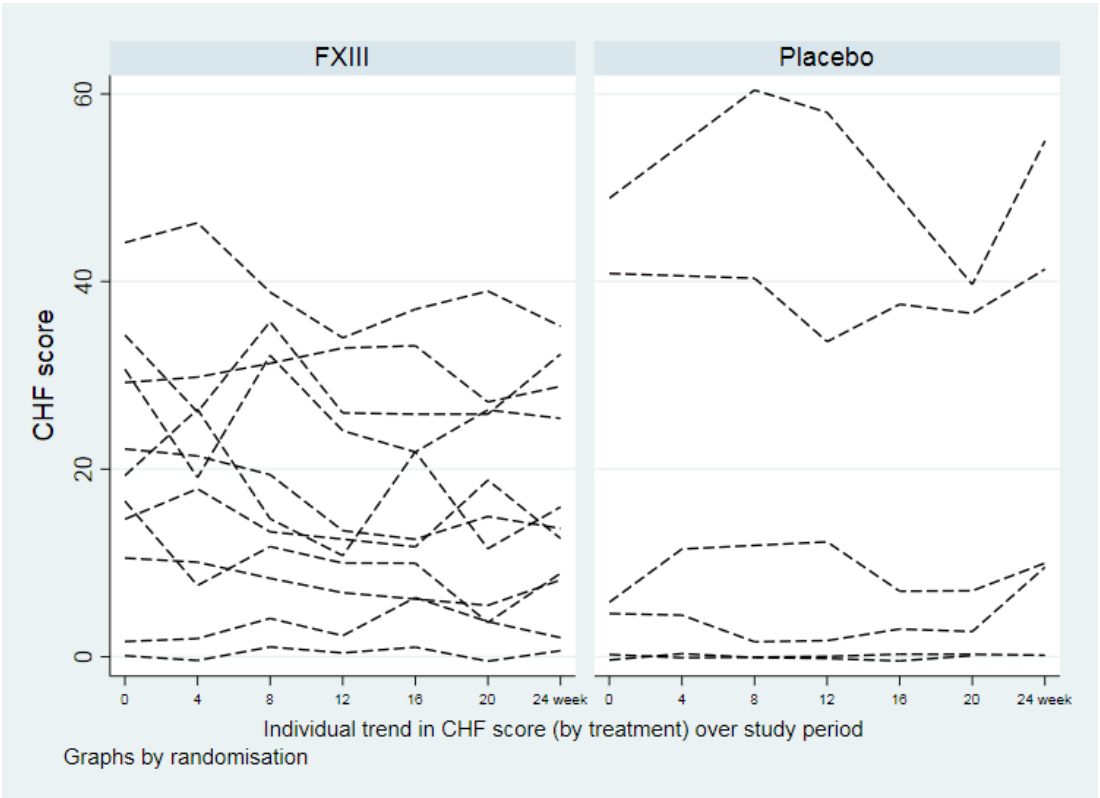


Figure 9 VAS for individual patients over 24 weeks

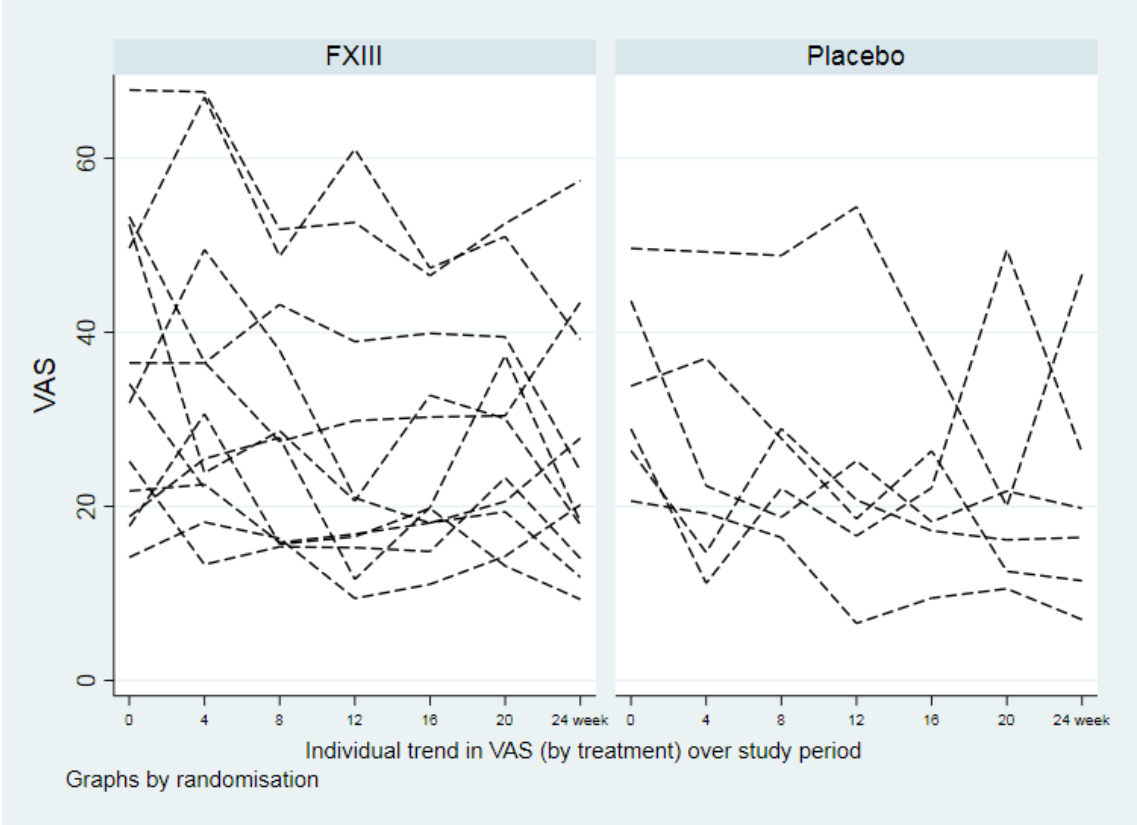


Figure 10 Digital ulcer count for individual patients over 24 weeks

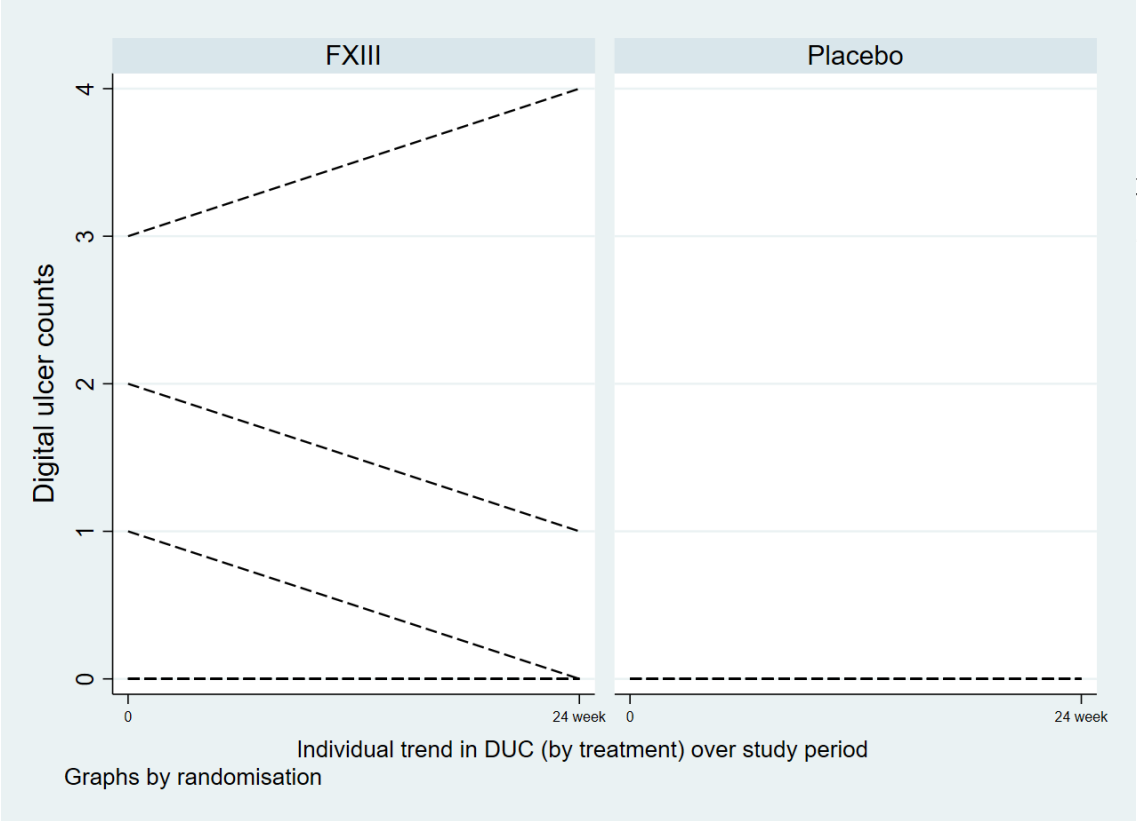


Table 3.8 Secondary endpoints and patient reported outcomes over time (mean (SD))

Outcome	Baseline		Week 4		Week 8		Week12		Week 16		Week 20		Week 24	
	FXIII	Placebo	FXIII	Placebo	FXIII	Placebo	FXIII	Placebo	FXIII	Placebo	FXIII	Placebo	FXIII	Placebo
VAS (%)	35.2 (17.1)	34.1 (11)	34.5 (18.3)	20.7 (10.1)	29.9 (13.8)	26.9 (13.2)	26.7 (17.3)	23.8 (16)	28 (13.3)	18.5 (6.3)	30 (13.8))	22 (13.9)	25.8 (14.9)	21.2 (14.3)
SHAQ	1.06 (0.69)	0.83 (0.93)	1.07 (0.78)	0.58 (0.86)	0.92 (0.84)	0.83 (1.07)	0.95 (0.76)	0.77 (0.97)	1.01 (0.81)	0.55 (0.84)	0.95 (0.8)	0.77 (0.96)	0.97 (0.8)	1.03 (0.93)
Digital ulcer count	0.55 (1.04)	0	0.75 (1.36)	0	0.73 (1.01)	0	0.55 (0.82)	0	0.2 (0.42)	0	0.55 (1.51)	0	0.55 (1.21)	0
SF36														

Physical function	64.3 (24.4)	50 (-)	58.3 (20.4)	50 (-)	57.1 (18.9)	50 (-)	-	-	-	-	-	-	50 (0)	100 (-)
Physical role function	56.3 (32.2)	75 (27.4)	56.8 (33.7)	80 (32.6)	52.3 (34.4)	75 (30.6)	-	-	-	-	-	-	50 (35.4)	75 (17.7)
Emotional role function	56.3 (32.2)	75 (27.4)	56.8 (33.7)	80 (32.6)	52.3 (34.4)	75 (30.6)	-	-	-	-	-	-	50 (35.4)	75 (17.7)
Energy/fatigue	50 (0)	50 (0)	50 (0)	50 (0)	50 (0)	50 (0)	-	-	-	-	-	-	50 (0)	50 (0)
Social function	50 (0)	50 (0)	50 (0)	50 (0)	50 (0)	50 (0)	-	-	-	-	-	-	50 (0)	50 (0)
Emotional well-being														

Pain	49.4 (29)	32.5 (24.6)	48.8 (30.3)	28 (29.3)	53 (30.9)	32.5 (27.6)	-	-	-	-	-	-	55 (31.8)	32.5 (15.9)
General health	48.8 (6.4)	45 (5.5)	48.6 (6.7)	44 (6.5)	49.5 (6.9)	45 (6.1)	-	-	-	-	-	-	50 (7.1)	45 (3.5)
Cochin hand function	19.3 (13.3)	16.8 (22.1)	18.7 (13.4)	3.8 (5.2)	19.1 (13.3)	20.4 (27.9)	15.7 (11.8)	17.7 (23.6)	18.2 (11.7)	9.6 (16.1)	16.1 (12.4)	14.5 (18.8)	16.7 (11.9)	23.2 (23.5)

Note: All outcomes are summarised as mean (SD).

3.3 Laboratory studies

3.3.1 Routine laboratory studies

The baseline (week 0) results of the routine laboratory studies (blood and urine) are presented below.

Table 3.9 Baseline results for routine laboratory studies (mean (SD))

	FXIII	Placebo
Haematology		
White Blood Cells, $\times 10^9$/L	7 (1.8)	6.4 (1.3)
Red Blood Count, $\times 10^{12}$/L	4.4 (0.4)	4.3 (0.5)
Haemoglobin, g/L	117.8 (9.6)	124.7 (12.8)
Haematocrit, L/L	0.4 (0.02)	0.4 (0.04)
MCV, fL	87 (7.2)	90.6 (5.3)
MCH, pg	27.3 (3.3)	29.3 (2.3)
MCHC, g/L	312.3 (15.6)	322.8 (7.5)
Platelets, $\times 10^9$/L	298.5 (76.1)	245 (44.7)
Neutrophils, $\times 10^9$/L	4.8 (1.5)	4.2 (1.2)
Lymphocytes, $\times 10^9$/L	1.5 (0.5)	1.5 (0.3)

Eosinophils, $\times 10^9$/L	0.5 (0.2)	0.5 (0.2)
Basophils, $\times 10^9$/L	0.2 (0.2)	0.2 (0.04)
Reticulocytes, $\times 10^9$/L	0.05 (0.04)	0.04 (0.01)
Biochemistry		
Sodium, mmol/L	140.8 (1.9)	140.7 (2.1)
Potassium, mmol/L	4.6 (0.4)	4.5 (0.4)
Chloride, mmol/L	101.7 (2.8)	101.8 (2.3)
Bicarbonate, mmol/L	23.6 (4.1)	22.5 (2.9)
Urea, mmol/L	5.7 (2.3)	4.9 (1.9)
Creatinine, umol/L	73 (17.3)	75 (27.5)
Total protein, g/L	68.9 (3.8)	66.3 (4)
Albumin, g/L	44.3 (14.4)	43.5 (3.2)
Alkaline phosphatase, U/L	66.4 (14.4)	65.8 (13.5)
ALT, U/L	17.8 (6)	19 (3.9)
AST, U/L	19.1 (5.8)	20.7 (3.2)
Calcium, mmol/L	2.4 (0.1)	2.3 (0.1)

GGT, U/L	19.6 (13.6)	13.3 (4.3)
eGFR, mL/min	75.1 (14.6)	72.5 (19.8)
Amylase, U/L	77.3 (34.5)	74.8 (33.2)
Total cholesterol, mmol/L	5.3 (1.1)	4.7 (1)
Glucose, mmol/L	4.9 (0.6)	4.6 (0.3)
Urinalysis		
Glucose		
Negative, No (%)	10 (83.3)*	6 (100)
Blood		
Negative	7 (70)	5 (83.3)
Trace	1 (10)	0
+	2 (20)	0
++	0	1 (16.7)
Protein		
Negative	10 (83.3)*	5 (83.3)
+	0	1 (16.7)

*Note that two patients did not have results recorded for glucose.

During the trial most results remained within the normal range. There were no significant differences in laboratory values for each patient when compared over the duration of the trial other than for one patient who experienced significantly deranged liver function tests which was reported as an SAE and investigated appropriately. The patient's blood tests recovered to within normal parameters. The patient was later found to be receiving placebo.

3.3.2 Factor XIII analyses

3.3.2.1 Sclero XIII clinical trial – treatment phase

Factor XIII levels were collected regularly throughout the trial as laid out in the schedule of assessments. The full results can be viewed in appendix 6.3.

As seen in the pharmacokinetic phase, endogenous factor XIII level was stable between screening and baseline, and there was a marked increase in factor XIII level following each administered dose in the FXIII group. Adjusted mean change between pre- and post-dose levels was +66.56 IU/dL in the FXIII group compared to -3.05 IU/dL in the placebo group.

Table 3.10 Descriptive statistics for Sclero XIII treatment phase FXIII levels. Data included for all factor XIII doses received by all patients during the clinical trials.

Group		**Factor XIII level (IU/dL)**	
		Pre-dose	**Post-dose**
FXIII	n	140	119
	Mean	170.78	228.6

	Median	158.5	226
	Min	102	123
	Max	330	394
	SD	45.46	40.38
	Adjusted mean change 1 hour following dose administration (95% CI)	66.56 (60.46 to 72.66)	
Placebo	n	72	67
	Mean	134.31	132.16
	Median	135	129
	Min	13	96
	Max	179	185
	SD	25.45	21.9
	Adjusted mean change 1 hour following dose administration (95% CI)	-3.05 (-8.25 to 2.16)	

3.3.2.2 Healthy controls

Factor XIII levels measured at 3 timepoints over a 24-week period suggest that there is minimal baseline variability in the endogenous factor XIII level in the healthy population, with a similar median and standard deviation at each time point, and an adjusted mean change of only -0.33 IU/dL over the 24-week period (table 3.11) (figure 11).

Figure 11 Factor XIII levels in healthy controls over a 24-week period

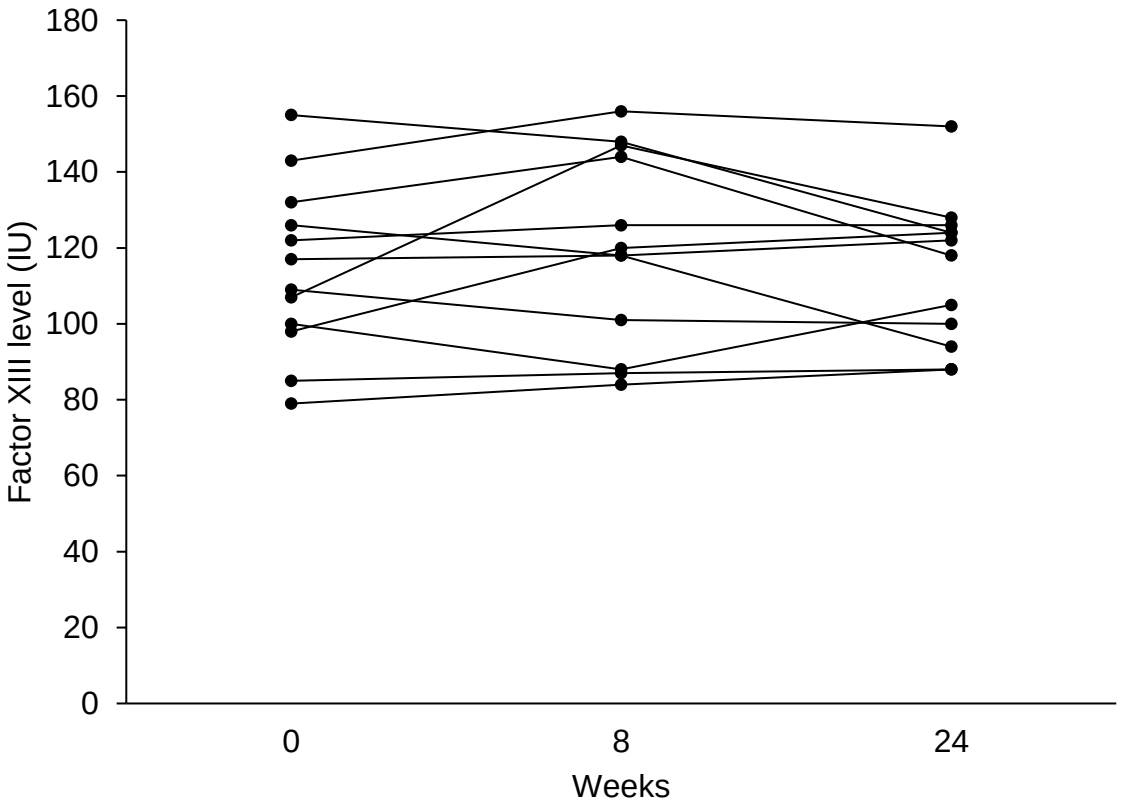
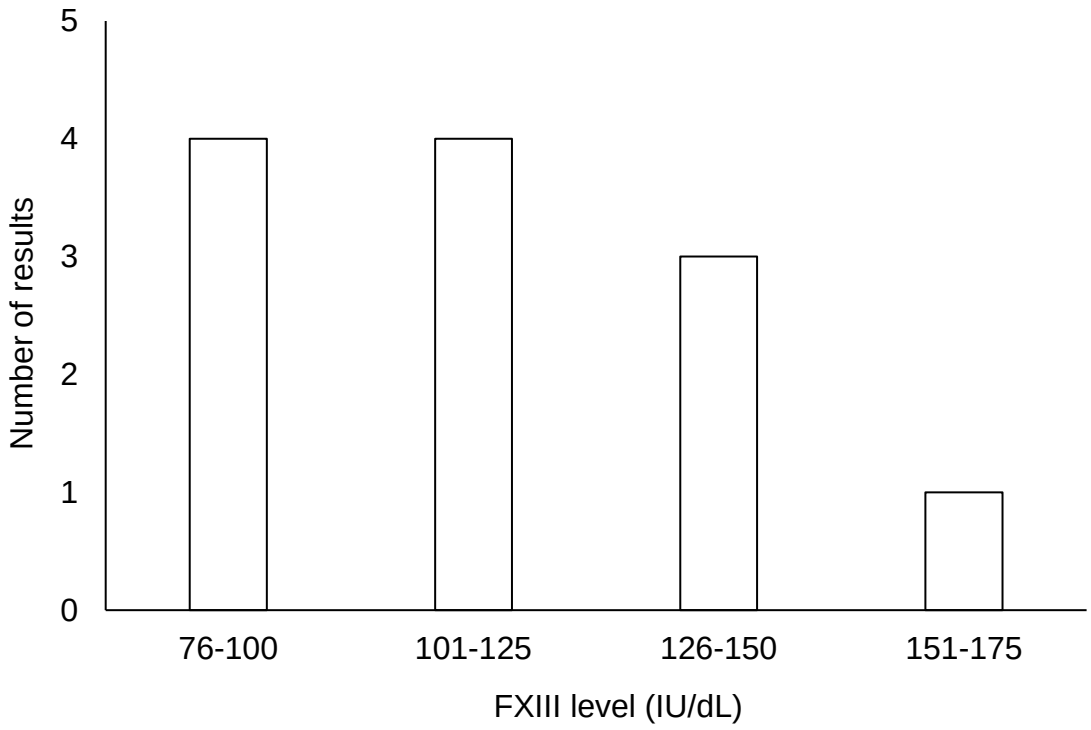


Table 3.11 Descriptive statistics of healthy control factor XIII levels (IU/dL)

	Week 0	**Week 8**	**Week 24**	**p-value**
n	12	12	11	
Mean	114.42	119.75	114.08	
Median	113.00	119.00	120.00	
Min	79.00	84.00	88.00	
Max	155.00	156.00	152.00	
SD	21.70	24.49	18.47	
Adjusted mean change from baseline week 0 to 24 (95% CI)	-0.33 (-9.44 to 8.78)			0.97

Figure 12 Distribution of FXIII levels in healthy controls



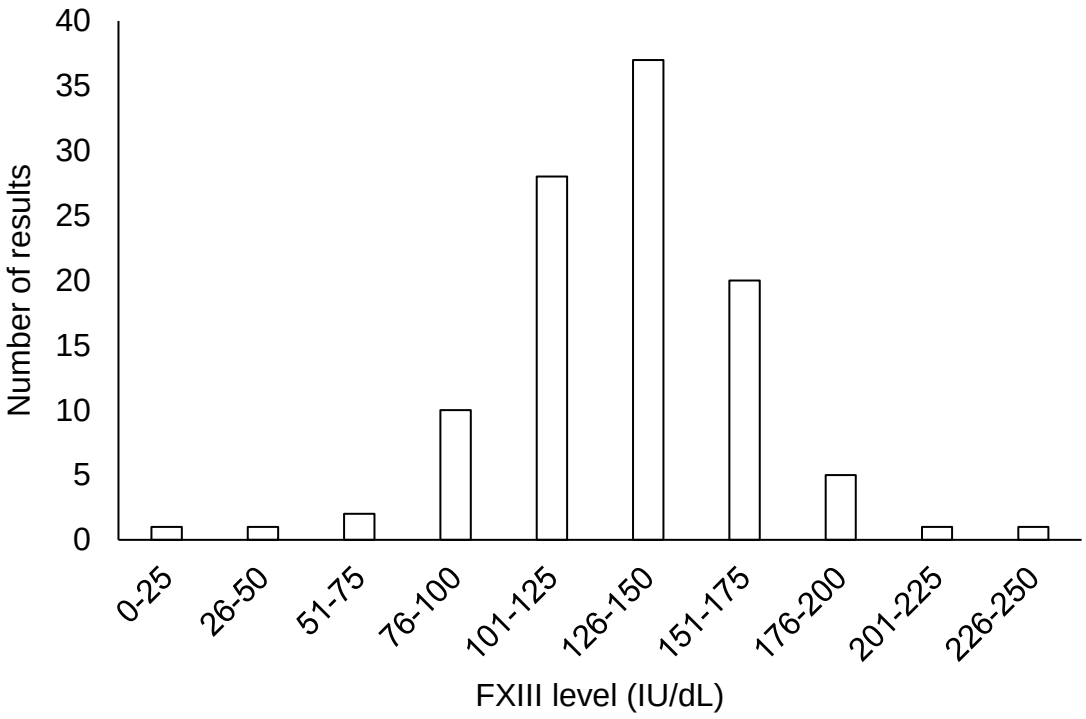
3.3.2.3 Systemic sclerosis patients

Data were collated from 106 SSc patients who gave a single factor XIII level. Descriptive statistics and the distribution of results are shown in table 3.12 and figure 13.

Table 3.12 Descriptive statistics of systemic sclerosis patients' factor XIII levels (IU/dL)

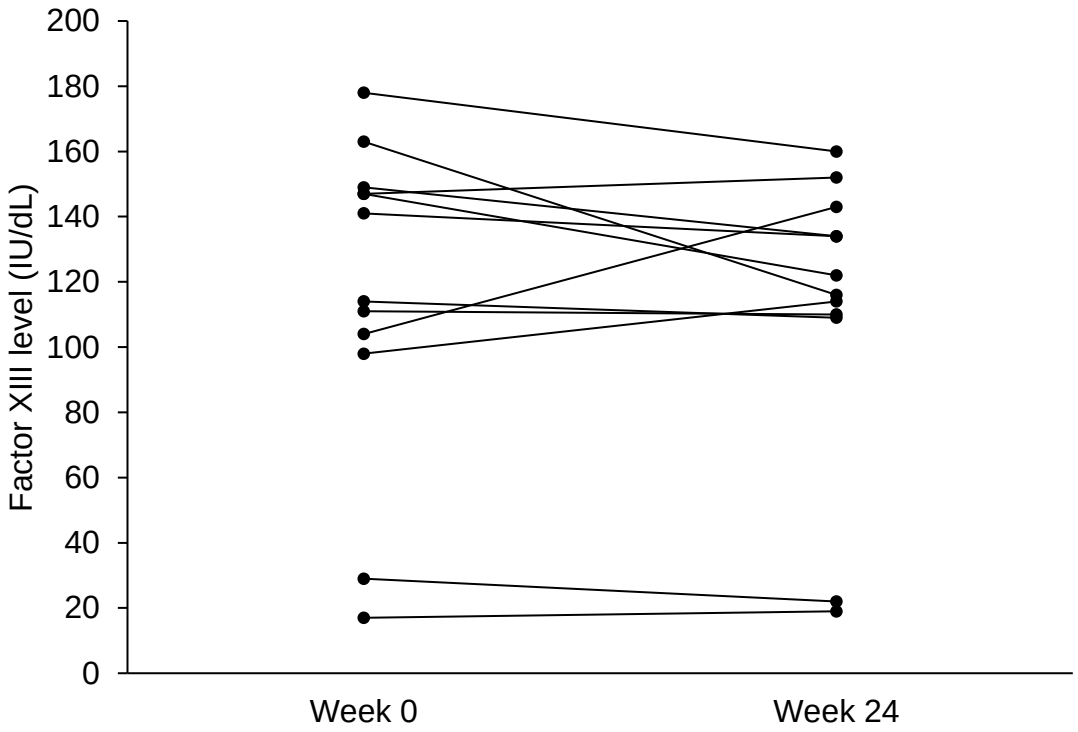
n	106
Mean	133.04
Median	135
Min	17
Max	281
SD	34.94

Figure 13 Distribution of FXIII levels in systemic sclerosis



Twelve of the 106 patients provided further samples for factor XIII levels at week 24. There was no significant variation in factor XIII levels in SSc patients over a 24-week period (p=0.97).

Figure 14 Factor XIII levels in SSc patients over a 24-week period



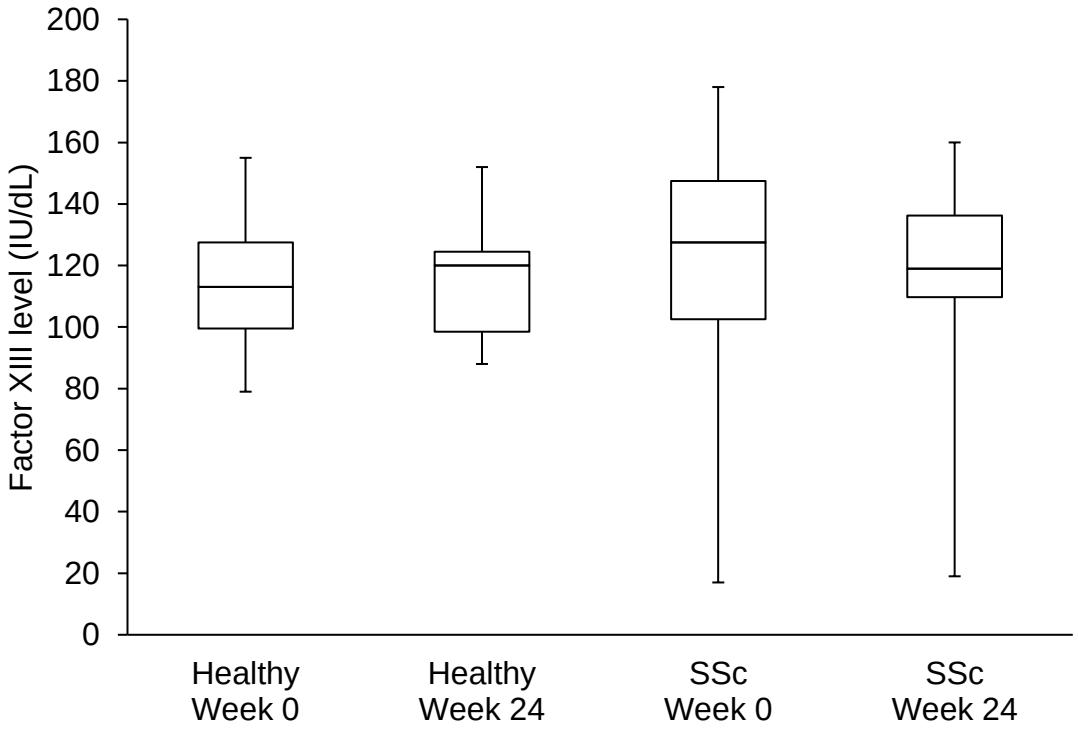
When the data for healthy controls and systemic sclerosis patients were compared, there were no significant differences between the groups.

Table 3.13 Descriptive statistics for factor XIII (IU) at Week 0 and Week 24 in SSc and healthy control groups

Treatment Group		n	Mean	SD	Min	Median	Max	p-value
Healthy	Week 0 baseline	12	114.42	21.70	79.00	113.00	155.00	
	Week 24	12	114.08	18.47	88.00	120.00	152.00	
	Change from baseline	12	-0.33	16.10	-31.00	3.50	26.00	
	Adjusted mean change from baseline (95% CI)	12	-0.33 (-9.44 to 8.78)					0.97
SSc	Week 0 baseline	12	116.50	47.92	17.00	127.50	178.00	
	Week 24	12	111.25	43.50	19.00	119.00	160.00	
	Change from baseline	12	-5.25	20.38	-47.00	-6.00	39.00	
	Adjusted mean change	12	-5.50 (-17.03 to 6.03)					0.26

	from baseline (95% CI)			
Difference between groups		Adjusted mean change from baseline (95% CI)	5.17 (-14.69 to 25.03)	0.53

Figure 15 Comparison of factor XIII levels at 0 and 24 weeks for SSc patients vs. healthy controls. Boxes represent median and interquartile range (IQR), whiskers represent maximum and minimum values.



3.3.3 Candidate biomarker studies

3.3.3.1 ELISAs

The following proteins were analysed using ELISA technology:

- COMP
- Fibronectin
- TSP-1
- VEGF-C
- VEGF-D

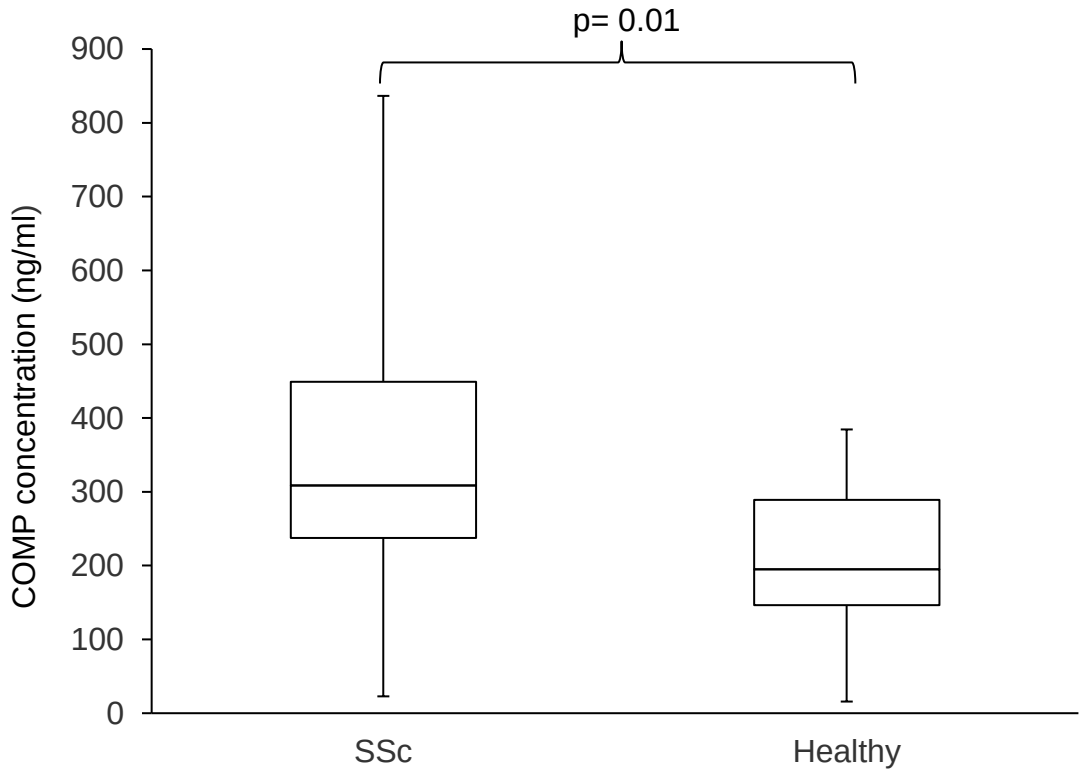
Graphical and tabulated representation of these results in full can be reviewed in appendix 6.5.1 and 6.5.2.

Statistical methods are discussed in section 2.13.

3.3.3.1.1 Cartilage oligomeric matrix protein

There was a statistically significant difference in COMP levels when comparing SSc patients to healthy controls at week 0 (p=0.01)

Figure 16 COMP concentration at Week 0: SSc vs. Healthy. Boxes represent median and IQR, whiskers represent maximum and minimum values.



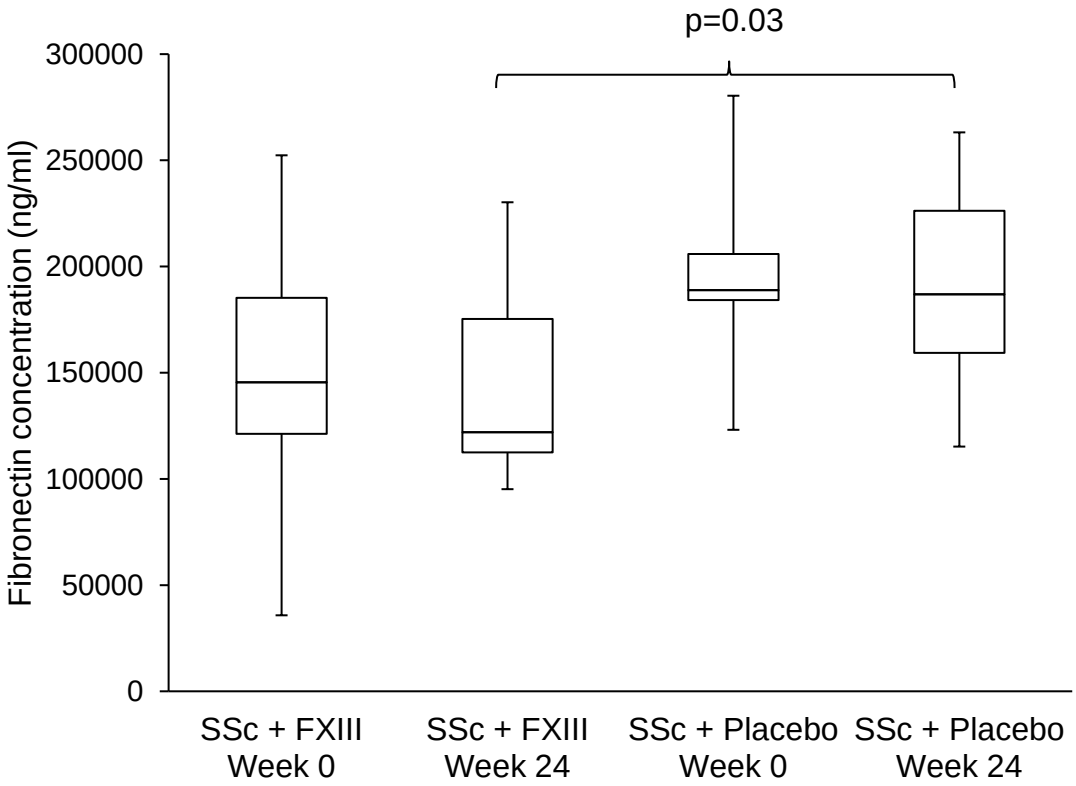
However, there was no significant difference in COMP levels between baseline and week 24 in the SSc + FXIII (p=0.53) or placebo groups (p=0.21). There was also no significant difference seen when comparing the changes in COMP levels in the FXIII and placebo groups at 24 weeks (p=0.39).

3.3.3.1.2 Fibronectin

There was a numeric but not statistically significant difference in fibronectin levels when the SSc group was compared to the healthy control group at week 0 (p=0.07).

There was no significant variation in fibronectin concentration in either the SSc + FXIII or the SSc + placebo group during the 24-week trial (p=0.08 and p=0.59 respectively). There was however a statistically significant difference when the groups were compared, with the FXIII group showing a larger reduction in fibronectin level compared to the placebo group (p=0.03).

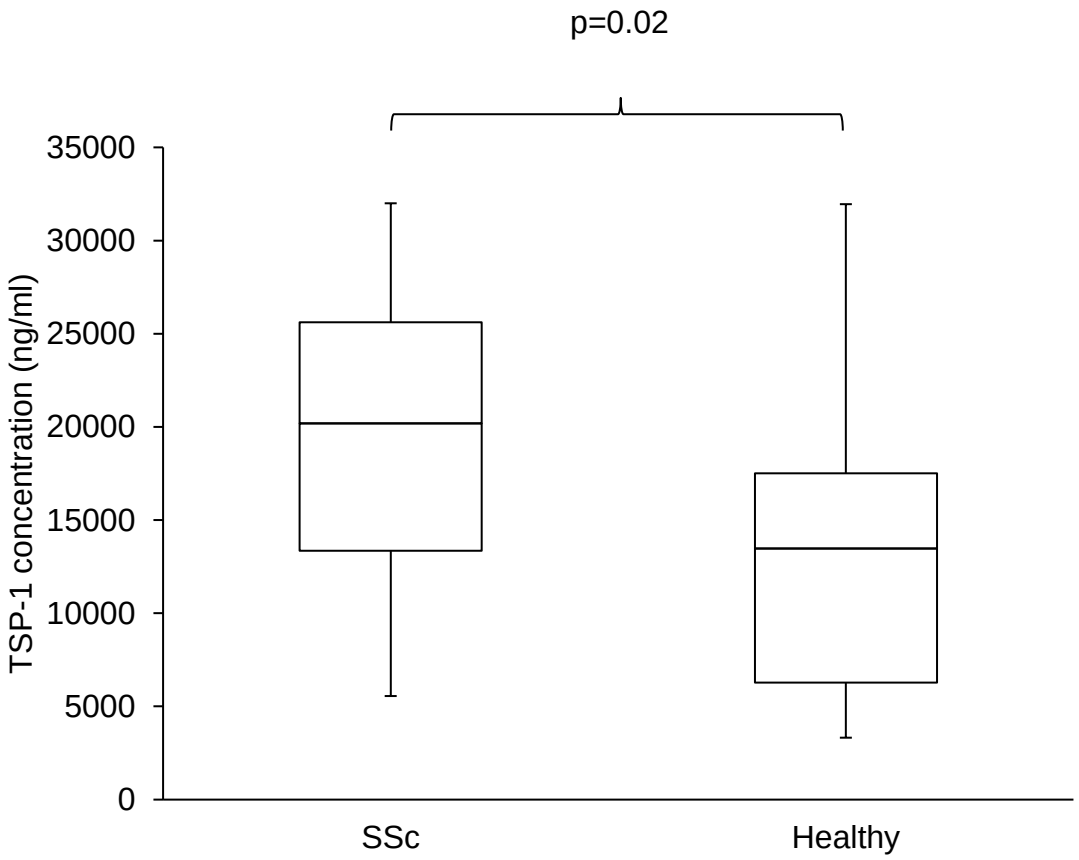
Figure 17 Distribution of fibronectin concentrations week 0 vs. week 24. Boxes represent median and IQR, whiskers represent maximum and minimum values.



3.3.3.1.3 Thrombospondin-1

There was a statistically significant difference in TSP-1 concentrations when systemic sclerosis patients and healthy controls were compared at baseline (p=0.02).

Figure 18 TSP-1 concentration at Week 0: SSc vs. Healthy. Boxes represent median and IQR, whiskers represent maximum and minimum values.



There was a statistically significant decrease in the TSP-1 level at week 24 compared to week 0 in the SSc + FXIII group (p=0.02) which was not present for the SSc+ placebo group (p=0.68) (table 3.14). However, there was no significant difference in the adjusted mean change from baseline when the groups were compared at week 24 (p=0.57). When the SSc FXIII-treated patient data at week

24 were compared to healthy controls, the difference between the SSc and healthy groups was no longer significant (p=0.09).

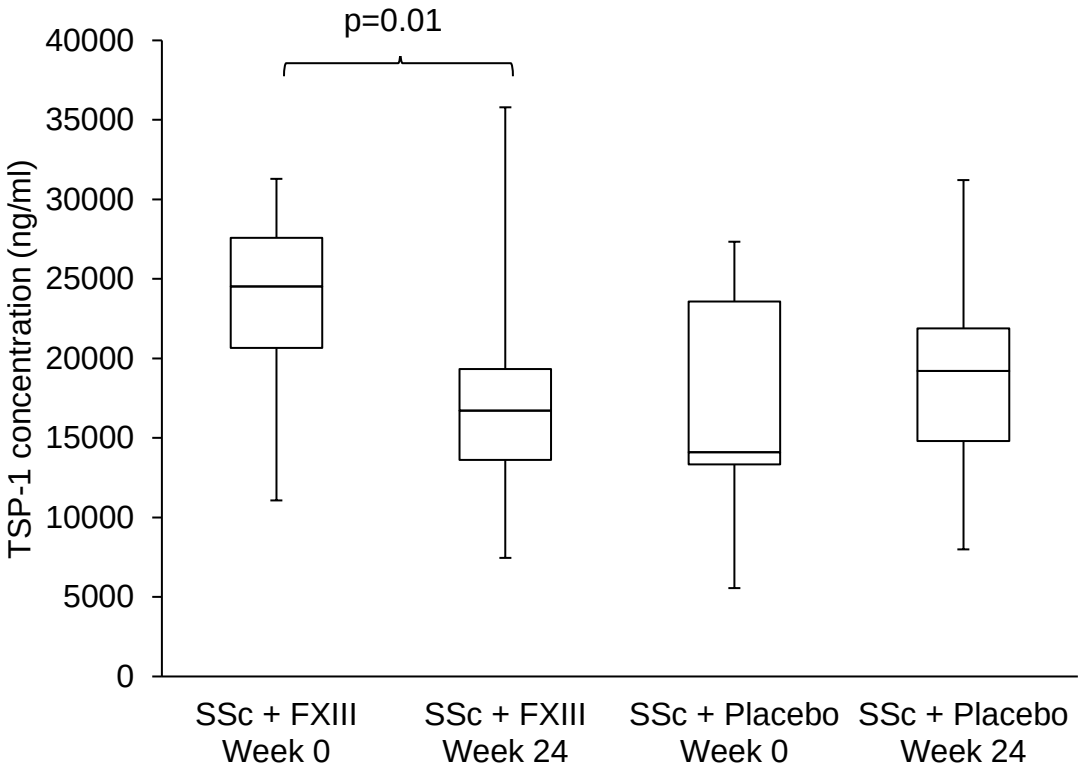
However, it should be noted that these p-values were calculated as part of a descriptive analysis and were not adjusted for multiple analyses.

Table 3.14 Descriptive statistics for TSP-1 (ng/ml) from week 0 to week 24 in SSc + FXIII, SSc + placebo and healthy control groups

Treatment Group		n	Mean	SD	Min	Median	Max	p-value
SSc + FXIII	Week 0 baseline	12	23753.3	5400.0	11070.7	24523.6	31293.8	
	Week 24	12	17179.8	7273.8	7457.6	16727.4	35792.9	
	Change from baseline	12	-6573.5	7732.3	-16835.0	-8964.4	8540.3	
	Adjusted mean change from baseline (95% CI)	12	-6573.5 (-10948.4 to -2198.6)					0.02
SSc + Placebo	Week 0 baseline	9	17146.8	6774.9	5557.9	14110.5	27338.5	
	Week 24	9	18175.6	6733.8	7992.6	19218.4	31216.8	

	Change from baseline	9	1028.8	5423.5	-5373.6	700.3	10778.6	
	Adjusted mean change from baseline (95% CI)	9	1028.8 (-2514.5 to 4572.1)					0.68
Difference between groups		Adjusted mean change from baseline (95% CI)	7602.3 (-19180 to 3975.7)					0.57
Healthy	Week 0	13	13061.6	7805.5	3323.3	13017.5	31952.2	

Figure 19 **Distribution of TSP-1 concentrations Week 0 vs. Week 24. Boxes represent median and IQR, whiskers represent maximum and minimum values.**

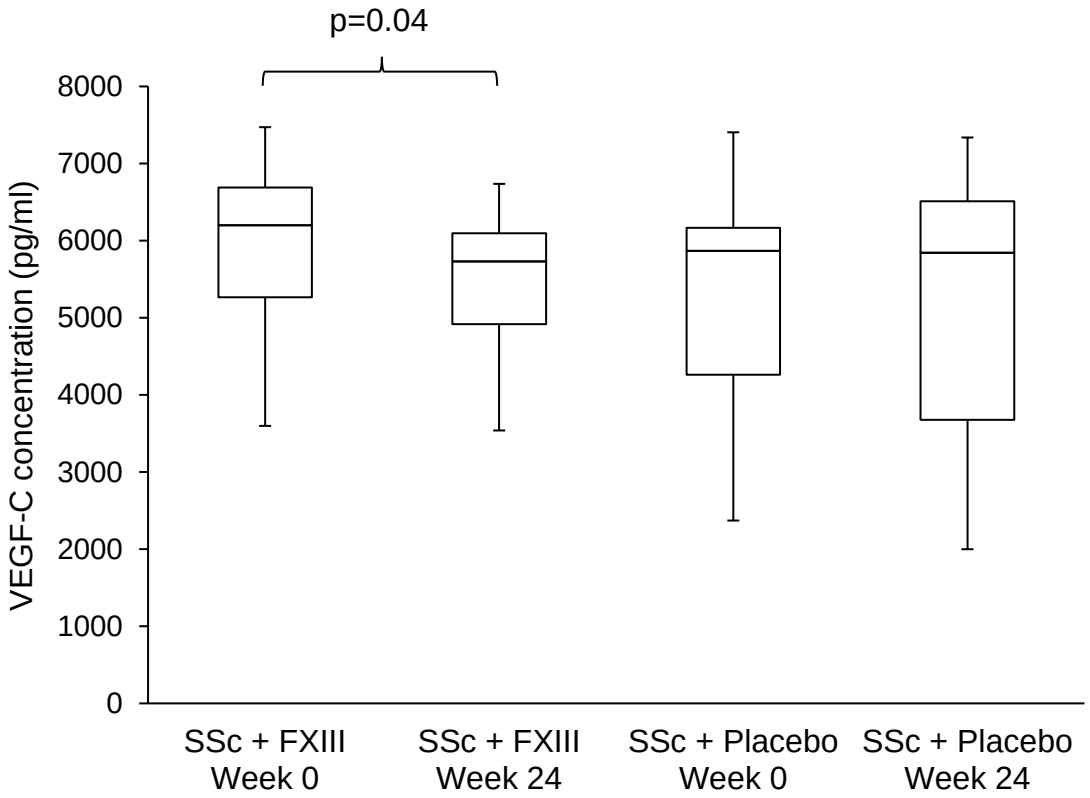


3.3.3.1.4 VEGF-C

There was no difference in VEGF-C levels between SSc patients and healthy controls at baseline (p=0.89).

There was a statistically significant decrease in VEGF-C levels in the SSc + FXIII group (p=0.04) which was not seen in the placebo group (p=0.91). However, there was no significant difference when the change from baseline in each group was compared at 24 weeks (p=0.86).

Figure 20 Distribution of VEGF-C concentrations week 0 vs. week 24. Boxes represent median and IQR, whiskers represent maximum and minimum values.

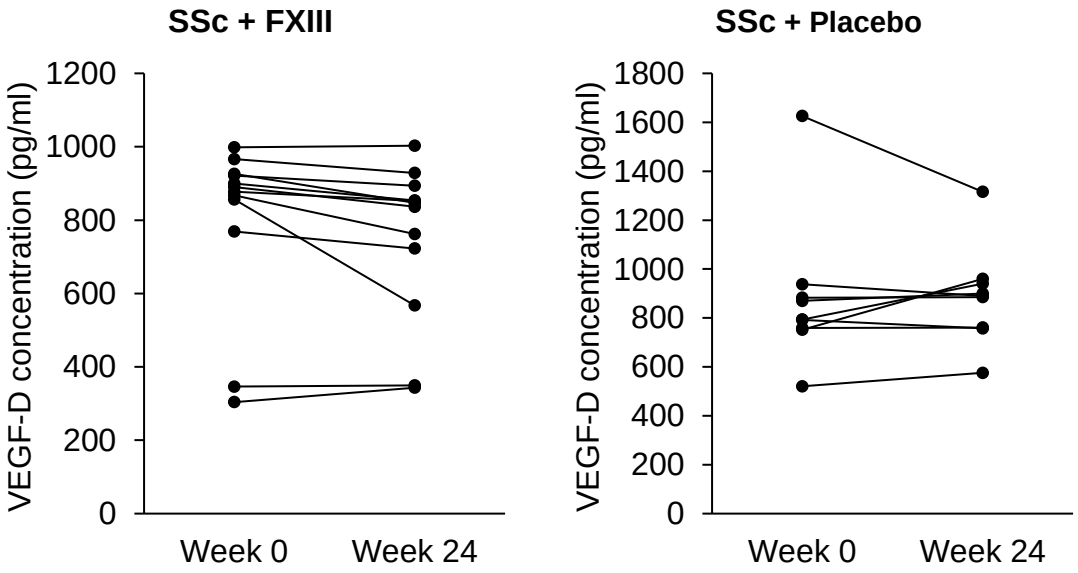


3.3.3.1.5 VEGF-D

There was no difference in VEGF-D concentrations when SSc patients were compared to healthy controls at baseline (p=0.97).

There was no significant difference between the change in VEGF-D levels in the FXIII and placebo groups over 24 weeks. However, there was a significant drop in VEGF-D levels in the placebo group. On review, there was a particular drop in one outlying patient's results which, although it was included in the adjusted mean change from baseline calculation, is likely to have skewed the result.

Figure 21 Change in VEGF-D concentration from week 0 to week 24



3.3.3.2 Combined ELISA and multiplex studies

Full results can be viewed in Appendix 6.5.2.

Statistical methods are discussed in section 2.13.

3.3.3.2.1 sCD40L

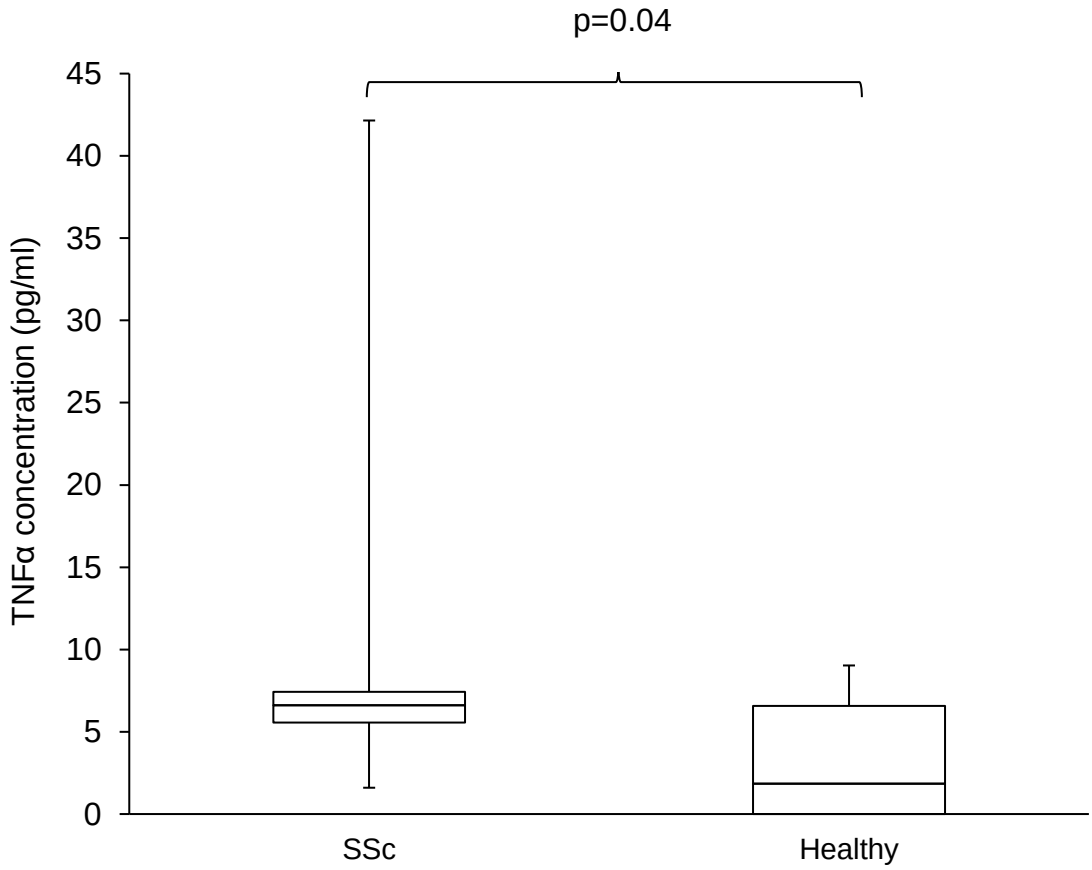
There was no significant difference in sCD40L levels between healthy people and patients with systemic sclerosis when compared at baseline (p=0.06).

There was no significant difference in sCD40L levels between week 0 and week 24 in either the SSc + FXIII (p=0.43) or the placebo group (p=0.95). There was also no difference in the values in the two groups when compared at week 24 (p=0.94).

3.3.3.2.2 Tumour necrosis factor alpha

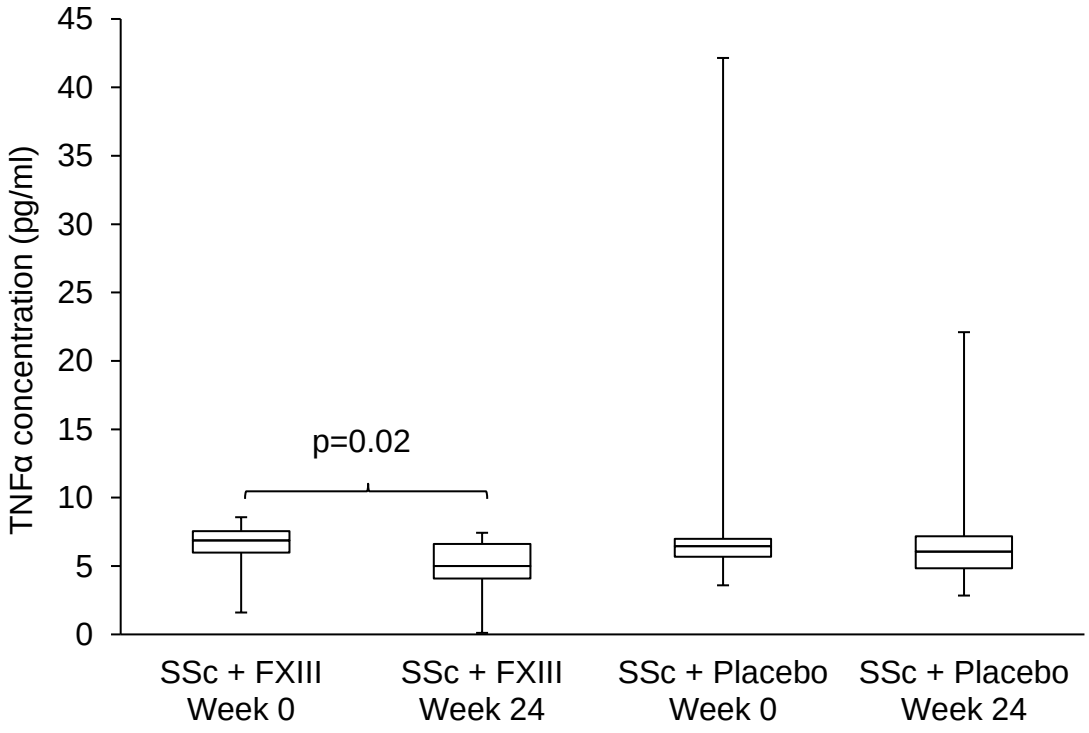
There was a statistically significant difference in TNFα levels between SSc and healthy controls when compared at baseline (p=0.04).

Figure 22 TNFα concentration at week 0: SSc vs. Healthy. Boxes represent median and IQR, whiskers represent maximum and minimum values.



There was also a significant decrease in TNFα levels in the FXIII + SSc groups during the 24-week trial period (p=0.02) which was not seen in the placebo group (p=0.17).

Figure 23 Distribution of TNFα concentrations week 0 vs. week 24. Boxes represent median and IQR, whiskers represent maximum and minimum values.



3.3.3.3 Multiplex assays

All multiplex assay results can be viewed in detail in Appendix 6.5.3.

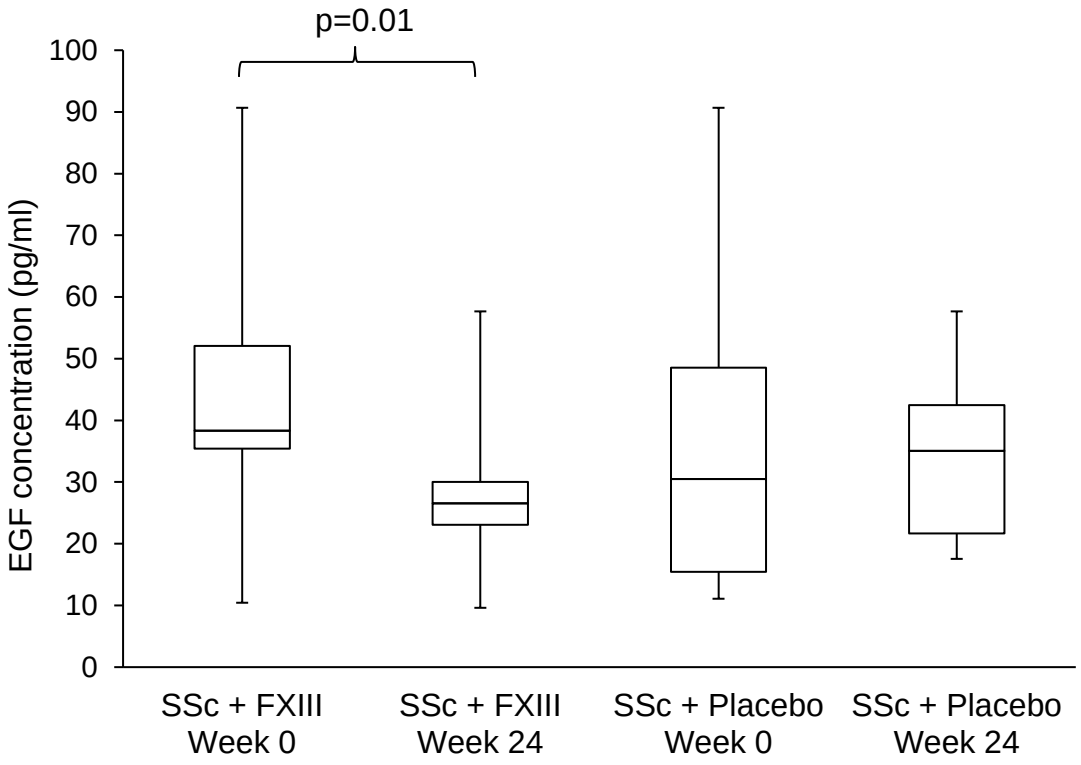
Statistical methods are discussed in section 2.13.

3.3.3.3.1 EGF

There was a numeric but not statistically significant difference in EGF levels when healthy people and patients with SSc were compared at baseline, with the maximum and median EGF level being higher among systemic sclerosis patients.

There was a statistically significant decrease in EGF levels from week 0 to week 24 in the SSc + FXIII group (p=0.01). There was also a decrease in EGF levels in the SSc + placebo group but this was not statistically significant. The mean change from baseline was not significantly greater in the FXIII group compared to placebo at week 24 (p=0.38).

Figure 24 **Distribution of EGF concentrations week 0 vs. week 24. Boxes represent median and IQR, whiskers represent maximum and minimum values.**

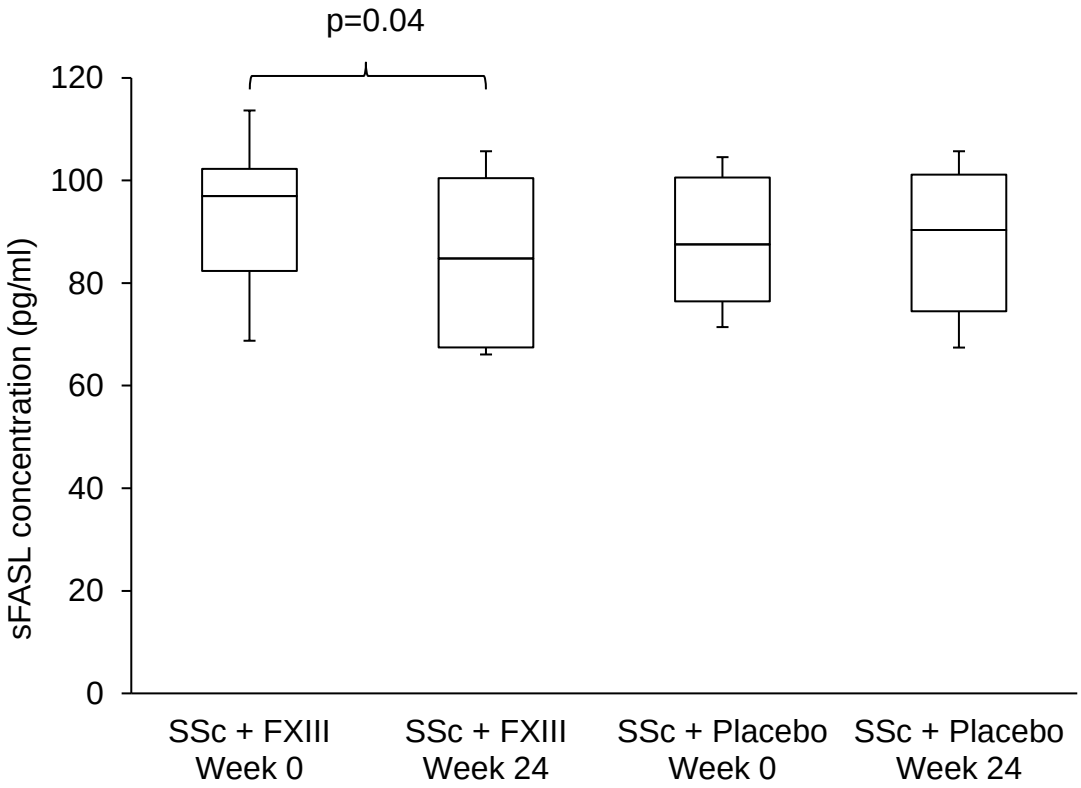


3.3.3.3.2 sFASL

There was no significant difference in sFASL levels when SSc patients were compared to healthy controls (p=0.36).

There was a significant decrease in sFASL levels in the SSc + FXIII group during the 24-week study (p=0.04) which was not seen in the placebo group (p=0.89), however there was no significant difference when the extent of change in sFASL levels in the two groups was compared over 24 weeks (p=0.59).

Figure 25 Distribution of sFASL concentrations week 0 vs. week 24. Boxes represent median and IQR, whiskers represent maximum and minimum values.



3.3.3.3.3 HB-EGF

There was no difference in HB-EGF levels when SSc patient were compared to healthy controls (p=0.85).

There was a statistically significant decrease in HB-EGF concentration in the SSc + FXIII group (p=0.04) which was not seen in the placebo group (p=0.60). When the two groups were compared at week 24 there was no significant difference in the change in HB-EGF over time (p=0.95). This also remained true when the outlying value in the placebo group was removed (p=0.51).

Table 3.15 Descriptive statistics for HB-EGF (pg/ml) at Week 0 and Week 24 in SSc + FXIII, SSc + placebo and healthy control groups

Treatment Group		n	Mean	SD	Min	Median	Max	p-value
SSc + FXIII	Week 0 baseline	9	64.85	5.96	52.29	65.17	73.81	
	Week 24	9	60.34	8.31	47.57	60.56	76.08	
	Change from baseline	9	-4.51	5.11	-13.53	-4.72	4.08	
	Adjusted mean change from baseline (95% CI)	9	-4.51 (-7.84 to -1.17)					**0.04**
	Week 0	6	66.83	29.87	46.52	55.59	132.56	

SSc + Placebo	baseline							
	Week 24	6	69.74	28.65	47.31	62.42	131.53	
	Change from baseline	6	2.91	7.31	-4.72	0.71	18.31	
	Adjusted mean change from baseline (95% CI)	6	-0.17 (-6.02 to 5.68)					0.60
Difference between groups		Adjusted mean change from baseline (95% CI)	-4.34 (-1.94 to -6.74)					0.95
Healthy	Week 0	5	64.62	8.06	54.63	64.65	77.60	

Figure 26 Change in HB-EGF concentration from week 0 to week 24

Dashed line represents outlying value (see text above)

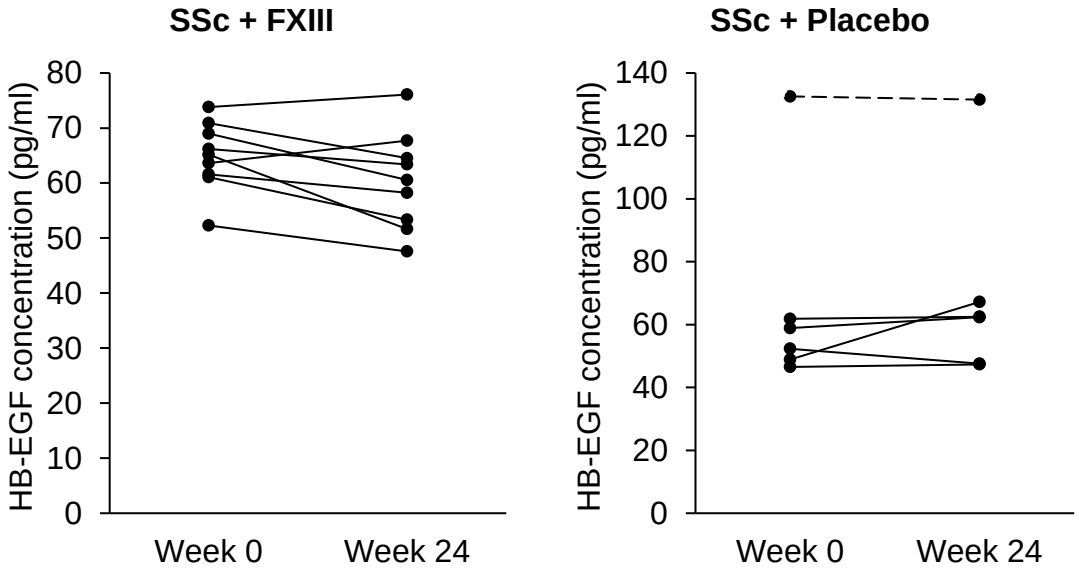
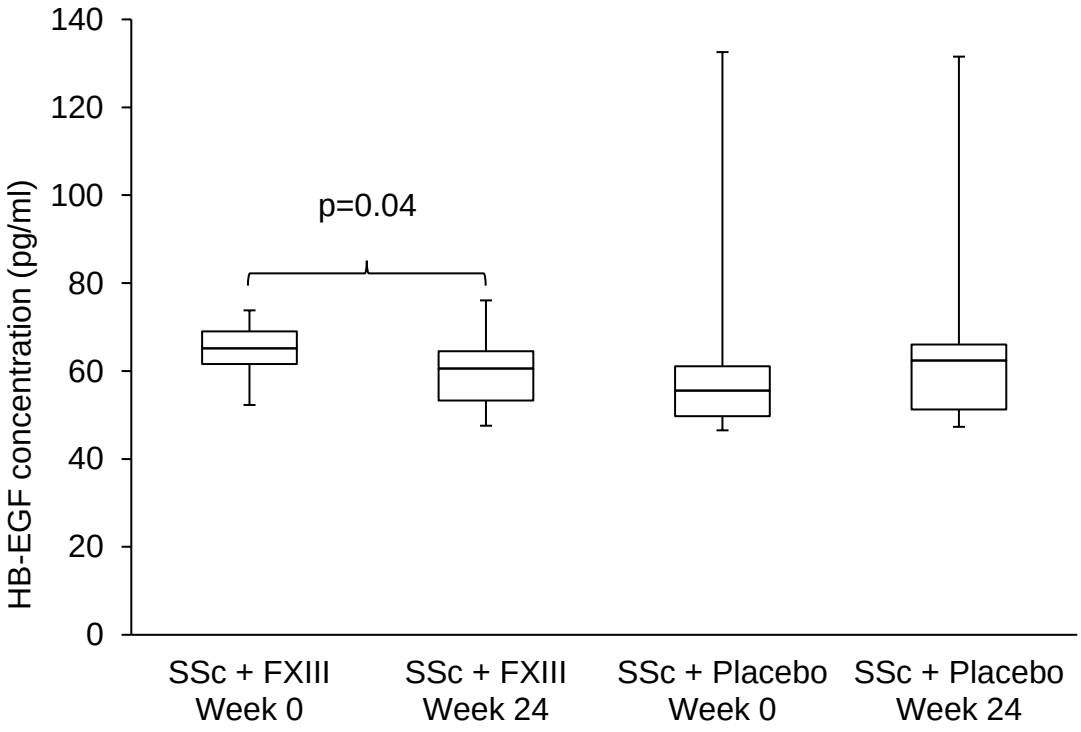


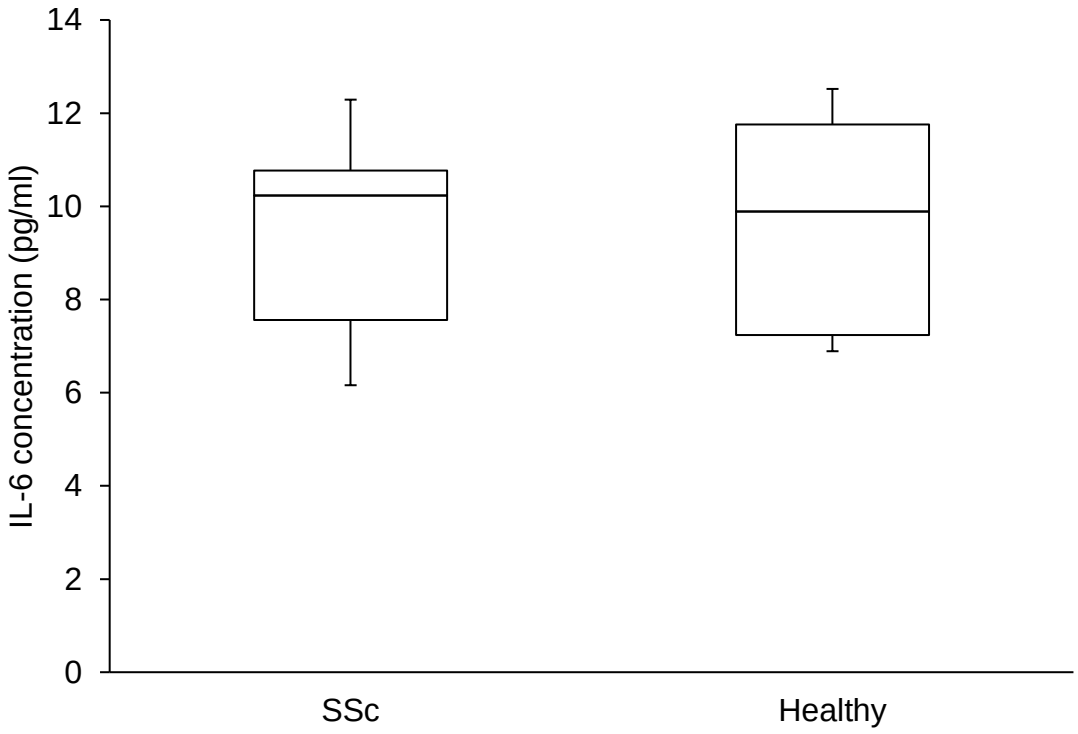
Figure 27 **Distribution of HB-EGF concentrations week 0 vs. week 24. Boxes represent median and IQR, whiskers represent maximum and minimum values.**



3.3.3.3.4 Interleukin-6

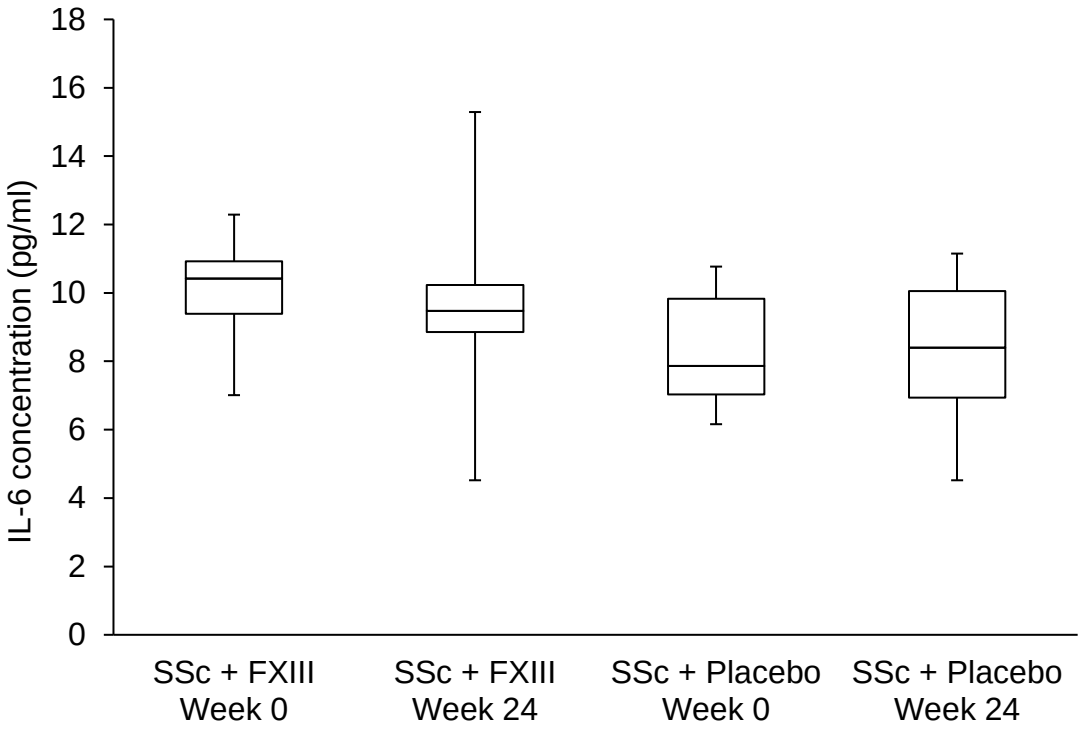
There was no difference in IL-6 concentrations between the SSc and healthy control groups at baseline (p=0.78).

Figure 28 IL-6 concentration at week 0: SSc vs. Healthy. Boxes represent median and interquartile range (IQR), whiskers represent maximum and minimum values.



There was no significant change in IL-6 concentration in the FXIII or placebo groups between week 0 and week 24 (p=0.21 and p=0.92 respectively).

Figure 29 **Distribution of IL-6 concentrations week 0 vs. week 24. Boxes represent median and IQR, whiskers represent maximum and minimum values.**



3.3.3.3.5 Transforming growth factor alpha

There was no significant difference in TGF-α concentration when SSc patients were compared to healthy controls at baseline (p=0.61).

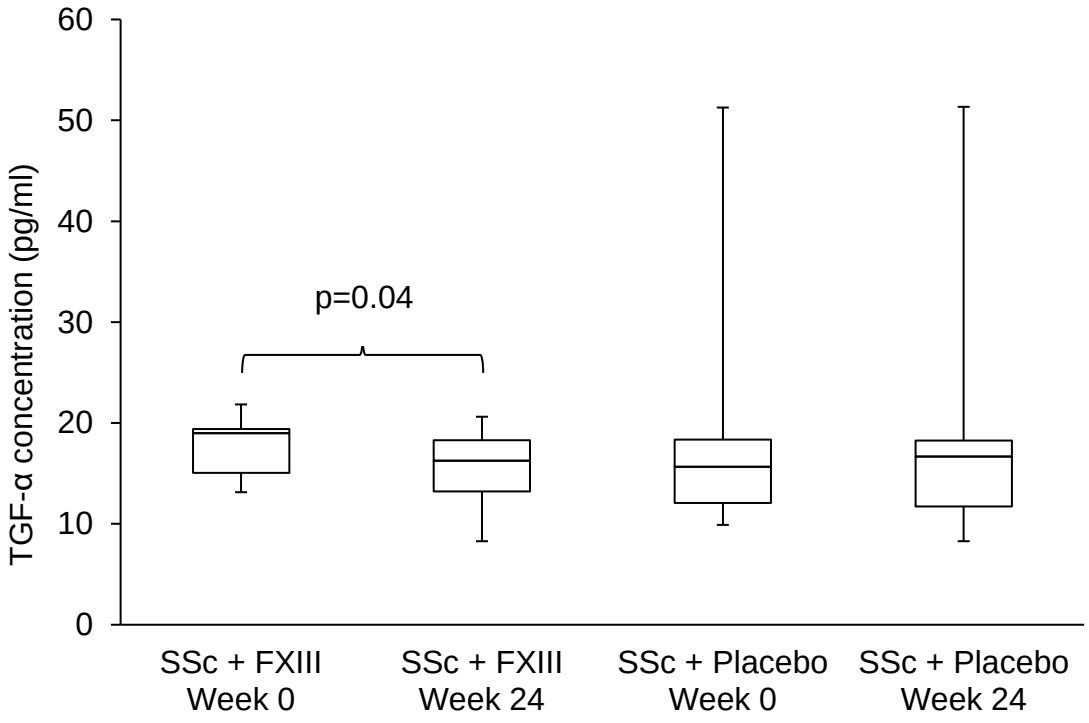
There was a significant decrease in TGF-α concentration during the 24-week trial in the SSc + FXIII group (p=0.04) which was not seen in the placebo group (p=0.92). However, there was no significant difference between the changes in TGF-α level in each group over the 24 weeks (p=0.86).

Table 3.16 Descriptive statistics for TGF-α (pg/ml) at Week 0 and Week 24 in SSc + FXIII, SSc + placebo and healthy control groups

Treatment Group		n	Mean	SD	Min	Median	Max	p-value
SSc + FXIII	Week 0 baseline	9	17.87	2.98	13.14	18.98	21.84	
	Week 24	9	15.59	3.70	8.28	16.26	20.62	
	Change from baseline		-2.28	2.44	-5.70	-1.48	1.23	
	Adjusted mean change from baseline (95% CI)		-2.28 (-3.87 to -0.68)					**0.04**
SSc + Placebo	Week 0 baseline	6	20.44	14.13	9.90	15.66	51.27	
	Week 24	6	20.31	14.37	8.28	16.67	51.34	

	Change from baseline		-0.13	2.93	-5.27	0.11	4.81	
	Adjusted mean change from baseline (95% CI)		-0.09 (-2.43 to 2.26)					0.92
Difference between groups		Adjusted mean change from baseline (95% CI)	-2.19 (-2.84 to -1.55)					0.86
Healthy	Week 0	5	16.27	3.92	11.52	15.99	22.12	

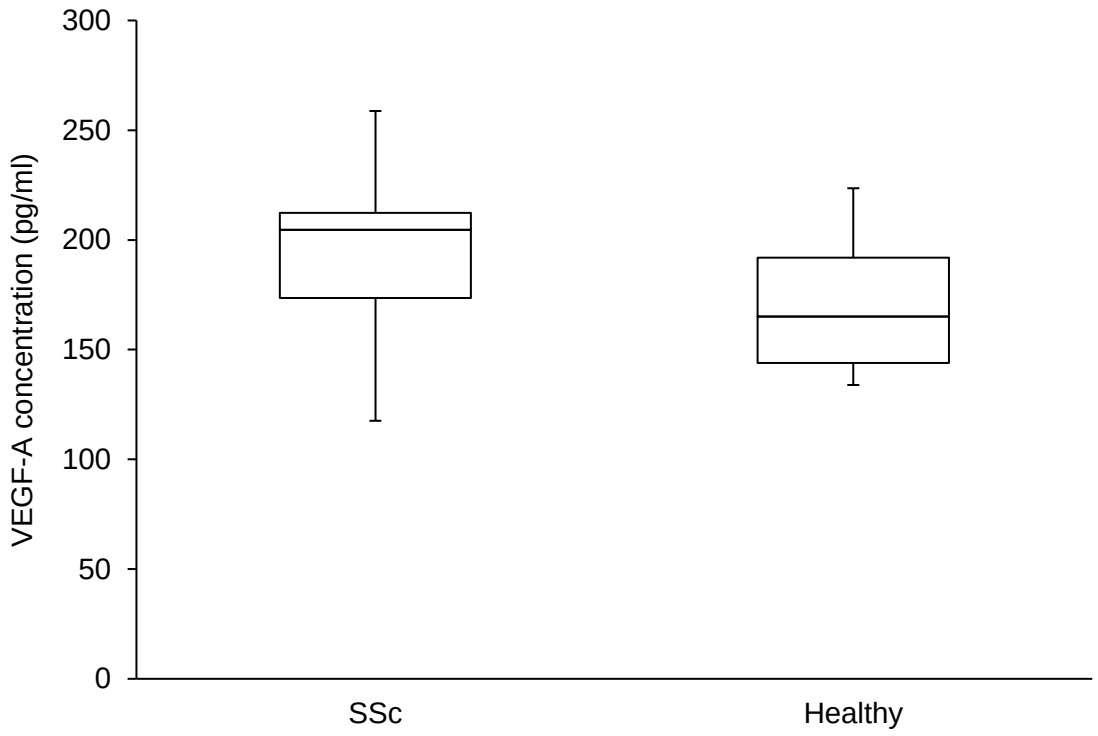
Figure 30 **Distribution of TGFα concentrations week 0 vs. week 24. Boxes represent median and IQR, whiskers represent maximum and minimum values.**



3.3.3.3.6 VEGF-A

There was no difference in VEGF-A levels when SSc patients and healthy controls were compared at baseline (p= 0.27).

Figure 31 VEGF-A concentration at Week 0: SSc vs. Healthy. Boxes represent median and IQR, whiskers represent maximum and minimum values.



There was no difference in VEGF-A levels over the 24-week trial period in either the FXIII or placebo groups (p=0.52 and p=0.25 respectively).

Table 3.17 Descriptive statistics for VEGF-A (pg/ml) at Week 0 and Week 24 in SSc + FXIII, SSc + placebo and healthy control groups

Treatment Group		n	Mean	SD	Min	Median	Max	p-value
SSc + FXIII	Week 0 baseline	9	201.30	14.57	170.82	204.80	220.12	
	Week 24	9	195.85	29.07	138.02	193.86	231.18	
	Change from baseline		-5.45	22.54	-38.50	-1.50	26.13	
	Adjusted mean change from baseline (95% CI)		-5.45 (-20.17 to 9.27)					0.52
SSc + Placebo	Week 0 baseline	6	187.36	53.75	117.58	180.26	258.76	
	Week 24	6	195.95	61.28	127.45	181.84	304.83	
	Change from baseline		8.60	18.36	-8.79	5.58	46.07	
	Adjusted mean change from baseline (95% CI)		1.10 (-13.59 to 15.80)					0.25
Difference between groups		Adjusted mean	-6.55 (-20.8 to 7.7)					0.77

		change from baseline (95% CI)						
Healthy	Week 0	5	171.65	32.68	133.90	165.02	223.56	

Figure 32 Change in VEGF-A concentration from week 0 to week 24

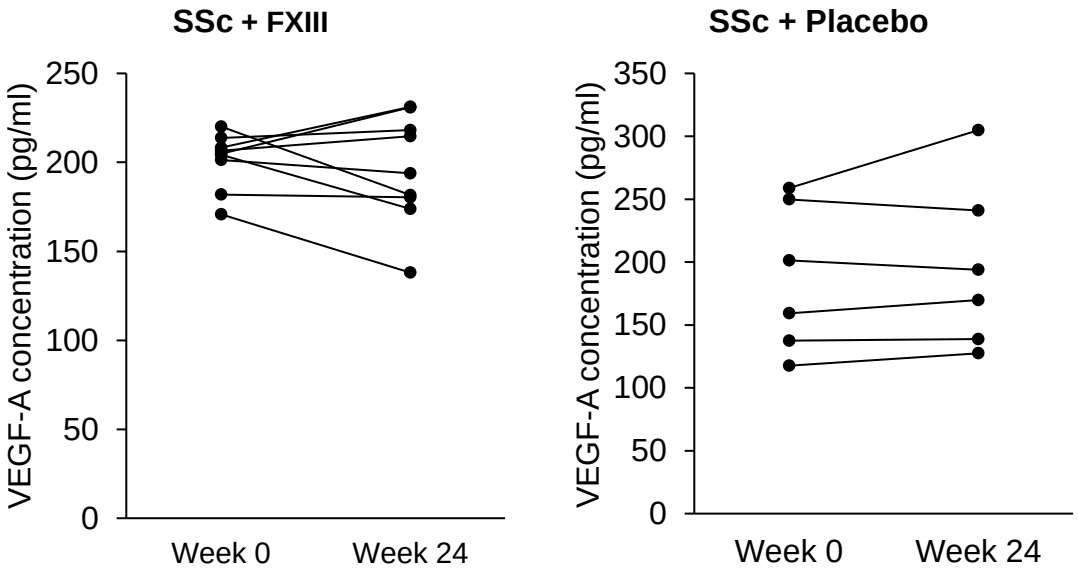
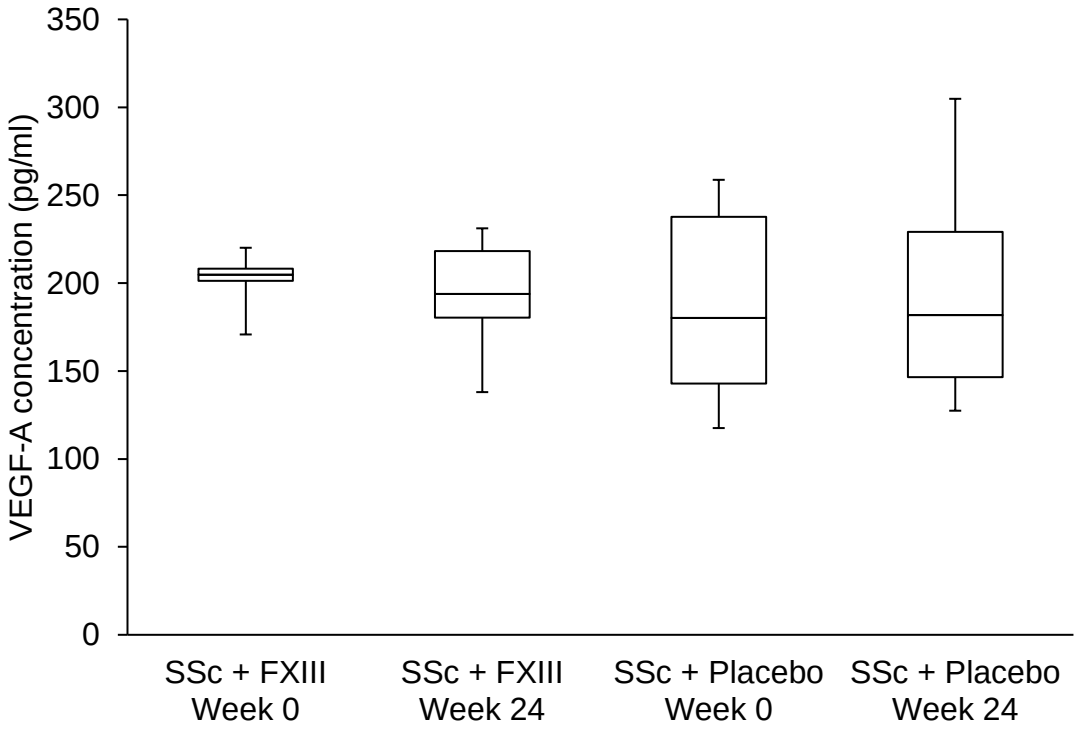


Figure 33 Distribution of VEGF-A concentrations week 0 vs. week 24. Boxes represent median and IQR, whiskers represent maximum and minimum values.



3.3.4 Explanted dermal fibroblast studies

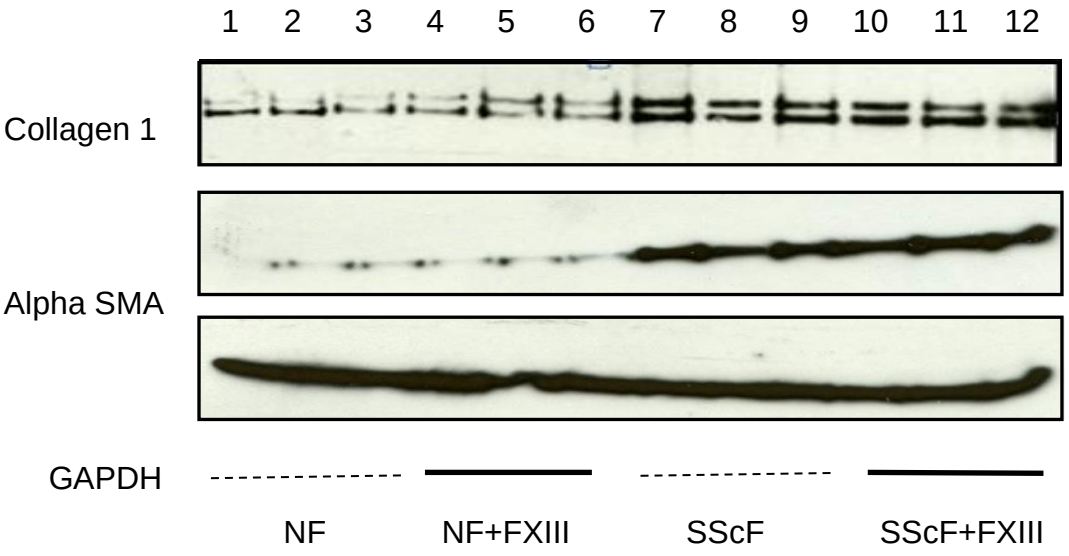
3.3.4.1 Western blot analysis

There was a more than 2-fold increase in collagen-1 and alpha-SMA protein expression in SSc fibroblasts compared with normal fibroblasts. These proteins have previously been shown to correlate with a pro fibrotic phenotype (Jimenez et al., 1986, Jinnin, 2010, Gilbane et al., 2013, Varga and Trojanowska, 2008). However, the degree of expression of these proteins was not altered by the addition of FXIII. This is in contrast to previously published data where addition of the same concentration of factor XIII inhibited SSc fibroblast collagen deposition *in vitro* (Paye et al., 1990).

Table 3.18 Densitometry assessment of Western blots

	NF	NF + FXIII	SScF	SScF + FXIII
n	6	6	6	6
Collagen 1 mean (SD)	7.53 (1.42)	7.75 (1.03)	21.08 (2.87)	20.65 (1.93)
Alpha SMA mean (SD)	4.8 (0.72)	4.95 (0.63)	19.5 (1.18)	18.53 (1.03)

Figure 34 Western blot showing collagen 1 and alpha SMA expression in normal fibroblasts (1, 2, 3) and SSc (7, 8, 9); and the extent of protein expression when factor XIII was added to normal (3, 4, 5) and SSc (10, 11, 12) fibroblasts



3.3.4.2 Collagen gel contraction assays

Gel contraction assays showed significantly greater contraction of the collagen gel matrix in SSc versus normal fibroblasts. Mean gel weight in SSc fibroblasts at 48 hours was 118 mg (SD 17 mg) versus 213 mg (SD 24 mg) in normal fibroblasts (p=<0.0001). This is in keeping with previous findings (Shi-wen et al., 2010, Shi-Wen et al., 2004, Aden et al., 2010).

In normal fibroblasts, FXIII significantly promoted collagen gel contraction. Normal fibroblasts supplemented with FXIII had a mean gel weight of 143 mg (SD 18 mg) at 48 hours, versus 213 mg without factor XIII (p=<0.0005). However, there was no promotion of contraction in SSc fibroblasts when factor XIII was supplemented. SSc fibroblast gel matrixes with FXIII had a mean weight of 117 mg (SD 14 mg) at 48 hours compared to the un-supplemented mean gel weight of 118 mg (SD 17 mg) (p=0.92).

Figure 35 Photographs of gel contraction assays showing normal fibroblasts (NF) and SSc fibroblasts (SScF) in collagen gel matrixes with and without factor XIII at 48 hours

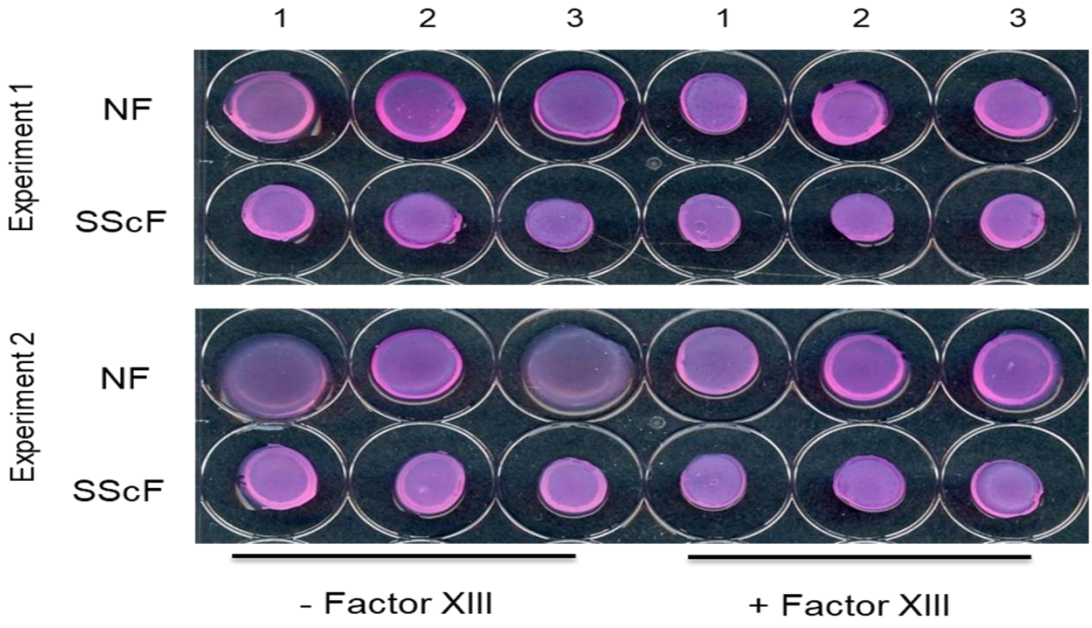
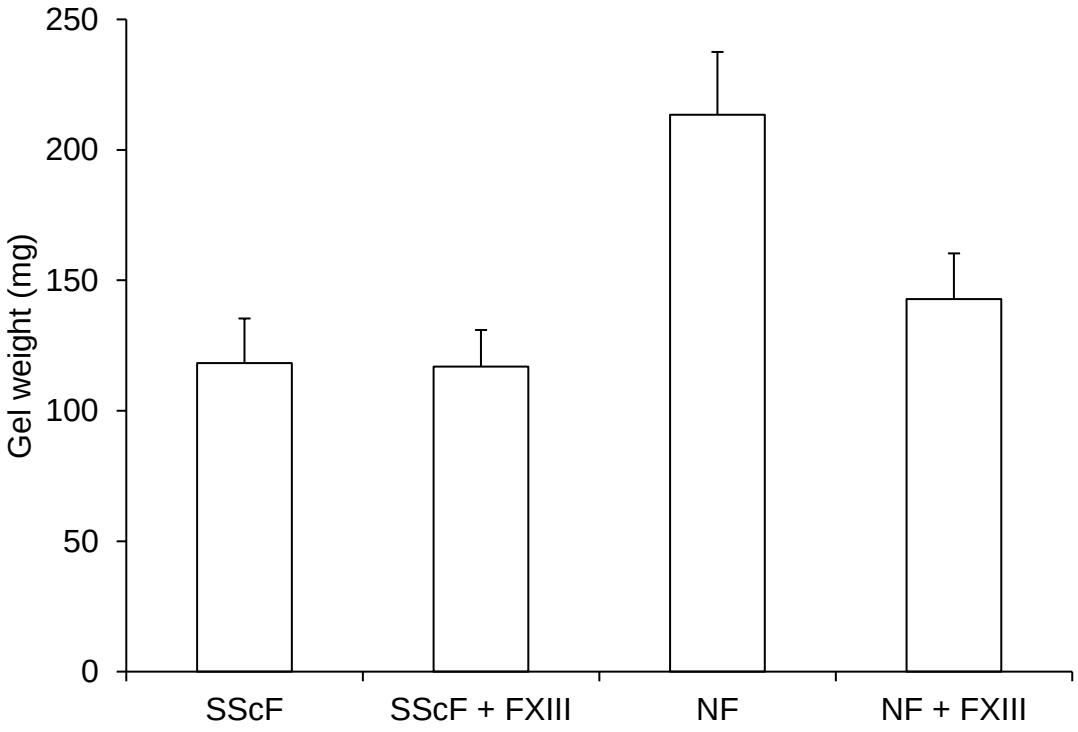


Figure 36 Mean gel weights for NF and SScF collagen gel matrixes with and without factor XIII at 48 hours

Bars represent mean gel weight and error bars represent standard deviation.



3.3.4.3 Fibroblast migration assays

The scratch migration assay without addition of factor XIII showed a greater migration of SSc fibroblasts compared to healthy fibroblasts. At 48 hours there was a mean gap size of 35.8% of the original in the SSc fibroblasts (SD 8.2%) compared to 68.0% (SD 7.9%) in the normal fibroblasts. This was statistically significant (p=<0.0001).

With the addition of FXIII, normal fibroblasts showed significantly increased migration similar to that seen in SSc fibroblasts. Mean gap size in normal fibroblasts plus FXIII was 41.4% at 48 hours (SD 9.5%) (p=0.01). However, there was no significant effect on SSc fibroblasts when FXIII was added (mean gap size 34.1% at 48 hours (SD 5.5%) (p=0.7)).

Figure 37 **Normal (NF) and SSc fibroblasts (SScF) at 0-hours and at 48-hours +/- factor XIII**

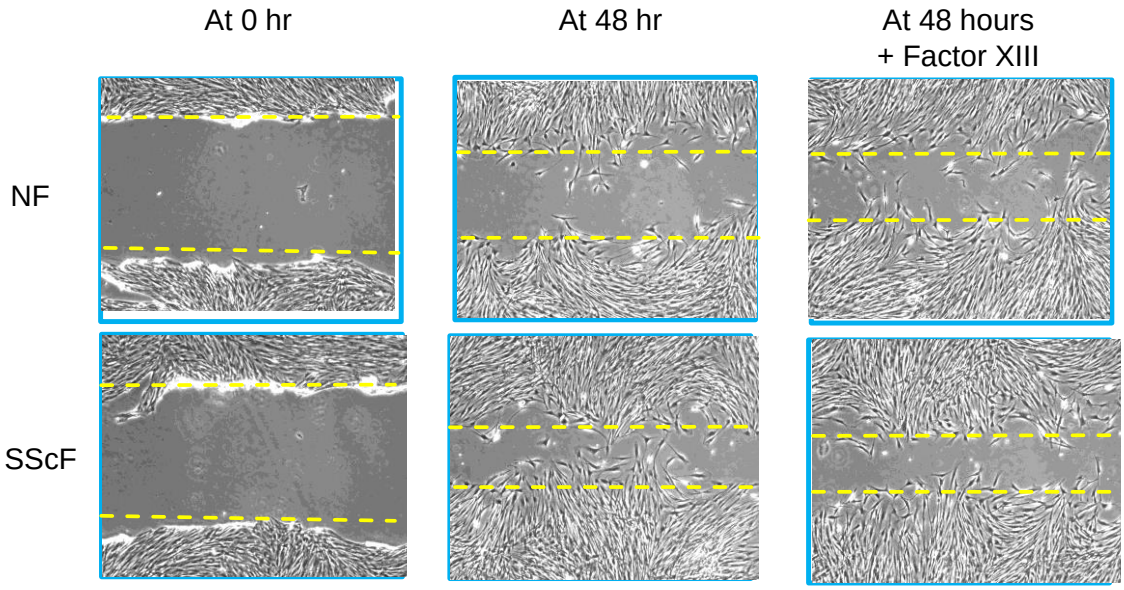
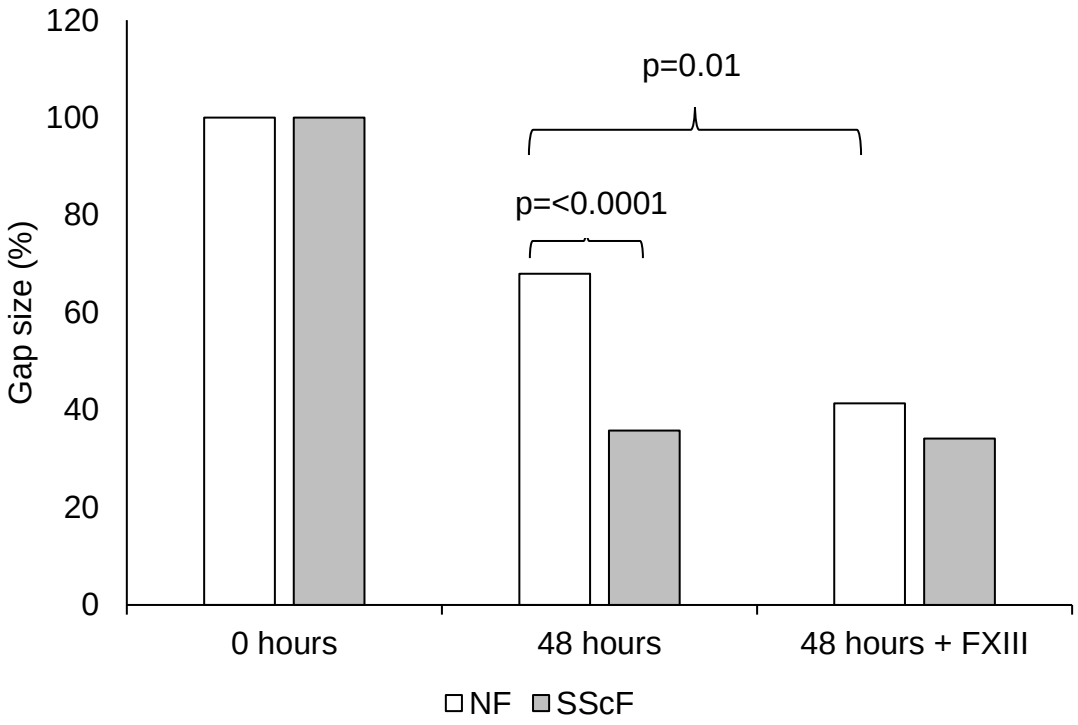


Figure 38 Graphical representation of percentage change in scratch wound migration assay gap sizes



3.2.4 Primary endpoints

Sixteen patients were included for the analysis of Raynaud's condition score due to significant missing data for one patient. Seventeen patients were included for the analysis of modified Rodnan skin score. Table 3.19 presents mean at baseline and 24 weeks, and mean change from baseline to 24 weeks by treatment group. The mean difference between arms is also computed.

Table 3.19 Primary endpoint results

	Baseline Mean (SD)		**24 weeks Mean (SD)**		**Change at 24 weeks Mean (95% CI)**		**Adjusted difference between arms Mean (95% CI)**
	Factor XIII (N=12)*	Placebo (N=6)	Factor XIII (N=11)	Placebo (N=6)	Factor XIII (N=11)	Placebo (N=6)	
Raynaud's condition score	3.55 (2.02)	4.33 (2.16)	2.27 (1.74)	3.5 (3.56)	-1.2 (-2.26, -0.14)	-0.83 (-3.44, 1.77)	0.49 (-2.68, 1.69)
Modified Rodnan skin score (mRSS)	10.67 (6.46)	8.5 (4.46)	9.45 (5.94)	7 (3.95)	-1.27 (-2.81, 0.26)	-1.5 (-2.95, -0.05)	0.6 (-1.4, 2.6)

*For Raynaud's condition score, the sample size at baseline for FXIII group is N=11.

The regression analysis for Raynaud's condition score suggested that there was a slightly greater reduction in the score for patients who received factor XIII compared to placebo (adjusted mean difference between arms was -0.49, 95% CI: -2.68 to 1.69). However, this difference was not statistically significant (p=0.63).

The regression analysis adjusted for baseline score in mRSS suggests that the treatment is not associated with a significant reduction in score (mean adjusted difference between arms 0.6, 95% CI: -1.4 to 2.6) with a p-value of 0.53.

The response rate for patients meeting the MCID (defined as a 20% change and at least 4 integer unit change in mRSS) was 16.7% (2/12) and 0% for the FXIII and placebo group, respectively.

Figure 39 presents the individual mRSS scores at baseline and 24 weeks for the FXIII and placebo groups. Figure 40 shows the Raynaud's condition score at the same time points for the two comparison groups.

Figure 39 Modified Rodnan skin score at baseline and 24 weeks for FXIII and placebo groups

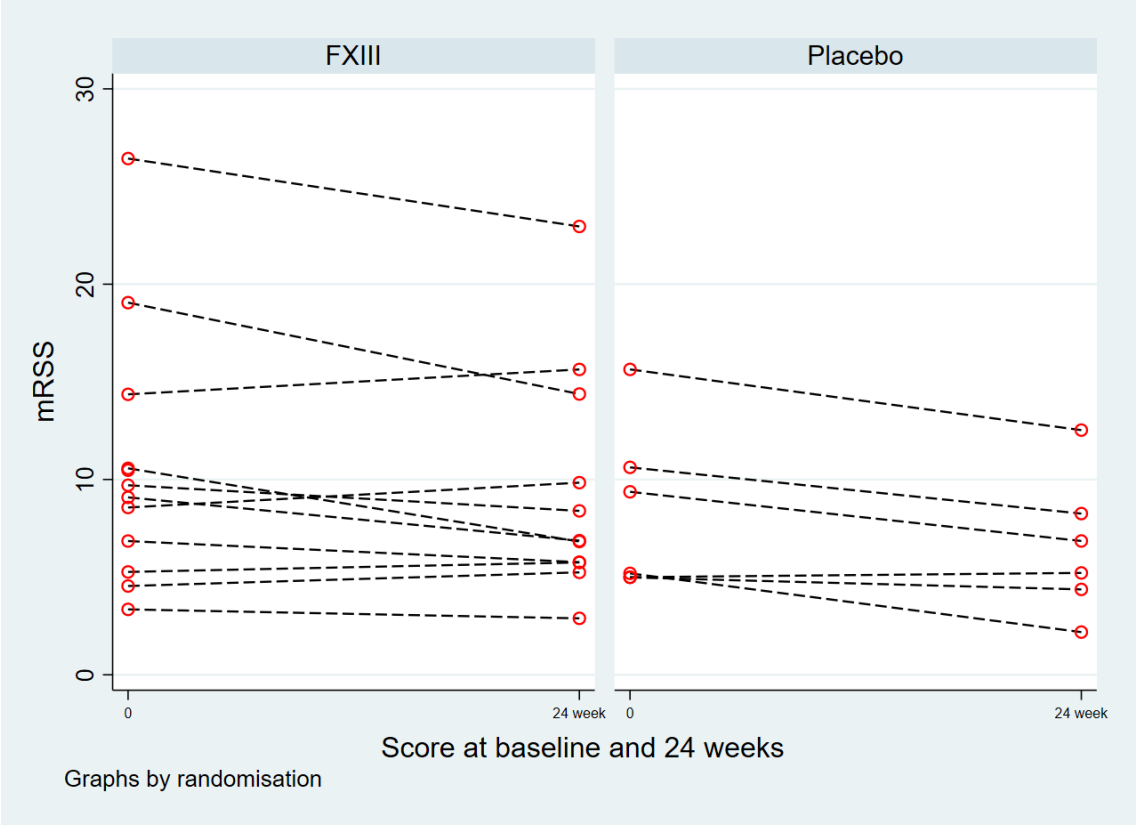
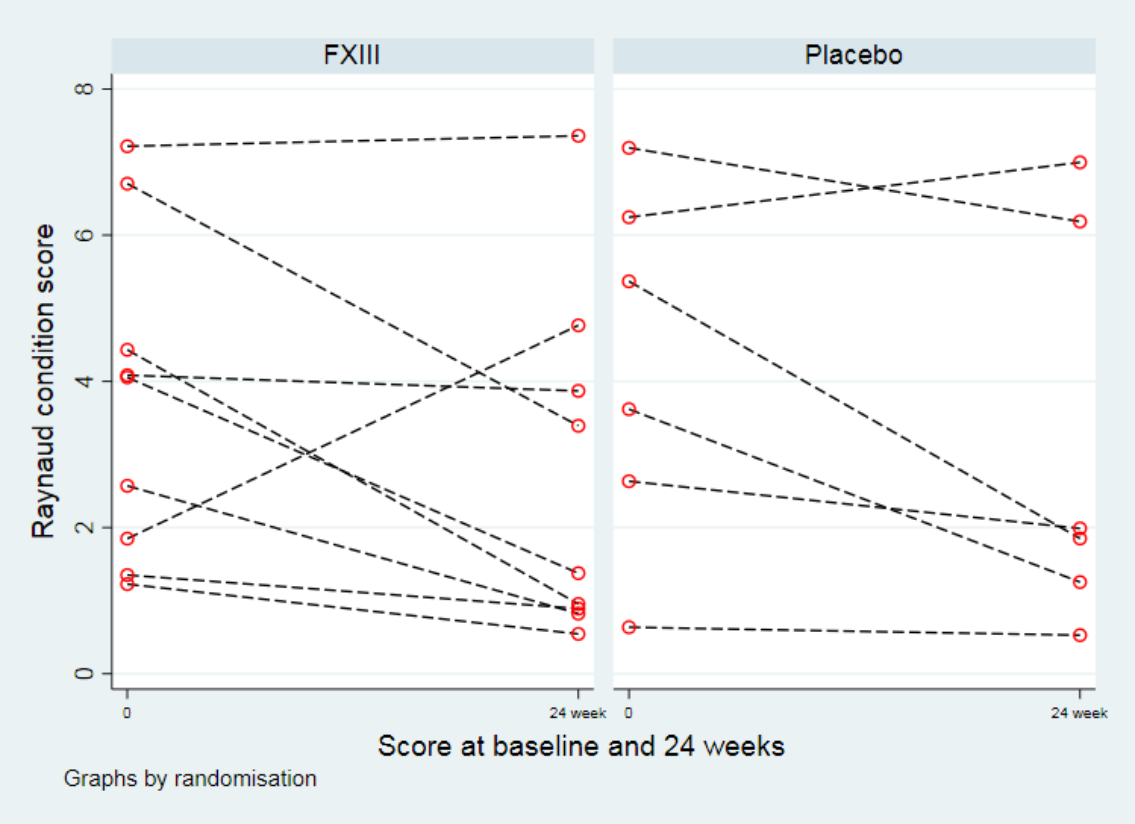


Figure 40 Raynaud's condition score at baseline and 24 weeks for FXIII and placebo groups



3.3.5 Coagulation studies

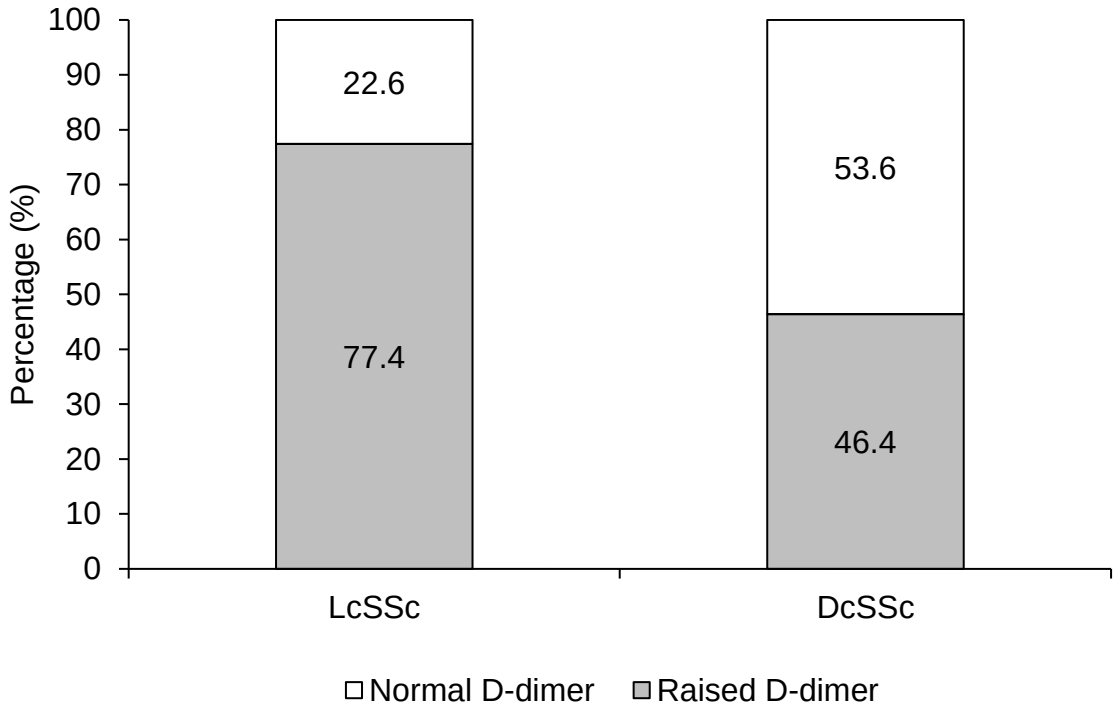
Of the 60 patients assessed, 83.3% were female. Age range was 20-82 years, with a mean age of 60.4 years. 51.7% had limited SSc. The duration of SSc ranged from 0.62 to 50.6 years, with a mean duration of 14 years.

Table 3.20 Demographics of participants in coagulation studies (n=60)

Age (years) (mean, SD)	60.4 (13.5)			
Gender (number (%))	Female	50 (83.3)	Male	10 (16.7)
Subtype (number (%))	LcSSc	31 (51.7)	DcSSc	29 (48.5)
Disease duration (years) (mean (SD))	14 (11.2)			
Digital ulceration (number (%))	Active	5 (8.3)	Historical or active	19 (31.7)
Overlap condition present (number (%))	15 (25)			

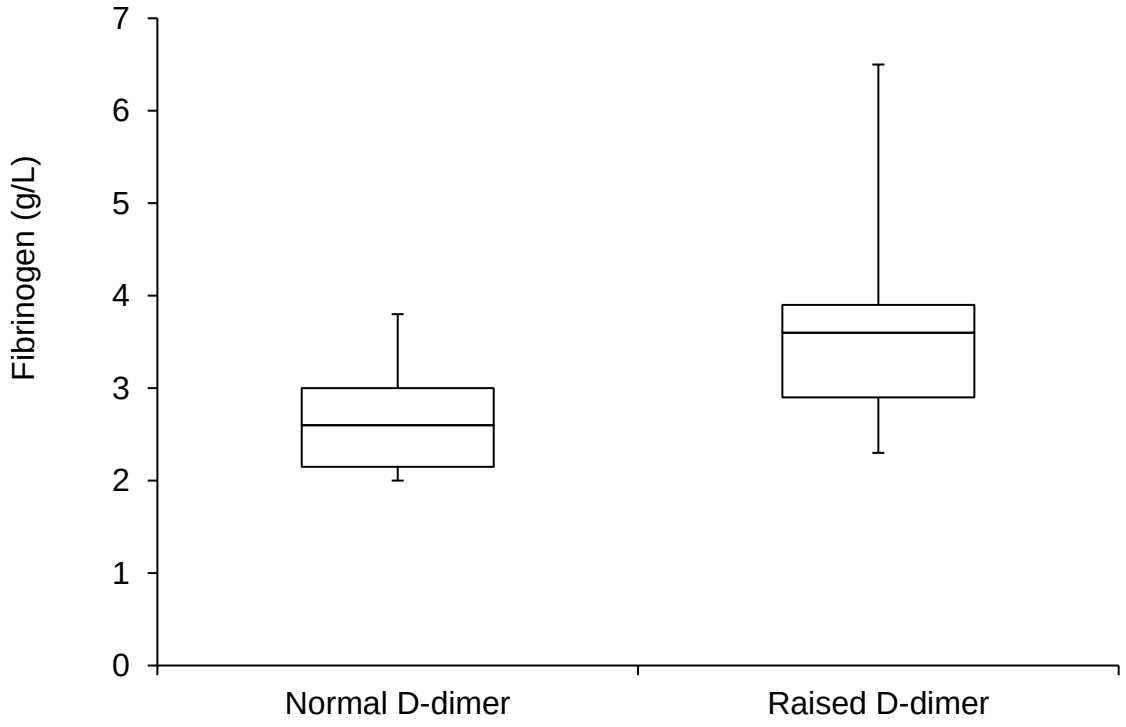
Analysis of coagulation profiles showed that D-dimer level is commonly elevated outside the normal range in systemic sclerosis patients. Overall, 61.9% of patients with SSc had a raised D-dimer. D-dimer was more commonly raised in lcSSc compared to dcSSc (p=0.02).

Figure 41 Distribution of D-dimer results elevated outside the normal range in lcSSc and dcSSc



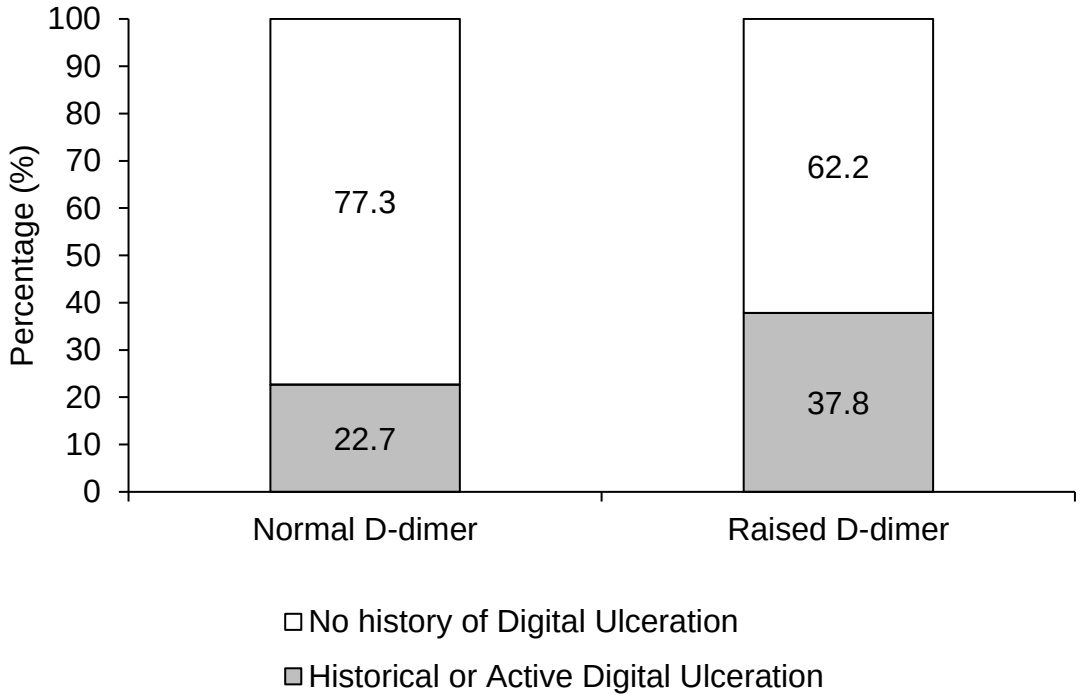
Fibrinogen was not raised in systemic sclerosis: 13.6% of patients assayed had a fibrinogen results higher than the reference range. However higher fibrinogen levels were associated with a raised D-dimer (OR 6.5, p=0.001). The mean fibrinogen for the normal D-dimer group was 2.7 g/L but for the raised D-dimer group it was 3.5 g/L.

Figure 42 Distribution of fibrinogen levels in SSc patients with normal and raised D-dimer. Boxes represent median and interquartile range (IQR), whiskers represent maximum and minimum values.



Raised D-dimer did not correlate with active or historical digital ulceration (p=0.264).

Figure 43 Percentage of SSc patients with a raised D-dimer and historical or active digital ulceration



4 Discussion

In this thesis I have presented the results of two inter-linked clinical trials assessing the effects of administering coagulation factor XIII to patients with systemic sclerosis. The inter-linked trial design has allowed me to gather previously unreported data showing the pharmacokinetics of factor XIII in this patient group. The trials recruited from both limited and diffuse disease subsets, and unlike most clinical trials in systemic sclerosis did not specify disease duration. This provides more generalisable data compared to most clinical trials in this disease. Close monitoring during the trial, coupled with extensive data collection for secondary endpoints, has generated valuable information regarding the safety, tolerability and efficacy of factor XIII in systemic sclerosis.

4.1 Safety and tolerability

There were low numbers of AEs in both clinical trials, likely reflecting the chronic duration of SSc in the trial participants and that background immunosuppressive therapy was permitted before and during the trial.

Although there were numerically more AEs in the treatment group compared to placebo (75 vs. 25) it should be noted that the allocation ratio of FXIII to placebo was 2:1, therefore the treatment group was double the size. Overall, there were 6.25 AEs per patient in the FXIII group and 4.17 in the placebo group.

Of the 100 reported AEs, the 12 events “digital ulcer” can be considered related to the underlying SSc. Due to the low numbers of patients experiencing digital ulcers in the trial it is not possible to draw conclusions on whether the new events of digital ulceration were related to study drug.

The AEs “headache” and “diarrhoea” occurred significantly more in the FXIII group (6 vs. 0 and 9 vs. 2 respectively), which was expected as these are documented adverse effects related to FXIII treatment. No patient withdrew due to AEs.

On review of the other safety parameters, such as vital signs and electrocardiography, there were no concerning findings relating to any of these.

From these data we can conclude that FXIII treatment in systemic sclerosis is safe and well tolerated.

4.2 Primary endpoints

The baseline mean mRSS in the FXIII group was 10.67 (SD 6.46) compared to the slightly lower 8.5 (SD 4.46) in the placebo group. This would be described as mild skin thickening, which was expected as the study population was not enriched for early-progressive or severe systemic sclerosis. Studies have demonstrated that skin thickening usually reaches a peak and then begins to plateau or decline within the first 2 years. Serial skin score assessments in patients with a disease duration of greater than 2 years therefore often show a gradual decline (Medsger, 2003).

At 24 weeks the mean mRSS in the FXIII group was 9.45 (SD 5.94) and in the placebo group 7 (SD 3.95). Neither of these changes in mRSS were statistically significant and were also not considered clinically significant.

The MCID in systemic sclerosis has been estimated at 3-4 units of change on the mRSS in lcSSc and 5 units in dcSSc (Khanna et al., 2019). The mean change in mRSS seen during the trial did not meet the MCID in either the FXIII or the placebo groups. Only two patients experienced a change in mRSS meeting the MCID criteria in the FXIII group, and none met the criteria in the placebo group.

The baseline Raynaud's condition score was higher in the placebo group, 4.33 (SD 2.16) compared to 3.55 (SD 2.02). There was a numerically greater reduction in RCS in the FXIII group compared to placebo, but this was not statistically significant (mean change of -1.2 (95% CI -2.26 to -0.14) versus -0.83 (95% CI -3.44 to 1.77). The MCID for the RCS has been estimated as a change of 1.4-1.5 on a 10-point Likert scale VAS score (Khanna et al., 2010) therefore the changes seen were also not considered to be clinically significant. The small numerical

improvement in the RCS seen in the FXIII group could suggest that further work is needed to establish whether FXIII treatment truly influences the severity of Raynaud's phenomenon in SSc.

As discussed above (section 1.3), the RCS has limitations as an outcome measure for the assessment of severity of Raynaud's phenomenon in clinical trials in SSc.

Furthermore, treatment with vasodilators such as CCB, angiotensin receptor blockers (ARBs), PDE5 inhibitors, and prostacyclin analogues was permitted throughout the trials. These medications are likely to have affected the results of the Raynaud's condition score. Although not study specified, the study participants on ARBs, CCBs and PDE5 inhibitors were established on these before screening and continued them for the duration of the trial. Dose adjustments were made during the study, which may have affected the severity of Raynaud's phenomenon. The medication group with the biggest influence on Raynaud's phenomenon is likely to have been prostacyclin analogues. The prostacyclin analogue used in our centre is intravenous iloprost. This is commonly used on a seasonal basis, with patients receiving a planned infusion in the middle of winter. Patients who received a planned iloprost infusion as part of their usual care did not meet the secondary endpoint for digital ulcer worsening. Consideration was given to the possible confounding effect of vasodilators during the study design. Vasodilatory medications are commonly used in the standard care of systemic sclerosis, and it was not felt to be ethically appropriate to prohibit their use for the duration of the studies.

4.3 Secondary endpoints

The background and validation of the PROs used to assess some of the secondary endpoints are discussed in section 2.16.3.

4.3.1 Pulmonary function

There was a greater reduction in the percentage forced vital capacity in the placebo group compared to the FXIII group at 24 weeks, with a mean reduction of -2.45 % (95% CI -10.52 to 5.62%) compared to -1.25 % (95% CI -7.34 to 4.83 %). However when the DLCO was assessed, there was a greater reduction in the FXIII group compared to placebo. The mean change in DLCO at 24 weeks was -4.05 % (95% CI -9.17 to 1.07%) in the FXIII group and -1.47 % (95% CI -9.05 to 6.11 %) in the placebo group. Worsening of either the FVC or the DLCO is associated with decreased pulmonary function and worsening symptoms in SSc-related lung disease. However, these small changes were not considered clinically relevant and there was also no statistically significant difference between the groups.

4.3.2 Digital ulceration

Several measures were used to assess the extent of digital ulceration during the Sclero XIII studies. The digital ulcer count was used to assess the number of new ulcers which developed during the treatment period, and therefore whether the therapy might be associated with the development of fewer ulcers than in the placebo group. Physician questioning gathered data pertaining to digital ulcer worsening, indicated by requiring additional therapies or surgical intervention. Pain caused by digital ulcers was assessed using the physician global VAS.

Three patients in the FXIII group experienced digital ulcers during the trial. No patients in the placebo group experienced ulcers. The sample size and lack of placebo comparator make any analysis difficult. Two patients receiving FXIII saw an improvement in the number of digital ulcers suffered, while one saw a worsening. The mean change in the ulcer count over 24 weeks was -0.1 in the FXIII group (95% CI -0.51 to 0.31) but these data are insufficient to draw any conclusions regarding the effect of FXIII on digital ulceration.

Enriching future trials by selecting patients with significant digital vasculopathy +/- digital ulceration would provide useful additional data. This technique has been

applied successfully in other trials, for example the phase 2 randomised double-blind placebo-controlled trial comparing bosentan with placebo required patients to have at least one active ulcer for inclusion (Matucci-Cerinic et al., 2011).

4.3.3 Hand function

Hand function was assessed using the Cochin Hand Function score (CHFS). There was a slight worsening in the hand function of the placebo group during the trial (mean change of 1.26 (95% CI -0.51 to 6.51)) though this was not statistically significant. Previous data suggest that a patient's CHFS will worsen over time, in keeping with the result seen in the placebo group (Nguyen et al., 2016). There was a small and not statistically significant numerical improvement in CHFS in the FXIII group (mean change of -3.64 (95% CI -6.03 to -1.25)).

Raynaud's phenomenon and digital ulceration cause significant pain and functional impairment in the hands. Therefore, as above, it would be interesting to reconsider the role of the CHFS as a PRO in future trials of FXIII in SSc, if such trials were enriched for patients with significant vasculopathy.

4.4 Laboratory studies

4.4.1 Routine laboratory studies

It was reassuring to see that there was no significant change in any of the routine blood monitoring parameters that were measured during the study. This supported the hypothesis that FXIII administration is safe in systemic sclerosis.

4.4.2 Factor XIII levels

Factor XIII levels behaved as expected during the studies. My data from healthy controls and SSc patients suggest that in both populations endogenous factor XIII level is within the normal range. There was no significant difference in FXIII level between the two populations. These results support previously published data

showing that endogenous factor XIII levels are normal in the systemic sclerosis population (Marzano et al., 2000).

There was little variability in endogenous FXIII level in either the healthy population or the SSc (placebo) group. The adjusted mean change in FXIII level in the healthy group was -0.33 and in the placebo group of SSc patients it was -5.05, a difference which was not statistically significant (p=0.53).

During the PK phase, I demonstrated a rapid rise in serum FXIII level following administration of purified FXIII concentrate. The mean time for levels to fall back to their endogenous level was 16.6 days. These data were used in calculating the dosing regimen for the treatment phase. The dosing regimen aimed to balance patient wellbeing against clinical need. Fortnightly trial visits were time-consuming for patients and more frequent visits would likely have hampered recruitment.

The treatment phase data suggest that by using fortnightly dosing we were able to maintain patients' FXIII levels above their baseline for the majority of the study: for patients receiving FXIII 78.1% of pre-dose FXIII levels were higher than the patient's endogenous level (either screening or baseline).

4.4.3 Candidate biomarkers

4.4.3.1 COMP

Cartilage oligomeric matrix protein (COMP) is an extracellular glycoprotein found in tendon and cartilage (Hedbom et al., 1992, DiCesare et al., 1994). It is produced by fibroblasts (Dodge et al., 1998). It has a role in vascular endothelial structure as well as collagen synthesis, and has binding sites for various types of collagen (Riessen et al., 2001, Rosenberg et al., 1998, Holden et al., 2001, Halasz et al., 2007).

Serum COMP has been shown to be elevated in systemic sclerosis, with levels correlating with the extent of skin fibrosis, and changing over time as the extent of

skin fibrosis changes (Hesselstrand et al., 2008). In SSc where interstitial lung disease is present, serum COMP may be a marker for mortality as well as morbidity. High serum COMP in early SSc correlates with an increased risk of mortality overall (Hesselstrand et al., 2012). COMP is found in excess in skin samples from patients with systemic sclerosis compared to normal skin and is also present at higher levels in systemic sclerosis fibroblasts compared to normal fibroblasts (Farina et al., 2006, Yamamoto et al., 2007).

My data are in keeping with previous publications, showing a higher serum COMP level in SSc patients compared to healthy controls (p=0.01). There was no change in COMP level in either the FXIII or placebo group during the trial. Since there was also no significant change in skin fibrosis in either group, this finding was expected.

4.4.3.2 sCD40L

Soluble cluster of differentiation 40 ligand (sCD40L) is the soluble ligand for the cluster of differentiation 40 (CD40) receptor. CD40 is a transmembrane receptor of the TNF receptor family (Croft et al., 2013). It is constitutively expressed on B-lymphocytes and is also expressed on monocytes and dendritic cells. It has roles in immune cell survival, cytokine release and B-cell activation (Clark and Ledbetter, 1986, Stout and Suttles, 1996). CD40 is expressed on a range of non-immune cell types as well, including fibroblasts and vascular endothelial cells. CD40 signalling in these cell types may play a role in endothelial cell activation and expression of adhesion proteins such as E-selectin (Fries et al., 1995, Hollenbaugh et al., 1995). The expression of CD40 by umbilical vein endothelial cells is upregulated by TNFα and IL-1, providing supporting evidence for a role for CD40 signalling within known inflammatory signalling pathways (Karmann et al., 1995).

CD40 ligand (also known as CD154) exists as both a soluble and membrane bound protein. The membrane-bound protein is expressed on the surface of activated T-cells and is involved in B-cell-T-cell interactions (Hollenbaugh et al., 1992, Noelle et al., 1992). The soluble form (sCD40L) is a truncated form of the

membrane-bound ligand, and is produced by the action of MMPs on said ligand (Yacoub et al., 2013, Choi et al., 2010). Binding of soluble CD40L to CD40 leads to the upregulation of the adhesion proteins E-selectin, vascular cell adhesion protein 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1) on endothelial cell membranes. Binding also triggers endothelial cells to release the cytokines interleukin-8 and monocyte chemoattractant protein 1 (MCP-1). This leads to the recruitment of other cellular components of the inflammatory response (Henn et al., 1998). In addition to its effect on adhesion proteins via CD40 signalling, sCD40L also binds various integrins directly (Michel et al., 2017). The interaction between CD40 ligand and fibroblast-expressed CD40 contributes to fibroblast proliferation, the expression of adhesion proteins on fibroblast surfaces and fibroblast-T-cell interactions (Loubaki et al., 2010, Schonbeck and Libby, 2001, Yellin et al., 1995). CD40 and its membrane-bound and soluble ligands therefore play a role in immune cell signalling, as well as stimulating endothelial behaviours that recruit immune cells from the blood. Furthermore, CD40 signalling activates the endothelium and recruits and amplifies fibroblasts. This signalling pathway may be part of the link between the immune response and tissue repair pathways.

Dysregulated sCD40L has been implicated in the pathogenesis of multiple autoimmune disease including rheumatoid arthritis, inflammatory bowel disease and primary Sjögren's syndrome (Danese et al., 2003, Harigai et al., 1999, Goules et al., 2006) In a mouse model of Sjögren's syndrome an anti-sCD40L injection prevents the development of symptoms (Mahmoud et al., 2016). CD40L signalling has also been shown to be important in other fibrotic autoimmune diseases including pulmonary fibrosis (Zhang-Hoover et al., 2001).

Serum sCD40L levels are raised in systemic sclerosis patients. This has been found to correlate with a raised c-reactive protein (CRP), in keeping with the concept that sCD40L is part of the inflammatory response. This is further supported by the finding that sCD40L levels are particularly raised in patients with early SSc, which is known to have an inflammatory component (Komura et al.,

2004). Other studies have linked raised sCD40L in systemic sclerosis with vascular manifestations (Allanore et al., 2005).

My data show a numeric but not statistically significant difference in sCD40L levels in SSc patients compared to healthy controls, with levels being higher in SSc ($p=0.06$). sCD40L signalling may play an important role in inflammatory signalling in early SSc. The Sclero XIII trials did not select patients based on disease duration and all patients in the trials had a disease duration of greater than 2 years. These patients would therefore not be classified as having early disease, and the inflammatory phase of their SSc pathogenesis had likely already passed. There is no clear link between CD40 signalling and the mechanism of action of FXIII. This fits with my findings that FXIII treatment did not affect sCD40L levels: there was no significant difference in levels between the treatment and placebo groups.

4.4.3.3 Fibronectin

Fibronectin (cold insoluble globulin) is a glycoprotein found throughout the extracellular matrix, particularly in highly active tissues such as embryonic or injured tissues. It binds to surface integrins and collagens (Magnusson and Mosher, 1998). Activated factor XIII stimulates fibronectin to cross link to fibrin, an important step in the clot stabilisation process during coagulation (Mosher, 1975, Aeschlimann and Paulsson, 1994, Kamykowski et al., 1981). This step has a downstream effect on fibroblast behaviour: it is essential for the normal migration of fibroblasts into the fibrin clot. The action of FXIII on fibronectin thereby facilitates the subsequent actions of fibroblasts in wound healing (Grinnell et al., 1980, Corbett et al., 1996, Knox et al., 1986). Furthermore fibronectin cross-links to collagen and may play a role in collagen deposition (Mosher and Schad, 1979). Overall fibronectin is an important protein in early wound healing.

It is therefore not surprising that SSc fibroblasts produce more fibronectin than their healthy counterparts (Jing et al., 2015, Fleischmajer et al., 1981). The excess

fibronectin is deposited particularly in the dermis and the vascular endothelium (Chen et al., 1985).

My data show a moderately reduced fibronectin level in the serum of SSc patients compared to healthy volunteers. This might be because the excess fibronectin produced by SSc fibroblasts is not a free serum protein but is rapidly incorporated into the extracellular matrix where it is integrin-bound. A difference in serum fibronectin levels between SSc patients and healthy controls has not been published previously.

There was a greater reduction in serum fibronectin in the FXIII groups compared to the placebo group during the trial, but neither change was statistically significant. This suggests that if the concentration or activity of fibronectin is altered *in vivo* by FXIII, then this change occurs in the extracellular matrix-bound fibronectin rather than in the serum. This hypothesis could be further assessed by immunohistochemical staining of skin samples for fibronectin in patients who had received factor XIII compared to those who had not.

4.4.3.4 Interleukin 6

Interleukin-6 (IL-6) is one of a group of cytokines involved in cell-cell signalling via the 130 kilodalton glycoprotein receptor (gp130). IL-6 signalling also requires the presence of the IL-6 receptor (Rose-John, 2020). In normal physiology IL-6 signalling is involved in body weight regulation and liver regeneration (Wallenius et al., 2002, Cressman et al., 1996). IL-6 signalling is also involved in inflammatory processes. High serum IL-6 levels are found in sepsis and autoimmune inflammatory conditions (Waage et al., 1989, Tanaka et al., 2014). Tocilizumab is a monoclonal antibody which inhibits the binding of IL-6 to the IL-6 receptor and has been licensed for therapeutic use in autoimmune diseases, for example rheumatoid arthritis (Kerschbaumer et al., 2020).

IL-6 is elevated in systemic sclerosis serum and produced in excess by SSc peripheral blood mononuclear cells (Hasegawa et al., 1999, Needleman et al.,

1992, Hasegawa et al., 2011). High IL-6 levels have been found in SSc, particularly in patients with early dcSSc. Levels also correlate with the inflammatory markers CRP and erythrocyte sedimentation rate (ESR). These findings suggest that IL-6 plays a role in the early inflammatory phase of dcSSc (Hasegawa et al., 1998, Khan et al., 2012). The degree of elevation of IL-6 has been shown to corelate with the extent of skin fibrosis, lending further support to the association with dcSSc (Sato et al., 2001).

The use of tocilizumab has also been explored in the treatment of systemic sclerosis. Clinical trial data has led to tocilizumab being licensed for use in the treatment of SSc in the USA and Europe (Khanna et al., 2016b, Khanna et al., 2020b). Data from SSc fibroblasts collected during the phase II trial have shown that IL-6 signalling blockade interrupts TGFβ signalling in SSc fibroblasts, and as discussed above TGFβ signalling plays a key role in the pathogenesis of SSc (Denton et al., 2018).

Patients enrolled in the Sclero XIII clinical trials were from both lcSSc and dcSSc subsets. The trials were not selective for patients with early SSc and in fact all participants had a disease duration greater than 2 years. My data showed no significant difference in IL-6 levels when SSc patients were compared to healthy controls, and IL-6 level was not modulated by the administration of FXIII. My data therefore support previous publications suggesting the role of IL-6 in SSc is predominantly in early disease, particularly early dcSSc.

4.4.3.5 Thrombospondin-1

Thrombospondin-1 (TSP-1) is a glycoprotein found in the extracellular matrix. It does not have a structural role but acts as a matricellular protein: a protein which binds other proteins and cell surface receptors in the ECM, and modulates the synthesis and activity of the ECM (Bornstein, 1995). TSP-1 has a broad and complex role in matricellular cell signalling.

There are many functions of TSP-1 that are relevant to the pathogenesis of systemic sclerosis, particularly its anti-angiogenic properties. This is discussed further in section 1.1.4 above. TSP-1 synthesis is induced by platelet derived growth factor, which is upregulated in systemic sclerosis (Trojanowska, 2008). As expected, therefore, TSP-1 levels are also raised in systemic sclerosis plasma (Macko et al., 2002).

TSP-1 activates TGFβ, as demonstrated by the low levels of TGFβ expression in TSP-1 null mice, and the re-accumulation of TGFβ in these mice once treated with a TSP-1 derived peptide (Crawford et al., 1998). Levels of TGFβ are elevated in the serum of SSc patients (Dantas et al., 2016). TGFβ plays an extensive role in the pathogenesis of systemic sclerosis, with functions demonstrated in fibroblast activation and the excess production of ECM components (van Caam et al., 2018, Lafyatis, 2014). It also upregulates COMP, for which a role in systemic sclerosis is discussed above, and further upregulates TSP-1 expression in an autocrine loop (Farina et al., 2009, Farina et al., 2010). TSP-1 has been suggested as possible biomarker to predict the prognosis of systemic sclerosis, due to its correlation with mRSS (Farina et al., 2010).

In keeping with previous work, my data show a statistically significant elevation in TSP-1 when SSc and normal serum samples are compared ($p=0.02$). The TSP-1 levels in the placebo group remained relatively static during the 24-week trial, with no significant difference between the baseline and week 24 levels ($p=0.68$). However, there was a significant decrease in the TSP-1 levels in those patients treated with FXIII over the 24 weeks ($p=0.02$). These data therefore support my hypothesis that TSP-1 is modulated by administration of FXIII in vivo.

4.4.3.6 Transforming growth factor beta

Although TGFβ plays a key role in the pathogenesis of systemic sclerosis, as discussed in sections 1.1.4 and 4.4.3, TGFβ was not selected as a candidate biomarker for analysis in this study. TGFβ has not been used as a biomarker in

other SSc trials because it is challenging to measure in a reliable manner. Commercially available ELISA kits measure total TGFβ which is not always representative of levels of active TGFβ, due to tissue sequestration of the active form of TGFβ. Previous studies have reported that levels of serum total TGFβ are unchanged in diffuse SSc patients compared to healthy controls, and that serum active TGFβ levels are in fact reduced in diffuse SSc compared to healthy controls. This suggests that that the tissue-bound form (perhaps in active SSc skin) makes up a significant component of the active TGFβ truly present in total (Dziadzio et al 2005).

4.4.3.7 Tumour necrosis factor alpha

TNFα (TNF, cachexin) is a cytokine produced by macrophages. It is involved in cell-cell signalling during the immune response by binding the TNF receptors, and is important in the defence against microbial organisms (Brenner et al., 2015). There are two TNF receptors. The TNF receptor 2 (TNFR2) is expressed on lymphoid and regulatory T-cell membranes and its activation is important in wound healing (Gough and Myles, 2020, Walczak, 2011, Chen et al., 2013). TNFα exists as both a soluble and membrane bound protein (Horiuchi et al., 2010). Membrane-bound TNFα binds TNFR2 with greater affinity than the soluble form, therefore may be more relevant to wound healing than the soluble form (Grell et al., 1995).

Excess or dysregulated TNFα has been implicated in the pathogenesis of a wide range of autoimmune diseases; and TNFα inhibitors such as infliximab are used extensively to treat diseases such as rheumatoid and psoriatic arthritis (Radner and Aletaha, 2015, Gossec et al., 2020).

TNFα is upregulated in systemic sclerosis. TNFα levels are higher in supernatants from peripheral blood mononuclear cells, and in serum, from SSc patients, compared to controls (Dantas et al., 2018, Zhu et al., 2019, Kantor et al., 1992).

TNFα is implicated in abnormal fibrosis in SSc via its relationship with TGFβ signalling. TGFβ induces CTGF expression in normal fibroblasts, and in turn this

leads to fibroblast growth and collagen deposition (Grotendorst et al., 1996, Grotendorst, 1997, Frazier et al., 1996). As discussed above, TGFβ is upregulated in SSc, and as expected CTGF levels are also higher in SSc serum and skin samples compared to controls. This is related to both TGFβ induction of CTGF and constitutive over-expression of CTGF by SSc fibroblasts (Igarashi et al., 1995, Sato et al., 2000, Denton and Abraham, 2001, Abraham et al., 2000).

In normal fibroblasts, TNFα supresses TGFβ-induced expression of CTGF, but in SSc fibroblasts TNFα does not have this effect. The constitutive expression of CTGF by SSc fibroblasts is also not affected by TNFα levels. It appears that TNFα is produced in excess in SSc, but SSc fibroblasts are resistant to its function of moderating fibrosis through TGFβ and CTGF signalling (Abraham et al., 2000).

In keeping with previously published data, my data show significantly higher levels of TNFα in serum from SSc patients compared to healthy controls ($p=0.04$). During the trial, serum TNFα levels fell significantly in the group treated with FXIII ($p=0.02$). There was also an overall reduction in TNFα in the placebo group, but this was non-significant ($p=0.17$). The FXIII-treated group displayed an adjusted mean change from baseline of -0.84, almost double the change in TNFα levels seen in the placebo group. These results support my hypothesis that FXIII modulates key biomarkers in systemic sclerosis.

The mechanism of action by which FXIII might reduce TNFα levels is less clear than with other candidate biomarkers. Alongside TSP-1, TNFα may be an important link between coagulation and inflammation. The release of TNFα is stimulated by high levels of fibrin, produced when the coagulation system is activated (see section 4.6 below). It may be that factor XIII-related cross-linking of fibrin slows down the release of TNFα and therefore has downstream anti-inflammatory effects. This could be self-amplifying, as TNFα release stimulates further activation of the coagulation system via tissue factor induction (Napoleone et al., 1997, Bevilacqua et al., 1986).

4.4.3.8 Vascular endothelial growth factor

The vascular endothelial growth factor (VEGF) family of growth factors comprises five cystine-knot based glycoprotein growth factors. Four of these were assessed as part of this project: VEGF-A, -C, -D and PLGF. The VEGF growth factors exert their effects by binding the 3 VEGF receptors, a group of tyrosine kinase receptors expressed on the surfaces of endothelial and haematopoetic cells. These signalling pathways modulate the behaviour of endothelial cells, promoting their growth and suppressing cell death by apoptosis; as well as stimulating the release of CTGF and HB-EGF. These actions lead to downstream effects on angiogenesis, endothelial function, MMP activity, vasodilation, lymphangiogenesis and vasculogenesis (Ferrara et al., 2003, Gerber et al., 1998a, Gerber et al., 1998b).

VEGF-A is required for normal angiogenesis. In normal physiology, VEGF-A signalling is essential in embryological development (Carmeliet et al., 1996, Ferrara et al., 1996). In post-natal physiology, it is important in normal ovarian angiogenesis as part of the menstrual cycle, in the normal longitudinal growth of bones, and in wound healing (Phillips et al., 1990, Ferrara et al., 1998, Gerber et al., 1999, Chintalgattu et al., 2003).

Serum VEGF-A has been found to be higher in SSc patients compared to healthy controls, particularly in those patients with dcSSc, early SSc, more severe skin fibrosis, pulmonary hypertension and positive anti Scl-70 antibody (Distler et al., 2002, Choi et al., 2003, Papaioannou et al., 2009). VEGF-A expression is also raised in cultured SSc fibroblasts (Kajihara et al., 2013). This has been linked to the abnormal angiogenesis seen in SSc (Koch and Distler, 2007, Distler et al., 2004).

Dysregulated VEGF-A signalling plays a role in the development of fibrosis. In transgenic mice, VEGF-A overexpression has been linked to the development of skin fibrosis. This is due to the induction of collagen synthesis by dermal fibroblasts

under VEGF-A stimulation (Maurer et al., 2014). In humans with SSc, VEGF-A levels correlate with the extent of skin and lung fibrosis (Kikuchi et al., 1998)

As discussed above, TGF-β plays an important role in the pathogenesis of systemic sclerosis. Part of this role is via upregulation of VEGF-A. We know that the excessive VEGF-A expression in SSc patients' skin is independent of the physiological driver of VEGF-A expression: hypoxia inducible factor 1 (HIF-1). However, fibroblasts supplemented with TGFβ produce excess VEGF-A. Other cytokine drivers of excess VEGF-A expression in SSc are IL-1 and PDGF. These signalling process likely contribute to not only dysregulated angiogenesis, but also the fibrotic phenotype seen in SSc. In SSc fibroblasts where TGFβ signalling is blocked, the expression of α2(I) collagen is normal. (Kajihara et al., 2013, Kikuchi et al., 1998, Ihn et al., 2001, Distler et al., 2004).

My data did not show a significant difference in serum VEGF-A levels when SSc patients and healthy controls were compared. Furthermore, there was no significant change in serum VEGF-A level in either the FXIII or the placebo group during the trial. Since I would expect TGFβ levels to be modulated by FXIII administration (via the effects of FXIII on TSP-1) these findings suggest that alternative cytokines which increase VEGF-A expression in SSc are playing an important role in controlling VEGF-A levels in these patients (such as IL-1 and PDGF).

VEGF-C and -D are involved in angiogenesis and lymphangiogenesis via their actions on VEGF receptors 2 and 3 (Joukov et al., 1996, Makinen et al., 2001, Joukov et al., 1997, Achen et al., 1998, Stacker et al., 2014, Bower et al., 2017). However neither VEGF-C or -D are indispensable in embryonic development unlike VEGF-A (Haiko et al., 2008, Koch et al., 2009).

VEGF-C signalling is essential for a normal lymphangiogenic response to injury; and blocking the VEGF-C signalling pathway halts lymphangiogenesis (Kajiya et al., 2009, Baluk et al., 2005, Hagura et al., 2014). VEGF-C signalling also

contributes to the normal pumping function of lymph vessels (Breslin et al., 2007). Overexpression of VEGF-C in transgenic mice is associated with cutaneous lymph vessel hyperplasia (Jeltsch et al., 1997, Lohela et al., 2008). VEGF-C is induced by IL-1 and TNFα; and, as discussed above, TNFα is upregulated in SSc (Ristimaki et al., 1998).

VEGF-D signalling is also implicated in the lymphangiogenetic response to tissue injury and subsequent wound healing. Transgenic knockout mice deficient for VEGF-D display abnormal wound healing with insufficient lymphatic drainage (Paquet-Fifield et al., 2013).

Lymph vessels are important in wound healing because they drain fluid and immune cells generated as part of the immune response away from the area of injured tissue (Alitalo and Carmeliet, 2002). Abnormal lymph vessel structure is a feature of various inflammatory skin pathologies, for example psoriasis (Henno et al., 2009). Dermal lymphatic vessels are significantly reduced or even absent in patients with SSc. This reduction is greatest in those with late-stage disease and those with digital ulceration (Leu et al., 1999, Manetti et al., 2011, Akhmetshina et al., 2010). This might be due to abnormal lymphangiogenesis.

Following these findings, VEGF-C and -D have been investigated in SSc. Serum levels of VEGF-C have been shown to be raised in SSc, particularly in lcSSc (Chitale et al., 2008). Levels of the VEGF receptor 3 are also increased in SSc skin and this receptor preferentially binds VEGF-C (Mackiewicz et al., 2002, Joukov et al., 1996). VEGF-D levels are also raised in systemic sclerosis skin tissue though not in SSc patient serum. Higher serum VEGF-D correlates with digital ulceration and pulmonary arterial hypertension (Honda et al., 2010, Tabata et al., 2021, Kylhammar et al., 2018).

I found no significant difference in VEGF-C levels when SSc patients and healthy controls were compared. However, in patients treated with FXIII there was a statistically significant decrease in VEGF-C levels in the SSc + FXIII group

(p=0.04) which was not seen in the placebo group (p=0.91). VEGF-C levels were not raised unlike previous publications, this could be due to the small sample size or the presence of late stage SSc in my patient population. Given that I showed a significant decrease in serum TNFα following administration of FXIII, it is possible that a decline in the TNFα-induced VEGF-C might be related, but this would require further clarification.

For VEGF-D I did not find a significant difference in levels when SSc patients were compared with controls, and there was no significant change in these levels following administration with FXIII.

4.4.3.9 Other proteins of interest

4.4.3.9.1 Epidermal growth factor

Epidermal growth factor (EGF) is a transmembrane glycoprotein involved in wound healing. In normal circumstances, serum EGF levels are undetectably low. When tissue injury occurs these levels rapidly rise. The rise is due to a release of stored EGF from platelets. High serum EGF stimulates cell migration and reproduction, including fibroblasts (Bennett and Schultz, 1993). It also leads to angiogenesis *in vitro* (Schreiber et al., 1986). Addition of topical EGF to human skin wounds leading to accelerated healing (Brown et al., 1989, Brown et al., 1991).

EGF is linked to TGF-β signalling, over expression of which is implicated in SSc. EGF upregulates TGF-β in normal human fibroblasts but not in SSc fibroblasts, suggesting that some of the EGF-related signalling pathways in fibroblasts are already activated in SSc (Yamane et al., 2003). This is supported by data showing that the EGF receptor is upregulated in SSc fibroblasts (Tokiyama et al., 1990).

My data show overall higher levels of serum EGF in SSc patients compared to healthy controls, but the difference is not statistically significant (p=0.07). There was a statistically significant decrease in EGF levels from week 0 to week 24 in the SSc + FXIII group (p=0.01). There was a decrease in EGF levels in the placebo

group as well, but this was not statistically significant. The mean change from baseline was not significantly greater in the FXIII group compared to placebo at week 24 (p=0.38). Serum EGF may be downregulated by FXIII as part of its effect on TGF-β, but this is unlikely to play an important role in the mechanism of action of FXIII.

4.4.3.9.2 Transforming growth factor alpha

Transforming growth factor alpha (TGF-α) is a member of the epidermal growth factor family. It is expressed predominantly in the skin (Derynck, 1990). As with EGF, resting serum concentration of TGF-α is low and rises during wound healing, likely to be due to secretion by macrophages (Bennett and Schultz, 1993). Supplementation with TGF-α causes the proliferation of alveolar epithelial cells *in vitro*, and transgenic mice whose alveolar epithelial cells overexpress TGF-α show increased tissue fibrosis. (Ryan et al., 1994, Hardie et al., 1997). As with EGF, TGF-α supplementation also accelerates wound healing (Schultz et al., 1987).

Serum levels of TGF-α have previously been shown to be raised in SSc patients compared to healthy controls but this area has not been studied in detail (Lalovac et al., 2014).

Given the pro-fibrotic nature of TGF-α in alveolar epithelial cells, overexpression of TGF-α may contribute to the fibrotic phenotype in SSc. TGF-α signalling increases cell proliferation in breast cancer cell lines with malignant phenotypes *in vitro*, and this seems to amplify the effect of TGF-β in these cell lines as well (Franco et al., 2016). In SSC, TGF-α may contribute to pathogenesis by increasing the well-known pro-fibrotic effects of TGF-β.

I did not see a significant difference in TGF-α levels when SSc patients were compared to healthy controls (p=0.61). However, there was a significant decrease in TGF-α concentration during the 24-week trial in the SSc + FXIII group (p=0.04) which was not seen in the placebo group (p= 0.92). The decrease in TGF-α may

be due to the interaction between TGF-α and TGF-β signalling, and the hypothesised effect on TGF-β signalling by factor XIII discussed above.

4.5 Explanted dermal fibroblast studies

Expression of pro-fibrotic proteins is upregulated in SSc fibroblasts. Addition of factor XIII to these fibroblasts *in vitro* does not affect expression of these proteins. Collagen-1 and αSMA synthesis by fibroblasts is controlled via several signalling pathways. Protein synthesis is upregulated by TGFβ, IL-4, CTGF and endothelin-1 (Kahari et al., 1990, Postlethwaite et al., 1992, Frazier et al., 1996, Horstmeyer et al., 2005). Collagen-1 synthesis is downregulated by epidermal growth factor 5 (EGF-5), TNFα, interferon gamma and extracellular signal related kinase-1/2 (ERK 1/2) (Kahari et al., 1990, Laato et al., 1987, Reunanen et al., 2000).

The hypothesised effect of FXIII supplementation on collagen-1 synthesis would be via the effects of FXIII on TSP-1, the VEGF receptor (VEGF-R) and TGFβ. TSP-1 modulates the VEGF signalling pathway by inhibiting VEGF-R via SHP-1, CD36 and CD 47 (Ferrari et al., 2009, Chu et al., 2013, Schultz-Cherry et al., 1994). This has downstream effects on TGFβ signalling. Combined with its effects on MMPs, the interaction between dysregulated TSP-1, VEGF and TGFβ is likely to contribute to the disordered and inappropriate angiogenesis seen in SSc.

FXIII mediated suppression of TSP-1 should in principle also lead to downregulation of TGFβ and a reduction in its downstream effects on fibroblasts, namely collagen-1 and alpha SMA production. However TGFβ is such a key player in signalling pathways relating to cellular growth and proliferation that it is activated by many other proteins in addition to TSP-1, including proteases, integrins and fibroblast growth factor 2 (FGF-2) (Sato and Rifkin, 1989, Yu and Stamenkovic, 2000, Munger et al., 1999, Mu et al., 2002). It may be that inhibition of TSP-1 signalling on TGFβ is insufficient to reduce its effects on collagen-1 and αSMA synthesis. However, the role of TSP-1 related signalling on VEGF and TGFβ may

be more important in control of angiogenesis. This is supported by my finding that serum TSP-1 levels are decreased following supplementation with FXIII.

Collagen gel contraction assays provide an opportunity to study fibroblast activity in three dimensions. The 3D gel exerts mechanical tension on fibroblasts and stimulates behaviour more similar to that seen *in vivo* (Kolodney and Wysolmerski, 1992, Delvoye et al., 1991). The reorganization of collagen matrixes into a denser, smaller gel by fibroblasts is used as a surrogate measure of fibrosis and scar tissue formation *in vivo* (Mochitate et al., 1991, Halliday and Tomasek, 1995). In my studies SSc fibroblasts acted on the collagen matrix to form a dense contracted matrix, in excess of the effects on the collagen matrix seen in normal fibroblasts. Supplementation with FXIII does not affect the SSc fibroblast activity. However, it does promote increased collagen reorganisation in normal fibroblasts. As with many aspects of inter and intracellular signalling, a physiologic balance may be required. It is known that FXIII is involved in wound healing, and the moderate increase in collagen reorganisation seen when normal fibroblasts are supplemented with FXIII may represent the promotion of transformation from resting fibroblasts to active collagen-depositing myofibroblasts needed for normal wound healing.

The lack of effect on SSc fibroblasts when FXIII is supplemented could be because the activation step has already been made. We know that SSc fibroblasts are constitutively activated and lay down excess collagen. The lack of effect of FXIII on SSc fibroblast activity suggests that FXIII is not a potent moderator of fibrosis in SSc. This is in keeping with previously published clinical data, and my serum COMP data and mRSS change seen in the Sclero XIII clinical trials. However, of note, these collagen gel matrix studies reviewed the effect of FXIII on SSc fibroblasts alone, whereas in SSc *in vivo* there are multiple other cell types present in the endothelium, as well as other coagulation proteins which interact with FXIII, the endothelial surface, collagen and the fibroblast surface and may affect the final outcome. For example, FXIII-related fibroblast activation has been linked particularly to the effects of FXIII on stabilising the cross-linked fibrin clot generated

during wound healing (Kasai S, 1983). Furthermore, fibroblasts in a collagen gel matrix do not behave exactly like fibroblasts *in vivo*, for example the tensile stresses of the collagen do not have a long term effect on fibroblast behaviour and the cells become quiescent after 24 hours and undergo abnormal apoptosis (Rosenfeldt and Grinnell, 2000, Fluck et al., 1998).

In the fibroblast migration assays, SSc fibroblasts showed greater and more rapid migration compared to normal fibroblasts. The migration rate of normal fibroblasts was increased when FXIII was added. In keeping with the gel contraction work, these results suggest that FXIII is involved in the activation step when normal fibroblasts convert to myofibroblasts involved in wound healing. The activated myofibroblasts would benefit from migrating more rapidly to fill a tissue defect as this would reduce the risk of infection or organ dysfunction. The constitutively activated fibroblast seen in SSc therefore may not respond to supplementation with FXIII because it has already adopted the activated state. Again, it seems that FXIII plays a role in this unidirectional activation step but does not modulate the cell fibroblast behaviour further.

Together these data suggest that factor XIII stimulates migration and gel contraction in normal fibroblasts towards that which is seen constitutively in SSc fibroblasts. However, SSc fibroblasts are refractory to supplementation with factor XIII *in vivo*.

4.6 Coagulation studies

My data confirm that a raised D-dimer level is frequent in SSc, as previously reported (Falanga et al., 1991). In contrast to previous studies, my data suggest raised D-dimer level is associated with lcSSc rather than dcSSc (Lippi et al., 2006).

The fibrin degradation product D-dimer was raised in SSc but fibrinogen itself was not. My data suggest that D-dimer in this context is a marker of inflammation rather than of a pro-coagulant state. Although fibrin is not measured in clinical practice,

the high levels of fibrin degradation products suggest that fibrin levels themselves were also raised in SSc patients.

There was no correlation between raised D-dimer level and duration of SSc. This is in contrast with other inflammatory markers such as CRP, which are raised in early SSc only (Muangchan et al., 2012). There was no association between D-dimer level and active or historical digital ulceration, further suggesting that this feature of vascular dysfunction in SSc is not related to a pro-coagulant state, in contrast to previous publications (Marie et al., 2008, Solfietti et al., 2016).

High levels of fibrin are known to stimulate inflammatory pathways by binding to integrins and triggering the release of pro-inflammatory cytokines such as TNFα and interleukin 1 beta (IL-1β). This has been shown to contribute to the pathogenesis of other inflammatory conditions including rheumatoid arthritis and colitis-associated malignancy (Perez and Roman, 1995, Perez et al., 1999, Flick et al., 2007, Steinbrecher et al., 2010). It may be that high fibrin levels contribute to inflammatory pathways in SSc, and this is reflected in the raised D-dimer levels I detected. This is also supported by my finding that higher fibrinogen levels are associated with a raised D-dimer in systemic sclerosis.

5 Conclusions and next steps

The Sclero XIII trials have shown that factor XIII administration is safe and well tolerated in systemic sclerosis. The adverse effect profile was minimal and predictable. There were no concerning safety signals. A key benefit of this trial was its inclusivity, meaning that the results are generalisable across the SSc patient population. The trial did not meet its primary endpoints, showing no significant improvement in skin fibrosis or Raynaud's phenomenon following administration of factor XIII. However, there are well-documented challenges in assessing Raynaud's phenomenon in clinical trials. There was a trend towards improvement in RP seen in the FXIII group which was not seen in the placebo group. Now that safety and tolerability have been proven in the general SSc population it would be

exciting to investigate the efficacy of FXIII in systemic sclerosis patients with significant digital vascular involvement in, to see whether this treatment may be more efficacious in this subset of SSc patients.

There was no benefit seen in the secondary endpoints of pulmonary function or digital ulceration, but the study was not powered to look at these outcomes. There was a numerical trend towards improvement in hand function, but this should be interpreted with caution given the small numbers. This finding is, however, in keeping with qualitative effects on hand function reported in earlier trials of FXIII in SSc. This result further supports a role for future research using FXIII in SSc looking at patients with significant vascular involvement, as in most patients this predominantly affects the hands.

Participants found the frequency of infusions and study visits challenging. Factor XIII must be administered intravenously so there is limited scope for home administration. In a rare disease with wide geographical spread, the frequency of travel to sites offering this treatment would be a limiting factor if factor XIII were to be licensed for the treatment of systemic sclerosis and might hinder recruitment in future studies.

As these studies were exploratory and there was a potential risk of deranged thrombosis formation following FXIII administration, a relatively low dose was chosen, and the patient population was selected to reduce those with pro-coagulant tendencies. The safety data from the trial suggest this potential risk is not a concern at the FXIII dosing specified, though of course only small patient numbers were studied. The therapeutic effect of FXIII in systemic sclerosis could be considered further with a study assessing the safety and efficacy of higher FXIII doses.

Previous trials assessing the efficacy of therapies in RP in SSc have selected their trial population to enrich for severe RP patients. For example, inclusion criteria have specified a minimum frequency of RP attacks or a history of digital ulceration

for trial inclusion (Shenoy et al., 2010, Wigley et al., 1994). Given that the Sclero XIII trial was an exploratory study focusing primarily on assessing the safety of FXIII therapy, the un-enriched sample was appropriate. However further trials in this area could consider using the inclusion criteria to select patients with significant vascular involvement, though then the generalisability of the results should be considered.

Similarly, future studies could consider restricting the use of vasodilators as concomitant medications, to reduce their potential confounding effect. Given the widespread use of these agents, a more ethically appropriate possibility might be to require the use of vasodilators in the eligibility criteria. If recruiting a patient subpopulation with severe vascular involvement, patients would be expected to be taking these agents already; and by making their use uniform across FXIII and placebo arm this would reduce their confounding effect on any conclusions drawn about the efficacy of FXIII in SSc.

Future studies could collect extended data on cardiovascular and thromboembolic risk, for example smoking status, lipid profile, anti-phospholipid antibodies and blood glucose could all be assessed at screening. In a larger study, the potential risk of existing thromboembolic clot extension with the administration of factor XIII could be assessed in more detail. If any new or worsening thromboembolic events were seen in this study, then understanding the participants' cardiovascular risk more thoroughly could be of benefit.

This project worked to maximise scientific benefit by aligning *in vitro* cellular and protein-based research in parallel alongside the clinical trials. This enabled the generation of longitudinal data allowing me to track changes in candidate biomarker concentrations over time following FXIII administration. This research has confirmed previous findings showing raised levels of COMP, TNFα and TSP-1 in systemic sclerosis patient serum when compared to healthy controls. I have demonstrated significant reductions in the levels of serum EGF, TGF-α, TNFα, TSP-1 and VEGF-C in SSc when patients are supplemented with FXIII. These data

have allowed me to consider and discuss the role of these proteins in the pathogenesis of SSc, and to hypothesise further on the mechanism of action of FXIII in SSc. I hope that this may lead to future studies investigating the interactions between these proteins in systemic sclerosis. An area of particular interest has been exploring the interplay between coagulation and inflammation which may be helpful for future in vitro and in vivo studies. The candidate biomarker changes will require further validation in large scale studies and are currently considered primarily hypothesis-generating.

6 Appendices

6.1 Patient-reported outcomes

SSc- HEALTH ASSESSMENT QUESTIONNAIRE

CSL SCLERO XIII (13/0417) – TREATMENT PHASE

Subject ID: _____ **Patient Initials:** ☐☐☐

Visit Type (circle one): **Baseline (visit 1)** **Visit 3 / 5 / 7 / 9**

Unscheduled visit **Early withdrawal** **End of therapy (visit 13)** **Follow up (visit 14)**

Date of completion: ___ ___ / ___ ___ ___ / 2 0 ___ ___

d d m m m y y y y

Please tick one response in the box which best describes your usual abilities over the past week

No	Item	Without ANY difficulty	With SOME difficulty	With MUCH difficulty	UNABLE to do
1.	**Dressing & Grooming** Are you able to:				
	• Dress yourself, including tying shoelaces and doing buttons	☐	☐	☐	☐
	• Shampoo your hair	☐	☐	☐	☐
2.	**Arising** Are you able to:				
	• Stand up from an armless straight chair	☐	☐	☐	☐
	• Get in and out bed?	☐	☐	☐	☐
3.	**Eating** Are you able to :				
	• Cut your Meat?	☐	☐	☐	☐
	• Lift a full cup or glass to your mouth?	☐		☐	☐
4.	**Walking** Are you able to:				
	• Walk outdoors on flat ground?	☐	☐	☐	☐
	• Climb up five stairs	☐	☐	☐	☐

Please tick any AIDS or DEVICES that you usually use for any of these activities:]

☐ Cane ☐ Devices used for dressing (buttonhooks, zipper pull, shoe horn)

☐ Walker ☐ Special utensils

☐ Crutches ☐ Special or built-up chair

☐ Wheelchair ☐ Other (specify).....

SSC- HEALTH ASSESSMENT QUESTIONNAIRE VERSION 1.0 PAGE 1 OF 5

SSc- HEALTH ASSESSMENT QUESTIONNAIRE

CSL SCLERO XIII (13/0417) – TREATMENT PHASE	
Subject ID: _____	Patient Initials: ☐☐☐

Please tick any categories for which you usually need ASSISTANCE FROM ANOTHER PERSON:

☐ Dressing &Grooming ☐ Eating

☐ Arising ☐ Walking

Please tick one response in the box which best describes your usual abilities over the past week

No	Item	Without ANY difficulty	With SOME difficulty	With MUCH difficulty	UNABLE to do
1.	**Hygiene** Are you able to:				
	• Wash and dry your entire body?	☐	☐	☐	☐
	• Take a bath?	☐	☐	☐	☐
	• Get on and off the toilet?	☐	☐	☐	☐
2.	**Reach** Are you able to:				
	• Reach and get down a 2 kilo object (E.g. a bag of sugar) from just above your head?	☐	☐	☐	☐
	• Bend down to pick up clothing off the floor?	☐	☐	☐	☐
3.	**Grip** Are you able to :				
	• Open car doors?	☐	☐	☐	☐
	• Open jars that have been previously opened?	☐	☐	☐	☐
	• Turn taps on and off?	☐	☐	☐	☐

SSc- HEALTH ASSESSMENT QUESTIONNAIRE

CSL SCLERO XIII (13/0417) – TREATMENT PHASE	
Subject ID: _____	Patient Initials: ☐☐☐

No	Item	Without ANY difficulty	With SOME difficulty	With MUCH difficulty	UNABLE to do
4.	**Activities** Are you able to:				
	• Run errands and shop	☐	☐	☐	☐
	• Get in and out of a car	☐	☐	☐	☐
	• Do everyday household cleaning	☐	☐	☐	☐

Please tick any AIDS or DEVICES that you usually use for any of these activities

☐ Raised toilet seat
☐ Bathtub seat
☐ Long handled appliances for reach
☐ Jar opener (for jars previously opened)
☐ Long handled appliances for bathroom
☐ Other (specify)……
☐ Bathtub bar

Please tick any categories for which you usually need help from another Person:

☐ Hygiene
☐ Gripping and opening things
☐ Reach
☐ Errands and chores

SSc- HEALTH ASSESSMENT QUESTIONNAIRE

CSL SCLERO XIII (13/0417) – TREATMENT PHASE	
Subject ID: _____	Patient Initials: ☐☐☐

We are also interested in learning whether or not you are affected by pain because of your illness.

How much pain have you had because of your illness in the past week?

PLACE A MARK ON THE LINE TO INDICATE THE SEVERITY OF THE PAIN

No pain (0) _____ Very severe pain (100)

Measured pain level ☐☐.☐

In the past week how much have your intestinal problems interfered with your daily activities?

PLACE A MARK ON THE LINE TO INDICATE THE LIMITATION OF ACTIVITY (INTESTINAL PROBLEMS)

Do not limit Activities (0) _____ Very severe Limitation (100)

Measured pain level ☐☐.☐

In the past week how much have your breathing problems interfered with your daily activities?

PLACE A MARK ON THE LINE TO INDICATE THE LIMITATION OF ACTIVITY (BREATHING PROBLEMS)

Do not limit Activities (0) _____ Very severe Limitation (100)

Measured pain level ☐☐.☐

In the past week how much has your Raynaud's interfered with your daily activities?

PLACE A MARK ON THE LINE TO INDICATE THE LIMITATION OF ACTIVITY (RAYNAUDS'S)

Do not limit Activities (0) _____ Very severe Limitation (100)

Measured pain level ☐☐.☐

SSc- HEALTH ASSESSMENT QUESTIONNAIRE

CSL SCLERO XIII (13/0417) – TREATMENT PHASE	
Subject ID: _____	Patient Initials: ☐☐☐

In the past week how much have your finger ulcers interfered with your daily activities?

PLACE A MARK ON THE LINE TO INDICATE THE LIMITATION OF ACTIVITY (FINGER ULCERS)

Do not limit Activities (0) _____ Very severe Limitation (100)

Measured pain level ☐☐.☐

Overall, considering how much pain, discomfort, limitation in your daily life and other changes in your body and life, how severe would you rate your disease today?

PLACE A MARK ON THE LINE TO INDICATE THE LIMITATION OF ACTIVITY (FINGER ULCERS)

No disease (0) _____ Very severe Limitation (100)

Measured pain level ☐☐.☐

RAYNAUDS CONDITION SCORE

CSL SCLERO XIII (13/0417) – TREATMENT PHASE	
Subject_ID: _____	Patient Initials: ☐☐☐

Visit Type (circle one): **Baseline (visit 1)** **Visit 3 / 5 / 7 / 9 / 11**

Unscheduled visit **Early withdrawal** **End of therapy (visit 13)** **Follow up (visit 14)**

Date of completion: __ __/__ __ __/ 2 0 __ __
d d m m m y y y y

Day (*circle*) Mon Tue Wed Thu Fri Sat Sun

How many times have you been exposed to outdoor temperatures today?

How many Raynaud's attacks have you had today? Please list below

Raynaud's attack number	Duration (minutes)	Raynaud's attack number	Duration (minutes)	Raynaud's attack number	Duration (minutes)
1		6		11	
2		7		12	
3		8		13	
4		9		14	
5		10		15	

Raynaud's condition score (RSC)

Circle below the number that best indicates the difficulty you had today with your Raynaud's condition:

1 2 3 4 5 6 7 8 9 **10**

No difficulty — Extreme difficulty

SF-36 QUESTIONNAIRE

CSL SCLERO XIII (13/0417) – TREATMENT PHASE	
Subject_ID: _____	Patient Initials: ☐☐☐

Visit Type (circle one): Baseline (visit 1) Visit 3 / 5

Unscheduled visit Early withdrawal End of therapy (visit 13) Follow up (visit 14)

Date of completion: __ __ / __ __ __ / 2 0 __ __

d d m m m y y y y

For each of the following questions, please tick the one box that best describes your answer.

1. In general, would you say your health is:

Excellent	Very good	Good	Fair	Poor
☐ 1	☐ 2	☐ 3	☐ 4	☐ 5

2. Compared to one year ago, how would you rate your health in general now

Much better now than one year ago	Somewhat better than one year ago	About the same as one year ago	Somewhat worse now than one year ago	Much worse now than one year ago
☐ 1	☐ 2	☐ 3	☐ 4	☐ 5

3. The following questions are about activities you might do during a typical day. Does your health now limit you in these activities? If so, how much?

Activities	Yes, limited A lot	Yes, limited a little	No, not Limited At all
a) **Vigorous activities** such as running, lifting heavy objects, participating in strenuous sports	☐ 1	☐ 2	☐ 3
b) **Moderate activities**, such as moving a table, pushing a vacuum cleaner, bowling or playing golf	☐ 1	☐ 2	☐ 3
c) Lifting or carrying groceries	☐ 1	☐ 2	☐ 3
d) Climbing **several** flights of stairs	☐ 1	☐ 2	☐ 3
e) Climbing **one** flight of stairs	☐ 1	☐ 2	☐ 3
f) Bending, kneeling, or stooping	☐ 1	☐ 2	☐ 3

SF-36 QUESTIONNAIRE

CSL SCLERO XIII (13/0417) – TREATMENT PHASE	
Subject ID: _____	Patient Initials: ☐☐☐

Activities	Yes, limited A lot	Yes, limited a little	No, not Limited At all
g) Walking **more than one kilometre**	☐1	☐2	☐3
h) Walking **half a kilometre**	☐1	☐2	☐3
i) Walking **100 metres**	☐1	☐2	☐3
j) **Bathing** or **dressing** yourself	☐1	☐2	☐3

During the <u>past 4 weeks</u>, how much of the time have you had any of the following problems with your work or other regular daily activities <u>as a result of your physical health?</u>

	All of the time	Most of the time	Some of the time	A little of the time	None of the time
a. Cut down on the amount of time you spend on work or activities	☐1	☐2	☐3	☐4	☐5
b. Accomplished less than you would like	☐1	☐2	☐3	☐4	☐5
c. Were limited in the kind of work or other activities	☐1	☐2	☐3	☐4	☐5
d. Had difficulty performing the work or other activities (for example, it took extra effort)	☐1	☐2	☐3	☐4	☐5

4. During the past 4 weeks, how much of the time have you had any of the following problems with your work or other regular daily activities as a result of any emotional problems (such as feeling depressed or anxious)?

	All of the time	Most of the time	Some of time	A little of the time	None of the time
a. Cut down on the **amount of time** you spent on work or other activities	☐1	☐2	☐3	☐4	☐5
b. **Accomplished less** than you would like	☐1	☐2	☐3	☐4	☐5
c. Did work or other activities **less careful than usual**	☐1	☐2	☐3	☐4	☐5

SF-36 QUESTIONNAIRE

CSL SCLERO XIII (13/0417) – TREATMENT PHASE	
Subject ID: _____	Patient Initials: ☐☐☐

5. During the past 4 weeks, to what extent has your physical health or emotional problems interfered with your normal social activities with family, friends, neighbours, or groups?

Not at all	Slightly	Moderate	Quite a bit	Extremely
☐1	☐2	☐3	☐4	☐5

6. How much bodily pain have you had during the past 4 weeks?

None	Very mild	Mild	Moderate	Severe	Very severe
☐1	☐2	☐3	☐4	☐5	☐6

7. During the past 4 weeks, how much did pain interfere with your normal work (including both work outside the home and housework)?

Not at all	Slightly	Moderate	Quite a bit	Extremely
☐1	☐2	☐3	☐4	☐5

8. These questions are about how you feel and how things have been with you during the past 4 weeks. For each question, please give the one answer that comes closest to the way you have been feeling. How much of the time during the past 4 weeks…

	All of the time	Most of the time	Some of the time	A little the time	None of the time
a) Did you feel full of life	☐1	☐2	☐3	☐4	☐5
b) Have you been very nervous	☐1	☐2	☐3	☐4	☐5
c) Have you felt calm and peaceful	☐1	☐2	☐3	☐4	☐5
d) Did you have a lot of energy	☐1	☐2	☐3	☐4	☐5
e) Have you felt downhearted and depressed	☐1	☐2	☐3	☐4	☐5
f) Did you feel worn out	☐1	☐2	☐3	☐4	☐5
g) Have you been happy	☐1	☐2	☐3	☐4	☐5
h) Did you feel tired	☐1	☐2	☐3	☐4	☐5

SF-36 QUESTIONNAIRE

CSL SCLERO XIII (13/0417) – TREATMENT PHASE	
Subject_ID: _____	Patient Initials: ☐☐☐

9. During the past 4 weeks, how much of the time has your physical health or emotional problems interfered with your social activities (like visiting with friends, relatives, etc.)?

All of the time	Most of the time	Some of the time	A little of the time	None of the time
☐1	☐2	☐3	☐4	☐5

10. How TRUE or FALSE is <u>each</u> of the following statements for you?

	Definitely true	Mostly true	Don't know	Mostly false	Definitely false
a) I seem to get sick a little easier than other people	☐1	☐2	☐3	☐4	☐5
b) I am as healthy as anybody I know	☐1	☐2	☐3	☐4	☐5
c) I expect my health to get worse	☐1	☐2	☐3	☐4	☐5
d) My health is excellent	☐1	☐2	☐3	☐4	☐5

Cochin Hand Function Score

CSL SCLERO XIII (13/0417) – TREATMENT PHASE	
Subject_ID: _____	Patient Initials: ☐☐☐

Visit Type (circle one): Baseline (visit 1) Visit 3 / 5 / 7 / 9 / 11

Unscheduled visit Early withdrawal End of therapy (visit 13) Follow up (visit 14)

Date of completion: ___ ___ / ___ ___ ___ / 2 0 ___ ___
d d m m m y y y y

Please indicate to what extent you can perform the following activities.

	Yes, without difficulty	Yes, with little difficulty	Yes, with some difficulty	Yes, with much difficulty	Nearly impossible to do	Impossible to do
1. Can you hold a bowl?	☐	☐	☐	☐	☐	☐
2. Can you grasp a full bottle and raise it?	☐	☐	☐	☐	☐	☐
3. Can you hold a plate full of food?	☐	☐	☐	☐	☐	☐
4. Can you pour liquid from a bottle into a glass?	☐	☐	☐	☐	☐	☐
5. Can you unscrew the lid from a jar that has been opened before?	☐	☐	☐	☐	☐	☐
6. Can you cut meat with a knife?	☐	☐	☐	☐	☐	☐
7. Can you prick things well with a fork?	☐	☐	☐	☐	☐	☐
8. Can you peel fruit?	☐	☐	☐	☐	☐	☐
9. Can you button your shirt?	☐	☐	☐	☐	☐	☐
10. Can you open and close a zipper?	☐	☐	☐	☐	☐	☐
11. Can you squeeze a new tube of toothpaste?	☐	☐	☐	☐	☐	☐
12. Can you hold a toothbrush effectively?	☐	☐	☐	☐	☐	☐
13. Can you write a short sentence with an ordinary pen?	☐	☐	☐	☐	☐	☐
14. Can you write a letter with an ordinary pen?	☐	☐	☐	☐	☐	☐

Cochin Hand Function Score

CSL SCLERO XIII (13/0417) – TREATMENT PHASE	
Subject_ID: _____	Patient Intials: ☐☐☐

	Yes, without difficulty	Yes, with little difficulty	Yes, with some difficulty	Yes, with much difficulty	Nearly impossible to do	Impossible to do
15. Can you turn a round door knob?	☐	☐	☐	☐	☐	☐
16. Can you cut a piece of paper with scissors?	☐	☐	☐	☐	☐	☐
17. Can you pick up coins from a table top?	☐	☐	☐	☐	☐	☐
18. Can you turn a key in a lock?	☐	☐	☐	☐	☐	☐

6.2 Physician assessments

MODIFIED RODNAN SKIN SCORE (MRSS)

CSL SCLERO XIII (13/0417) – TREATMENT PHASE	
Subject_ID: _____	Patient Intials: ☐☐☐

Visit Type (circle one):	Screening	Baseline (visit 1)	Visit 3 / 5 / 7 / 9 / 11
Unscheduled visit	Early withdrawal	End of therapy (visit 13)	Follow up (visit 14)

MODIFIED RODNAN SKIN SCORE(MRSS)

Date of assessment __ __ / __ __ __ / 2 0 __ __
d d m m m y y y y

Body site	Normal skin	Slight thickening	Moderate thickening	Severe thickening
	0	1	2	3
Face	☐	☐	☐	☐
Anterior chest	☐	☐	☐	☐
Abdomen	☐	☐	☐	☐
Upper arm – left	☐	☐	☐	☐
Upper arm – right	☐	☐	☐	☐
Forearm – left	☐	☐	☐	☐
Forearm – right	☐	☐	☐	☐
Hand – left	☐	☐	☐	☐
Hand – right	☐	☐	☐	☐
Fingers – left	☐	☐	☐	☐
Fingers – right	☐	☐	☐	☐
Thigh – left	☐	☐	☐	☐
Thigh – right	☐	☐	☐	☐
Leg – left	☐	☐	☐	☐
Leg – right	☐	☐	☐	☐
Foot – left	☐	☐	☐	☐
Foot – right	☐	☐	☐	☐
			Score	☐☐ /51

SCLERODERMA PHYSICIAN GLOBAL VISUAL ANALOGUE SCALE

CSL SCLERO XIII (13/0417) – TREATMENT PHASE	
Subject_ID: _____	Patient Intials: ☐☐☐
Visit Type (circle one): Baseline (visit 1)	Visit 3 / 5 / 7 / 9 / 11
Unscheduled visit Early withdrawal	End of therapy (visit 13) Follow up (visit 14)

Date of completion: __ __ / __ __ __ / 2 0 __ __
d d m m m y y y y

SCLERODERMA PHYSICIAN GLOBAL VISUAL ANALOGUE SCALE
☐ Tick if not Applicable **Overall, considering how much pain, discomfort, limitation in the patient's daily life, how severe would you rate their disease today?** PLACE A MARK ON THE LINE TO INDICATE PHYSICIAN GLOBAL ASSESSMENT Very well (0) ——— Very poor (100)

Office use only Measured pain level	☐☐ . ☐ cm

DIGITAL ULCER COUNT

CSL SCLERO XIII (13/0417) – TREATMENT PHASE	
Subject_ID: _____	Patient Intials: ☐☐☐
Visit Type (circle one): Baseline (visit 1) Visit 3 / 5 / 7 / 9 / 11 Unscheduled visit Early withdrawal End of therapy (visit 13) Follow up (visit 14)	

Date of observation: ------/-------/-------
(dd/mmm/yyyy)

Number of DUs on fingers at this visit? ☐☐

DU-associated Interventions on fingers since last visit:

Overnight Hospitalisation(s) for DU: ☐ Yes ☐ No

If yes, total number of weeks or days? ☐ Weeks ☐ Days ☐ Unknown

Additional surgical treatment for digital ulcers? ☐ Yes ☐ No

Gangrene? ☐ Yes ☐ No

Amputation? ☐ Yes ☐ No

Local Sympathectomy? ☐ Yes ☐ No

Botulinum Toxin A? ☐ Yes ☐ No

Systemic Antibiotic required? ☐ Yes ☐ No

IV Iloprost required? ☐ Yes ☐ No

Any AE or SAE reported? ☐ Yes ☐ No

If Yes, AE recorded in AE/SAE form? ☐ Yes ☐ No

6.3 Treatment phase factor XIII levels – patients receiving factor XIII (lcSSc unshaded boxes, dcSSc shaded boxes)

Patient		Screening	Baseline	Visit 02	Visit 03	Visit 04	Visit 05	Visit 06	Visit 07	Visit 08	Visit 09	Visit 10	Visit 11	Visit 12
01	Pre-dose	105	117	144	135	127	136	132	150	130	Missed visit	102	110	130
	Post-dose		173	179	150	188	212	202	230	180		138	205	242
07	Pre-dose	172		170	188	190	178	189	220	160	170	170	172	184
	Post-dose		206	217	220	236	214	212	198	240	250	272	262	231
08	Pre-dose	176	156	169	155	144	172	173	158	170		184	152	158

Patient		Scree-ning	Base-line	Visit 02	Visit 03	Visit 04	Visit 05	Visit 06	Visit 07	Visit 08	Visit 09	Visit 10	Visit 11	Visit 12
08	Post-dose		212	190	190	215	272	246	214	224	269	226	188	200
09	Pre-dose	164	150	162			172	144	173	200	160	148	179	178
	Post-dose		210	231	270	232	234	224	300	278	160	255	343	243
10	Pre-dose	135	127	153	156	153	132	144	150	163	156	148	148	160
	Post-dose		190	179	208	225	192	234	220	255	212	196	196	245
11	Pre-dose	122	228	161	157	159	161	152	149	158	155	159	141	162

Patient		Screening	Baseline	Visit 02	Visit 03	Visit 04	Visit 05	Visit 06	Visit 07	Visit 08	Visit 09	Visit 10	Visit 11	Visit 12
11	Post-dose		123	209	234	230	242	230	242	250	248	216	213	252
14	Pre-dose	111	108	136	140	154	158	154	150	156	163	156	144	144
	Post-dose		206	238	154	140	234	226	232	258	214	213		245
15	Pre-dose	281	258	304	268	292	296	321	330	314	260	314	301	290
	Post-dose			348	312	394	336	No further doses administered as per protocol due to high pre-dose levels						
Patient		Screening	Baseline	Visit 02	Visit 03	Visit 04	Visit 05	Visit 06	Visit 07	Visit 08	Visit 09	Visit 10	Visit 11	Visit 12

16	Pre-dose	189	160	176	Patient withdrew									
	Post-dose		162											
17	Pre-dose	114	119	149	159	152	134	147	160	156	153	148	156	169
	Post-dose		212	251	232	222	212	243	282	226		240	254	244
19	Pre-dose	166	157	155	165	152	154	167	148	Miss-ed visit	147	146	144	156
	Post-dose		226	204	224	225	250	274	254		191	242	190	234
Patient		Scree-ning	Base-line	Visit 02	Visit 03	Visit 04	Visit 05	Visit 06	Visit 07	Visit 08	Visit 09	Visit 10	Visit 11	Visit 12

21	Pre-dose	197	172	168	192	182	180	192	188	198	188	202	204	178
	Post-dose		224	218	300	217	240	222	254	256	230	274		

6.4 Treatment phase factor XIII levels – patients receiving placebo (lcSSc unshaded boxes, dcSSc shaded boxes)

Patient		Scree-ning	Base-line	Visit 02	Visit 03	Visit 04	Visit 05	Visit 06	Visit 07	Visit 08	Visit 09	Visit 10	Visit 11	Visit 12
02	Pre-dose	149	146	144	125	142		120	118	134	161	133	120	134
	Post-dose		134	132	125	134	117	114	118	138	152	124	120	124
03	Pre-dose	111	105	106	116	105	107	112	121	114	115	133	122	110
	Post-dose		96	105	102	104	104	103	120	108	100	106	120	108
Patient		Scree-ning	Base-line	Visit 02	Visit 03	Visit 04	Visit 05	Visit 06	Visit 07	Visit 08	Visit 09	Visit 10	Visit 11	Visit 12

12	Pre-dose	98	101	105	103			117	125	112	125	115	134	114
	Post-dose		106	105	101			119	123	114	114	114	125	116
13	Pre-dose	141	13	150	153	157	146	156	160	152	142	142	143	134
	Post-dose		127	141	150	155	158	159	142	144	156	157	142	136
18	Pre-dose	147	138	141		149	141		136	134		146	123	122
	Post-dose		133	135		129	135		118	131		139	122	126
Patient		Scree-ning	Base-line	Visit 02	Visit 03	Visit 04	Visit 05	Visit 06	Visit 07	Visit 08	Visit 09	Visit 10	Visit 11	Visit 12

20	Pre-dose	178	170	170	166	179	156	154	168	176	149	166	160	160
	Post-dose		166	158	160	185	174	162	158	180	148	162	160	162

6.5 Candidate biomarker results

6.5.1 ELISAs

6.5.1.1 Cartilage oligomeric matrix protein

Figure 44 COMP concentration at Week 0: SSc vs. Healthy. Boxes represent median and IQR, whiskers represent maximum and minimum values.

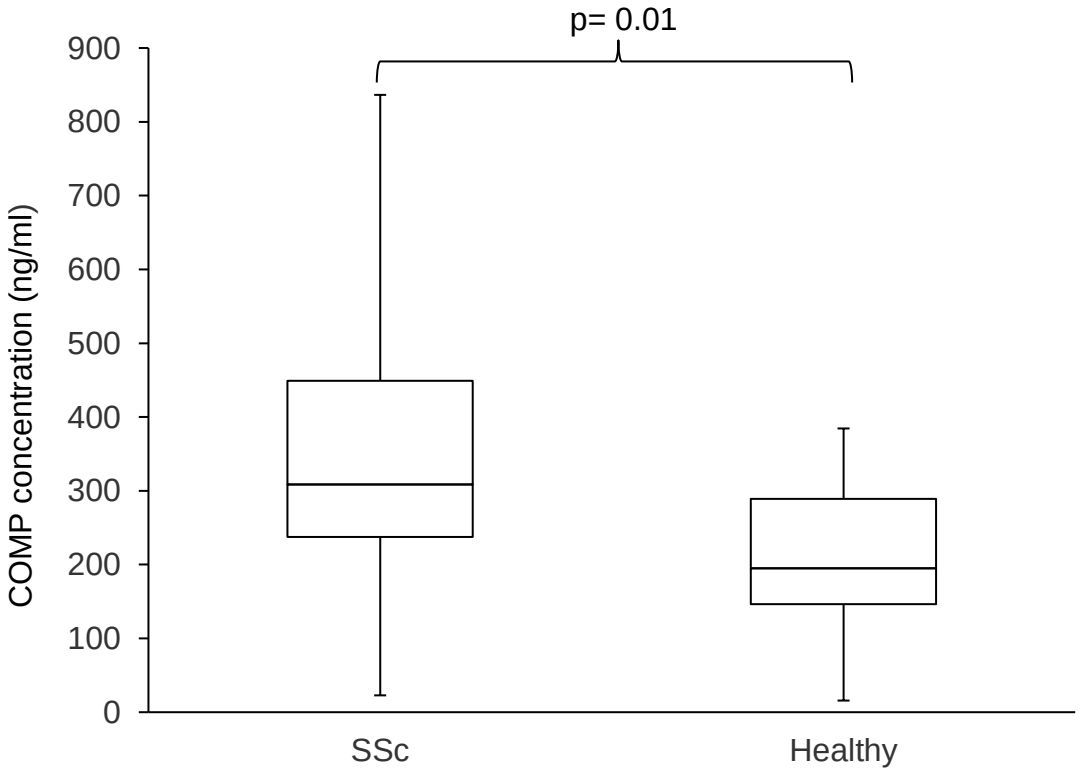


Table 6.1 Descriptive statistics for COMP (ng/ml) at Week 0 and Week 24 in SSc + FXIII, SSc + placebo and healthy control groups

Treatment Group		n	Mean	SD	Min	Median	Max	p-value
SSc + FXIII	Week 0 baseline	12	421.23	197.14	233.04	354.61	836.50	
	Week 24	12	419.38	242.14	70.42	326.54	772.55	
	Change from baseline	12	-1.85	159.66	-238.31	-26.04	322.42	
	Adjusted mean change from baseline (95% CI)	12	-62.86 (-153.19 to 27.48)					0.53
SSc + Placebo	Week 0 baseline	9	397.44	133.83	186.71	443.82	555.24	
	Week 24	9	495.35	241.82	182.13	473.67	826.89	
	Change from baseline	9	97.91	224.84	-373.12	111.36	376.74	

	Adjusted mean change from baseline (95% CI)	9	156.79 (9.89 to 303.68)					0.21
Difference between groups		Adjusted mean change from baseline (95% CI)						0.39
Healthy	Week 0	12	209.86	105.59	15.79	203.84	384.50	

Figure 45 Change in COMP concentration from week 0 to week 24

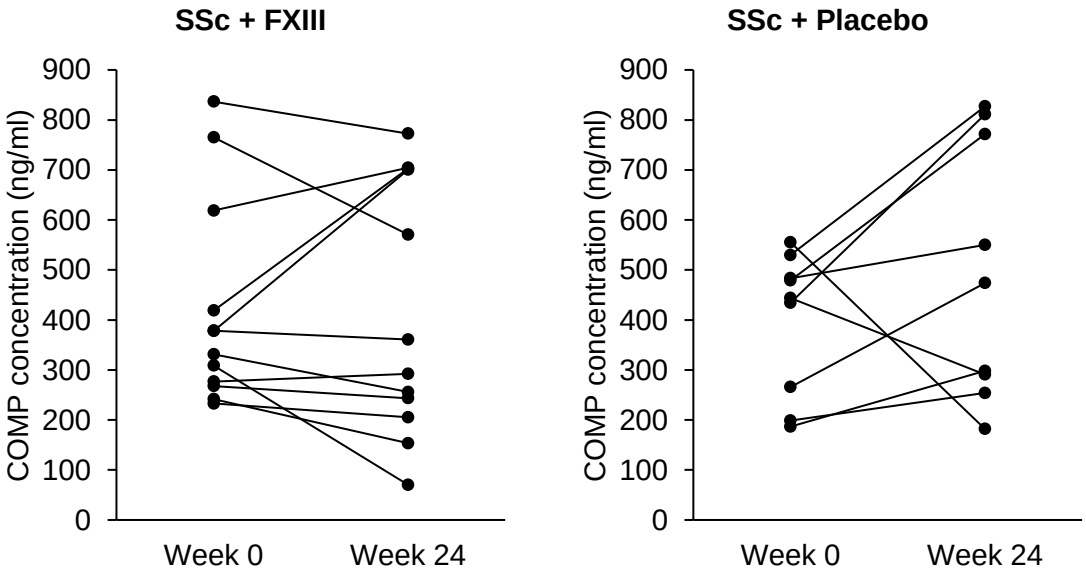
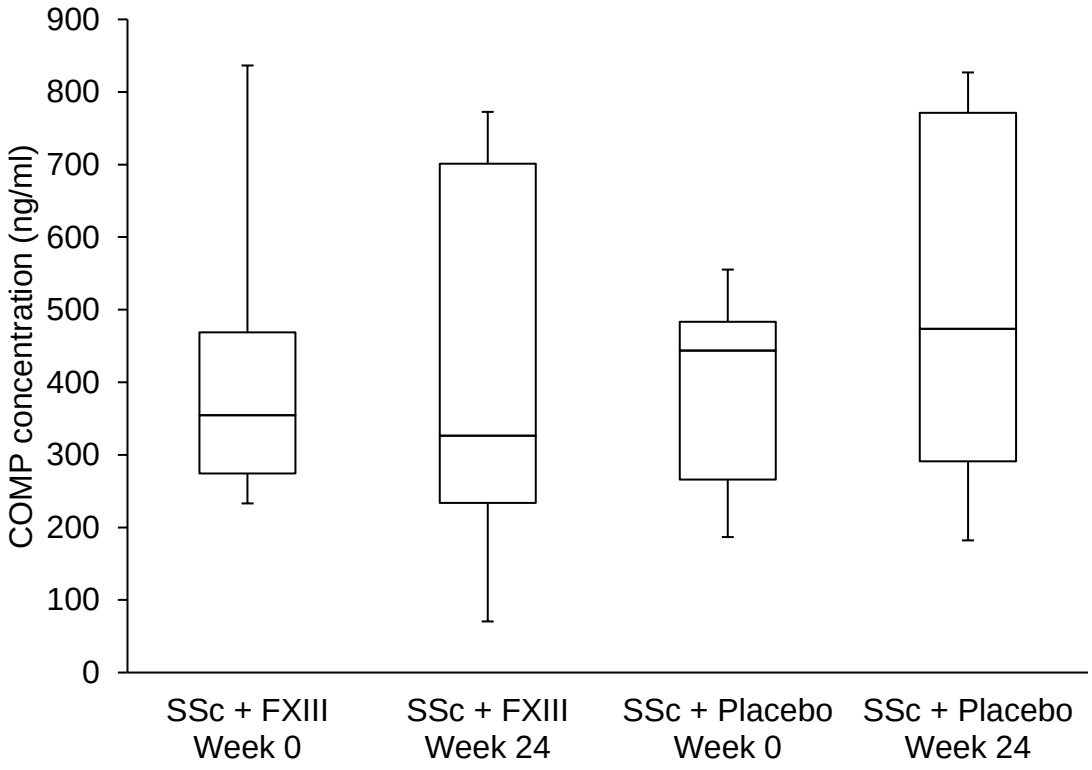


Figure 46 Distribution of COMP concentrations week 0 vs. week 24. Boxes represent median and IQR, whiskers represent maximum and minimum values.



6.5.1.2 Fibronectin

Figure 47 Fibronectin concentration at Week 0: SSc vs. Healthy. Boxes represent median and IQR, whiskers represent maximum and minimum values.

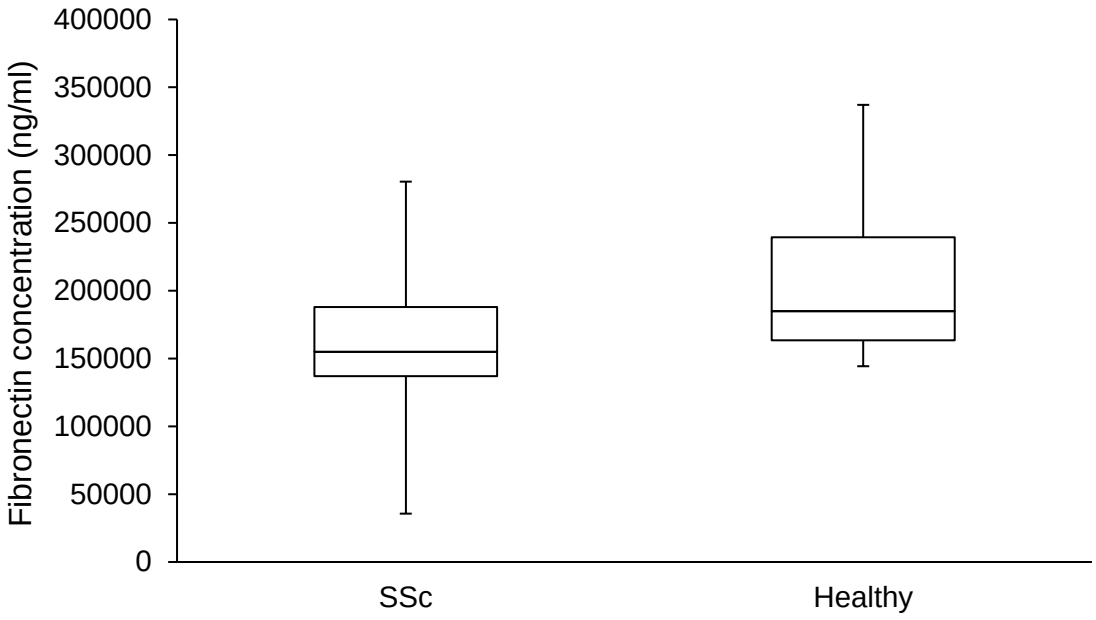


Table 6.2 Descriptive statistics for Fibronectin (ng/ml) at Week 0 and Week 24 in SSc + FXIII, SSc + placebo and healthy control groups

Treatment Group		n	Mean	SD	Min	Median	Max	p-value
SSc + FXIII	Week 0 baseline	12	152680	57843	35787	145409	252367	
	Week 24	12	144105	42684	95170	121975	230256	
	Change from baseline	12	-8574	64001	-138202	-11728	165414	
	Adjusted mean change from baseline (95% CI)	12	-13010 (-49222 to 23201)					0.08
SSc + Placebo	Week 0 baseline	9	194116	41863	123122	188921	280424	
	Week 24	9	189189	43392	115223	186968	263166	
	Change from baseline	9	-4927	34870	-53523	-279	52578	
	Adjusted mean change	9	-4927 (-27708 to 17855)					0.59

	from baseline (95% CI)							
Difference between groups		Adjusted mean change from baseline (95% CI)		-8084 (-42782 to 26615)				**0.03**
Healthy	Week 0	13	203135	61185	144341	178560	337041	

Figure 48 Change in fibronectin concentration from Week 0 to Week 24

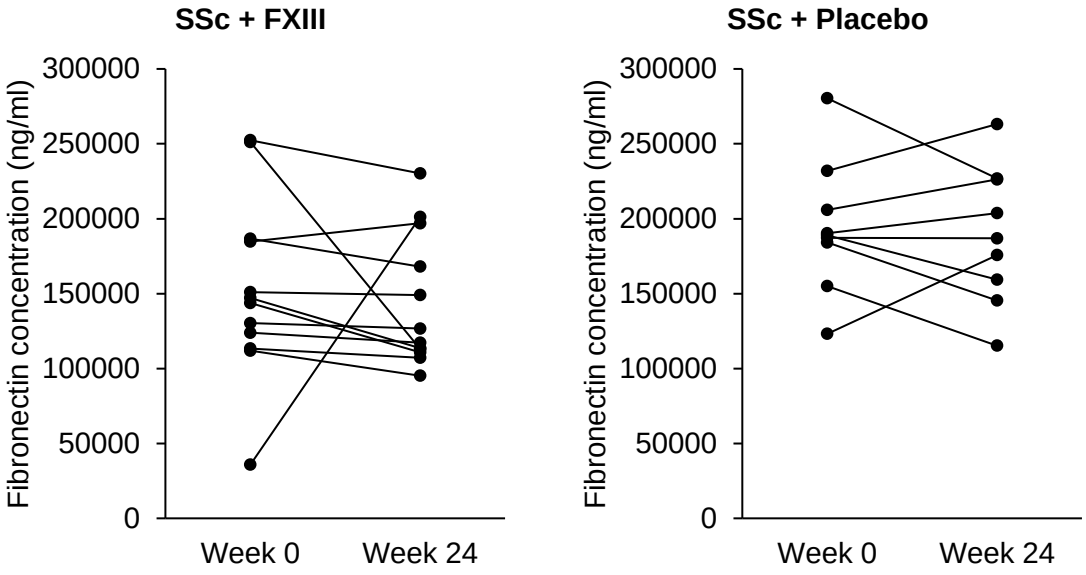
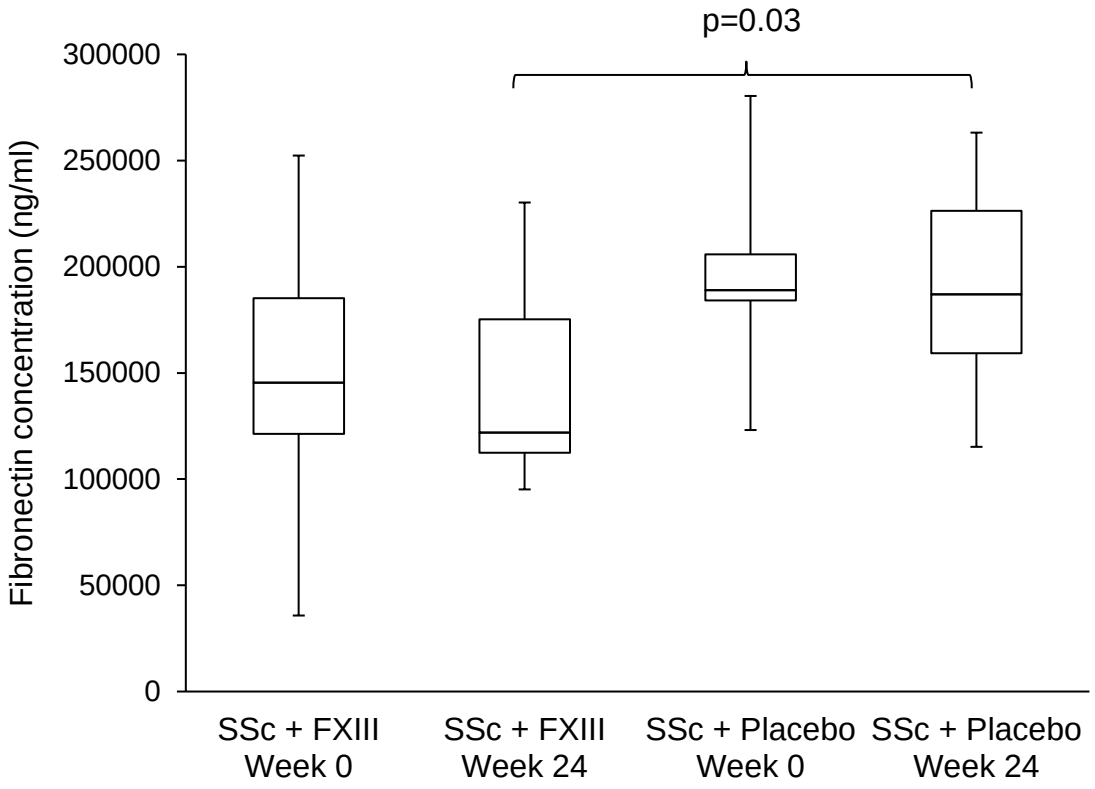


Figure 49 **Distribution of fibronectin concentrations week 0 vs. week 24. Boxes represent median and IQR, whiskers represent maximum and minimum values.**



6.5.1.3 Thrombospondin-1

Figure 50 TSP-1 concentration at Week 0: SSc vs. Healthy. Boxes represent median and IQR, whiskers represent maximum and minimum values.

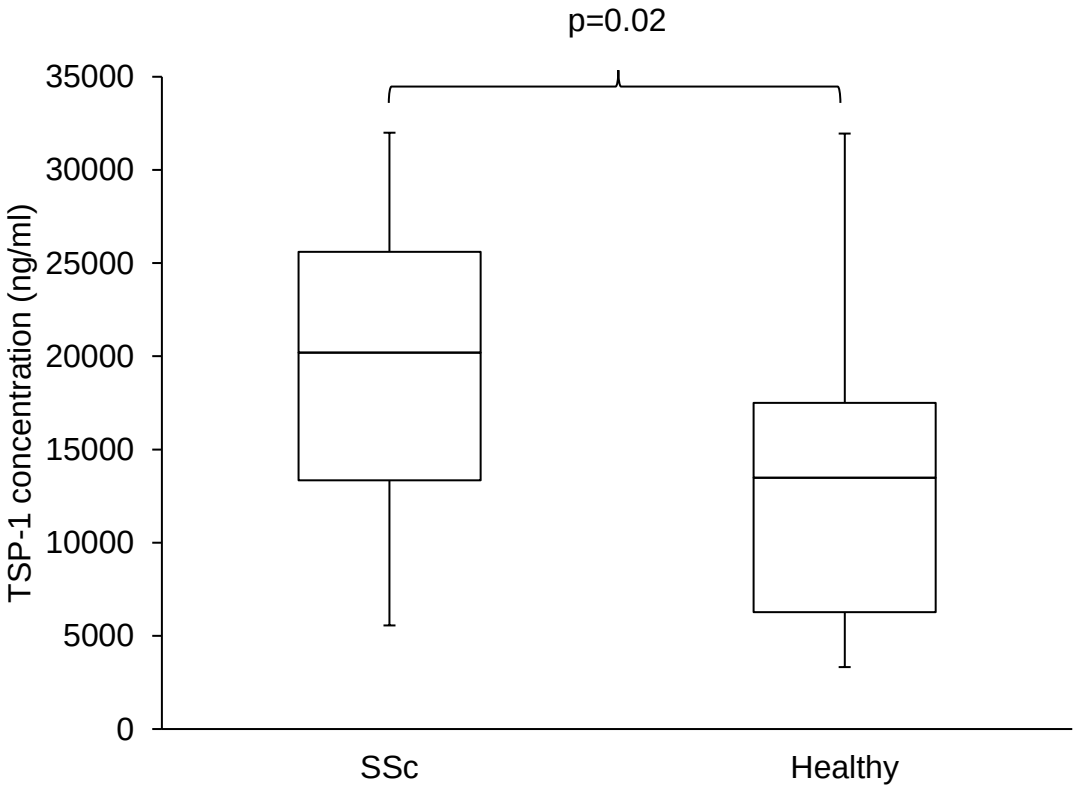


Table 6.3 Descriptive statistics for TSP-1 (ng/ml) from week 0 to week 24 in SSc + FXIII, SSc + placebo and healthy control groups

Treatment Group		n	Mean	SD	Min	Median	Max	p-value
SSc + FXIII	Week 0 baseline	12	23753.3	5400.0	11070.7	24523.6	31293.8	
	Week 24	12	17179.8	7273.8	7457.6	16727.4	35792.9	
	Change from baseline	12	-6573.5	7732.3	-16835.0	-8964.4	8540.3	
	Adjusted mean change from baseline (95% CI)	12	-6573.5 (-10948.4 to -2198.6)					0.02
SSc + Placebo	Week 0 baseline	9	17146.8	6774.9	5557.9	14110.5	27338.5	
	Week 24	9	18175.6	6733.8	7992.6	19218.4	31216.8	
	Change from baseline	9	1028.8	5423.5	-5373.6	700.3	10778.6	
	Adjusted mean change	9	1028.8 (-2514.5 to 4572.1)					0.68

	from baseline (95% CI)							
Difference between groups		Adjusted mean change from baseline (95% CI)		7602.3 (-19180 to 3975.7)				0.57
Healthy	Week 0	13	13061.6	7805.5	3323.3	13017.5	31952.2	

Figure 51 TSP-1 concentration: change from Week 0 to Week 24

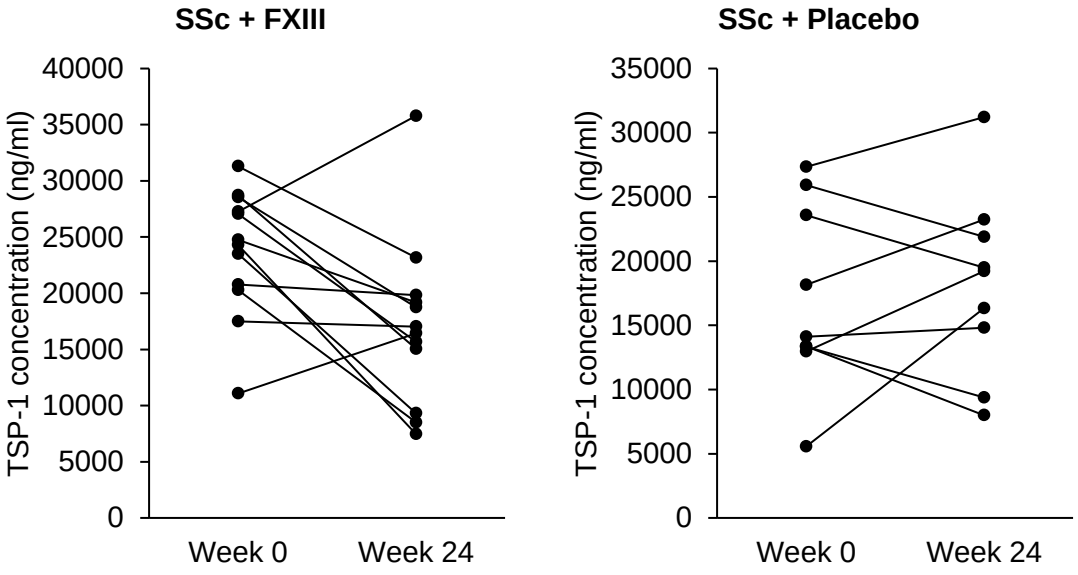
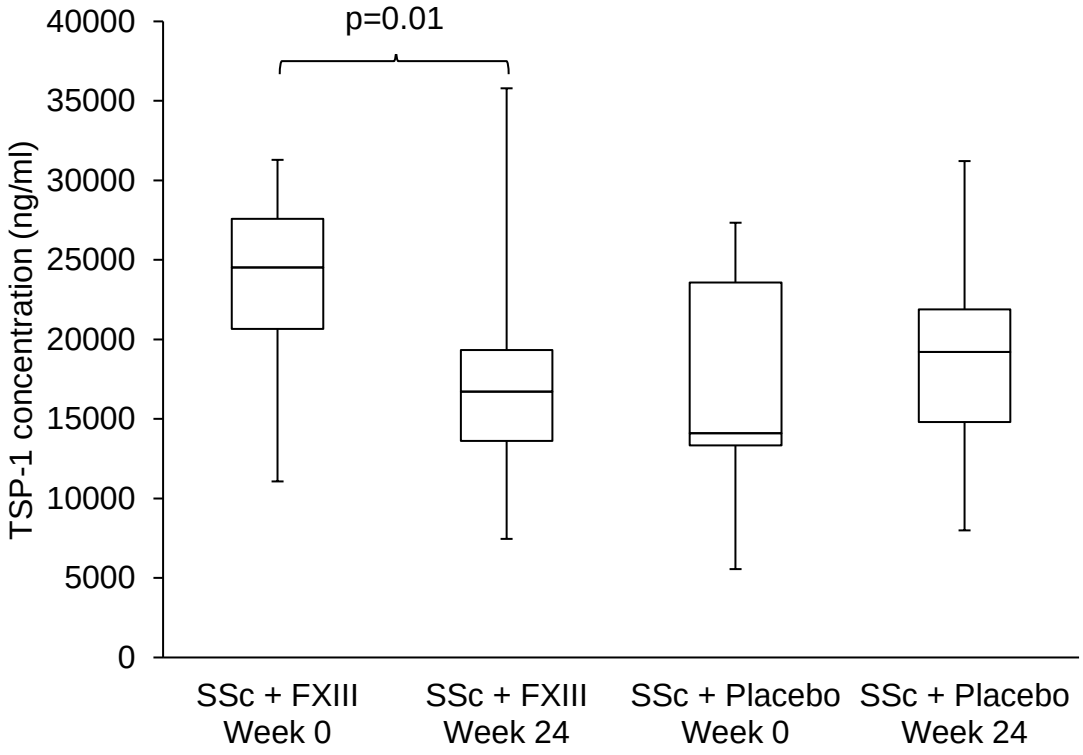


Figure 52 Distribution of TSP-1 concentrations Week 0 vs. Week 24. Boxes represent median and IQR, whiskers represent maximum and minimum values.



6.5.1.4 VEGF-C

Figure 53 VEGF-C concentration at week 0: SSc vs. Healthy. Boxes represent median and IQR, whiskers represent maximum and minimum values.

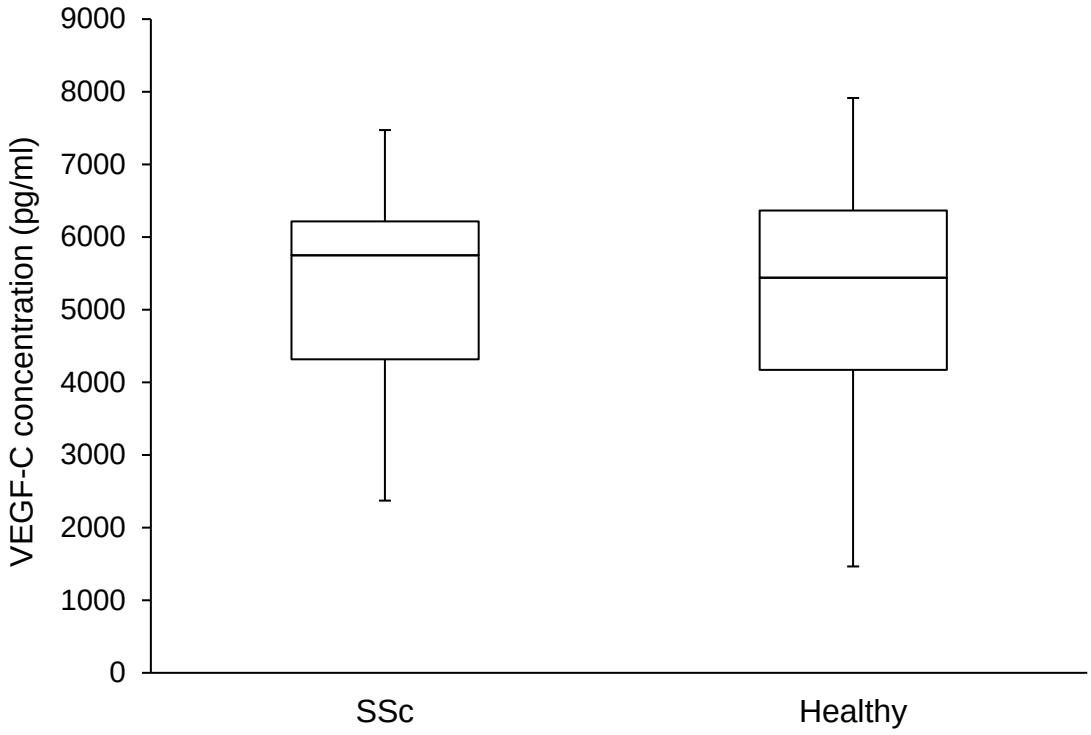


Table 6.4 Descriptive statistics for VEGF-C (pg/ml) at Week 0 and Week 24 in SSc + FXIII, SSc + placebo and healthy control groups

Treatment Group		n	Mean	SD	Min	Median	Max	p-value
SSc + FXIII	Week 0 baseline	12	5842.5	1196.6	3598.5	6198.5	7472.1	
	Week 24	12	5427.3	988.2	3538.9	5731.5	6736.8	
	Change from baseline	12	-415.2	596.6	-1770.7	-414.5	503.6	
	Adjusted mean change from baseline (95% CI)	12	-415.2 (-752.8 to -77.7)					**0.04**
SSc + Placebo	Week 0 baseline	9	5234.6	1718.7	2370.8	5866.6	7405.8	
	Week 24	9	5249.7	1708.3	1999.0	5843.6	7338.2	
	Change from baseline	9	15.1	590.8	-911.6	32.0	1307.0	
	Adjusted mean change	9	-146.4 (-532.4 to 239.6)					0.91

	from baseline (95% CI)							
Difference between groups		Adjusted mean change from baseline (95% CI)		-268.83 (512.80 to -24.86)				0.86
Healthy	Week 0	11	5349.6	1780.9	1464.2	5439.6	7913.6	

Figure 54 Change in VEGF-C concentration from week 0 to week 24

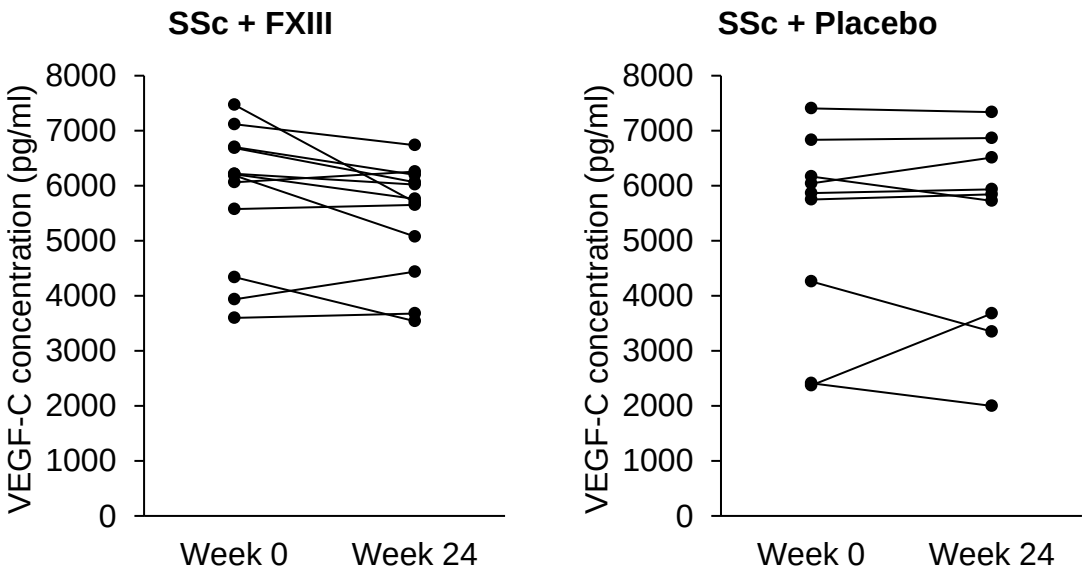
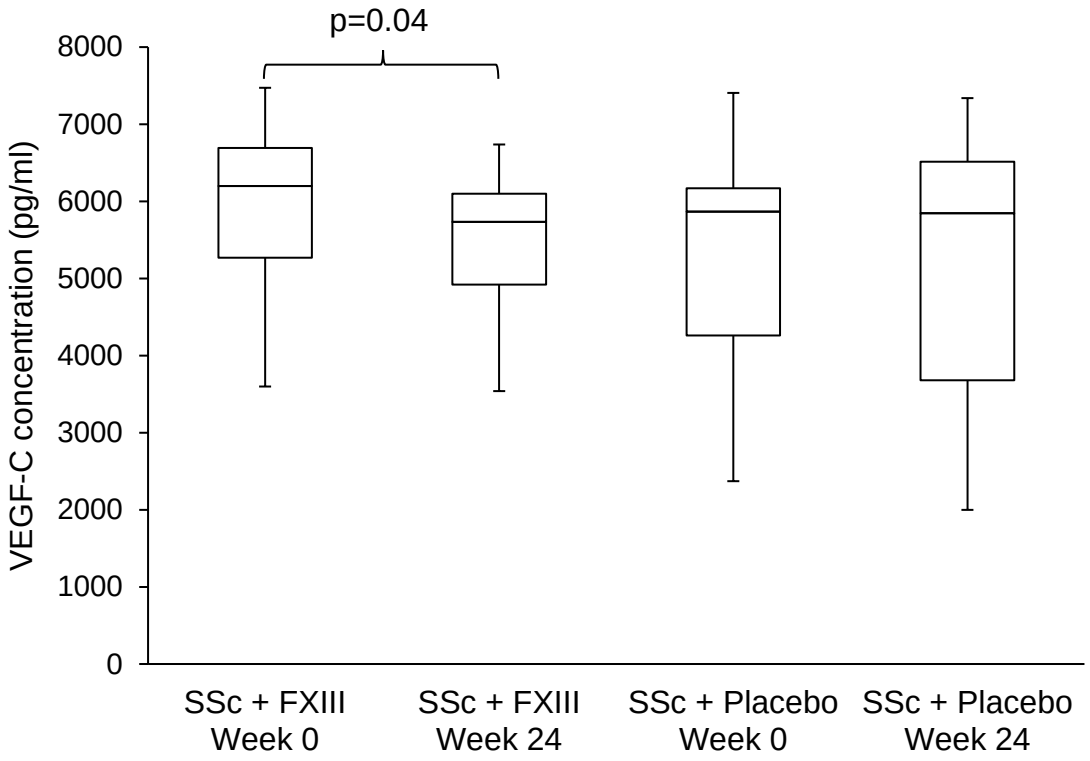


Figure 55 **Distribution of VEGF-C concentrations week 0 vs. week 24. Boxes represent median and IQR, whiskers represent maximum and minimum values.**



6.5.1.5 VEGF-D

Figure 56 VEGF-D concentration at week 0: SSc vs. Healthy. Boxes represent median and IQR, whiskers represent maximum and minimum values.

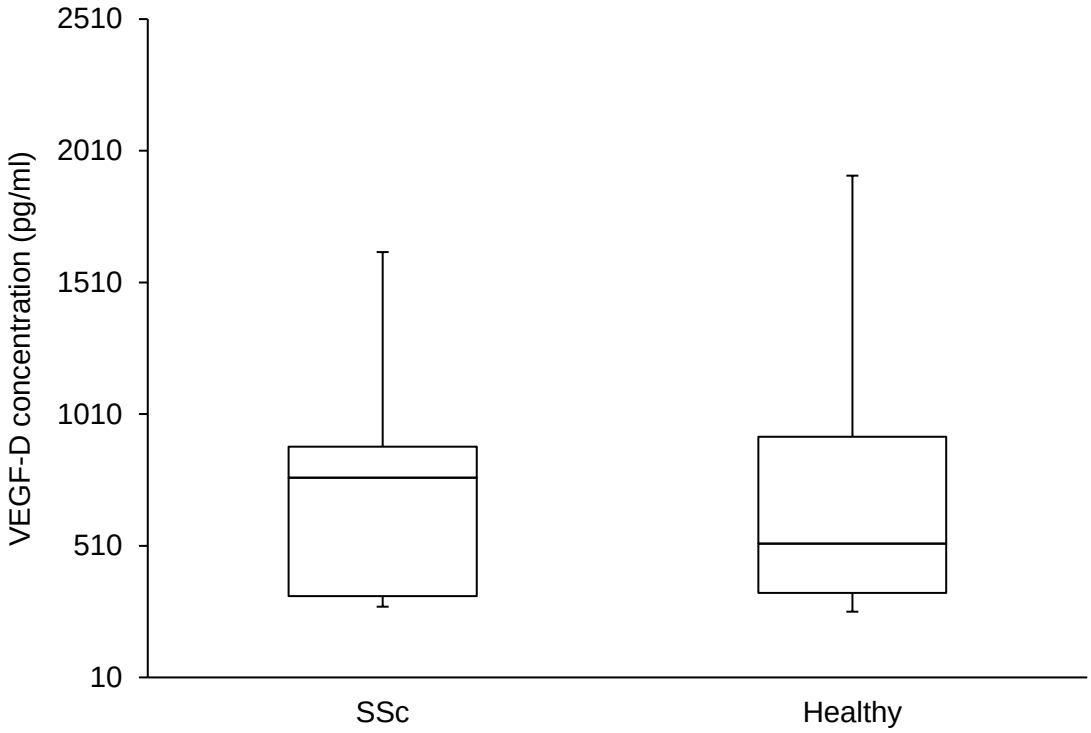


Table 6.5 Descriptive statistics for VEGF-D (pg/ml) at Week 0 and Week 24 in SSc + FXIII, SSc + placebo and healthy control groups

Treatment Group		n	Mean	SD	Min	Median	Max	p-value
SSc + FXIII	Week 0 baseline	12	801.88	220.31	303.77	883.76	998.48	
	Week 24	12	746.54	207.07	343.19	841.82	1002.8	
	Change from baseline	12	-55.34	79.56	-289.38	-42.10	39.42	
	Adjusted mean change from baseline (95% CI)	12	-34.07 (-79.08 to 10.94)					0.59
SSc + Placebo	Week 0 baseline	9	881.35	285.67	520.61	793.28	1625.3	
	Week 24	9	887.11	188.99	575.80	889.96	1315.4	
	Change from baseline	9	5.76	136.65	-309.89	2.48	208.26	
	Adjusted mean change	9	21.92 (-67.35 to 111.20)					**0.02**

	from baseline (95% CI)							
Difference between groups		Adjusted mean change from baseline (95% CI)		-55.99 (-99.98 to -12)				0.18
Healthy	Week 0	11	706.55	464.76	259.61	518.24	1915.5	

Figure 57 Change in VEGF-D concentration from week 0 to week 24

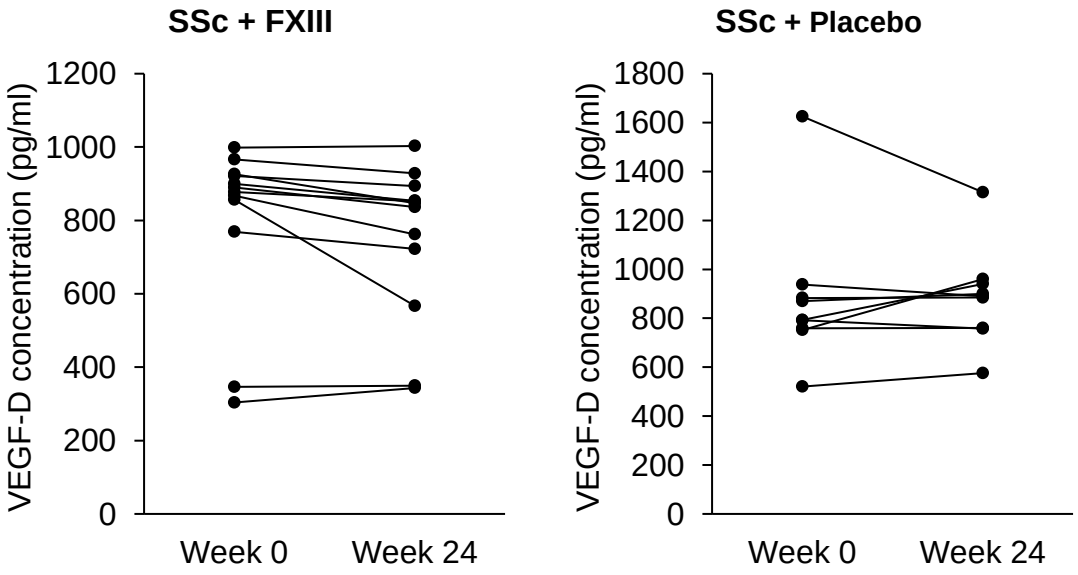
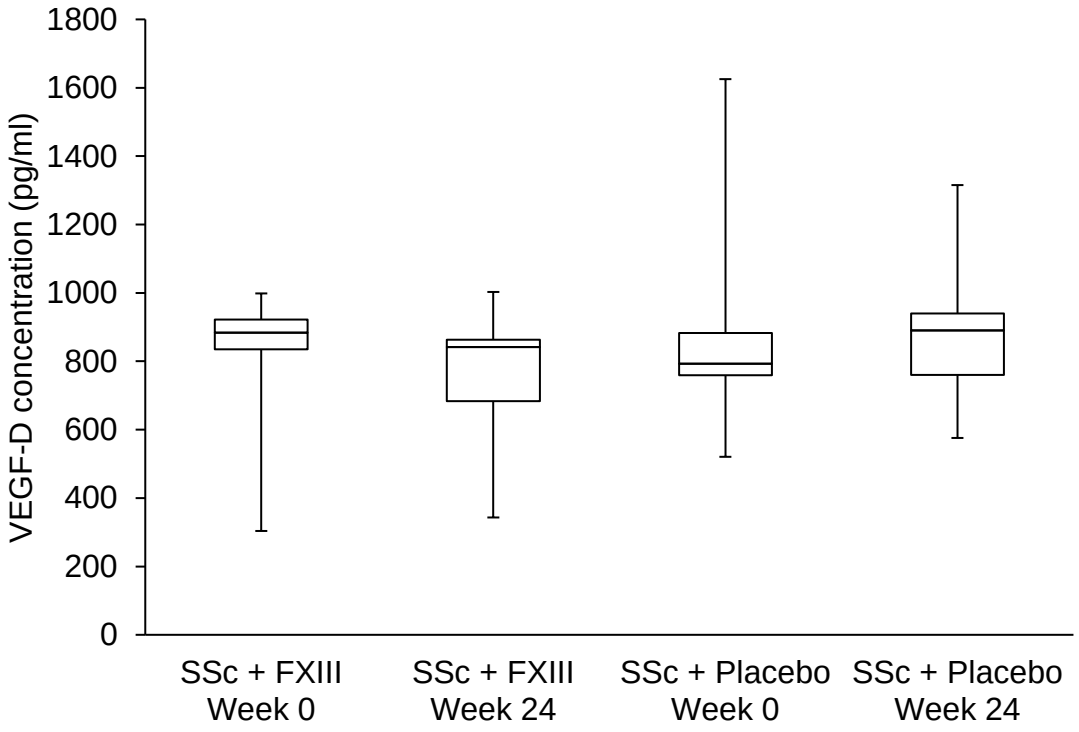


Figure 58 **Distribution of VEGF-D concentrations week 0 vs. week 24. Boxes represent median and IQR, whiskers represent maximum and minimum values.**



6.5.2 Combined ELISA and multiplex assays

6.5.2.1 sCD40L

Figure 59 **sCD40L concentration at week 0: SSc vs. Healthy. Boxes represent median and IQR, whiskers represent maximum and minimum values.**

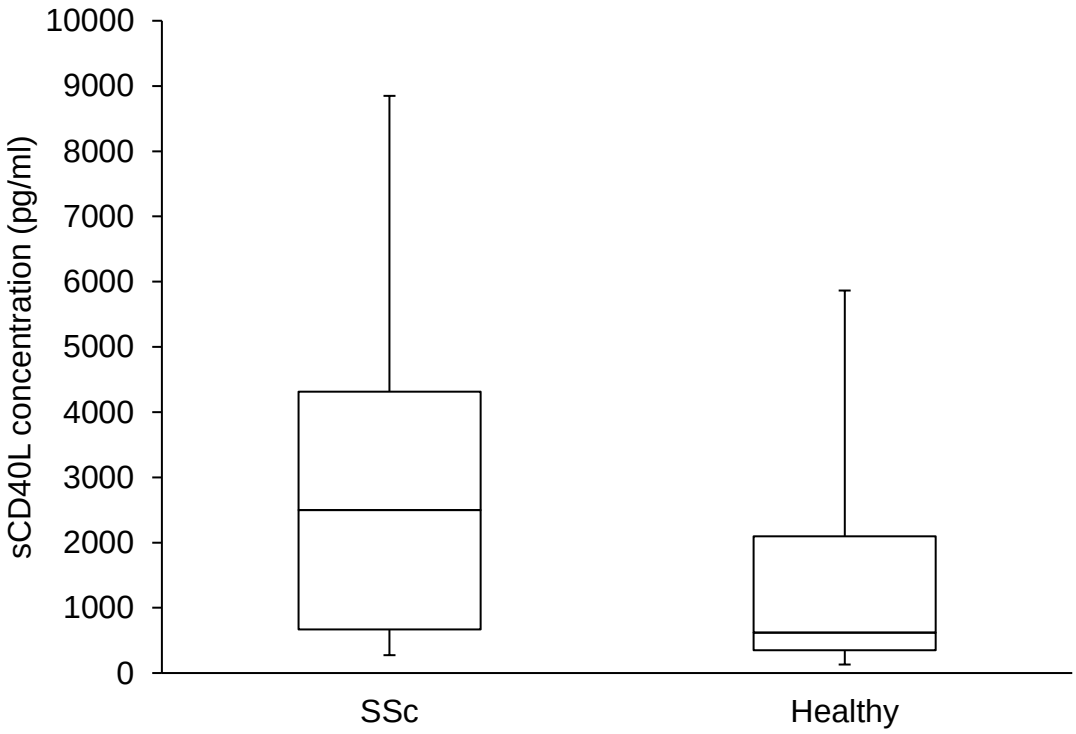


Table 6.6 Descriptive statistics for sCD40L (pg/ml) at week 0 and week 24 in SSc + FXIII, SSc + placebo and healthy control groups

Treatment Group		n	Mean	SD	Min	Median	Max	p-value
SSc + FXIII	Week 0 baseline	12	1799.2	1666.7	475.6	730.3	5673.1	
	Week 24	12	1793.1	1929.3	481.4	657.7	6509.9	
	Change from baseline	12	-6.1	686.8	-888.6	-43.0	1741.3	
	Adjusted mean change from baseline (95% CI)	12	-195.9 (-584.5 to 192.7)					0.43
SSc + Placebo	Week 0 baseline	9	1196.4	1255.5	273.8	723.8	4274.4	
	Week 24	9	1234.4	1047.0	440.9	769.0	3959.6	
	Change from baseline	9	38.0	654.1	-1147	-40.4	1518.2	
	Adjusted mean change	9	-4.1 (-431.4 to 423.2)					0.95

	from baseline (95% CI)							
Difference between groups		Adjusted mean change from baseline (95% CI)		-191.8 (-375.49 to -8.11)				0.94
Healthy	Week 0	11	2682.1	3568.9	130.3	1433.3	12571	

Figure 60 Change in sCD40L concentration from week 0 to Week 24

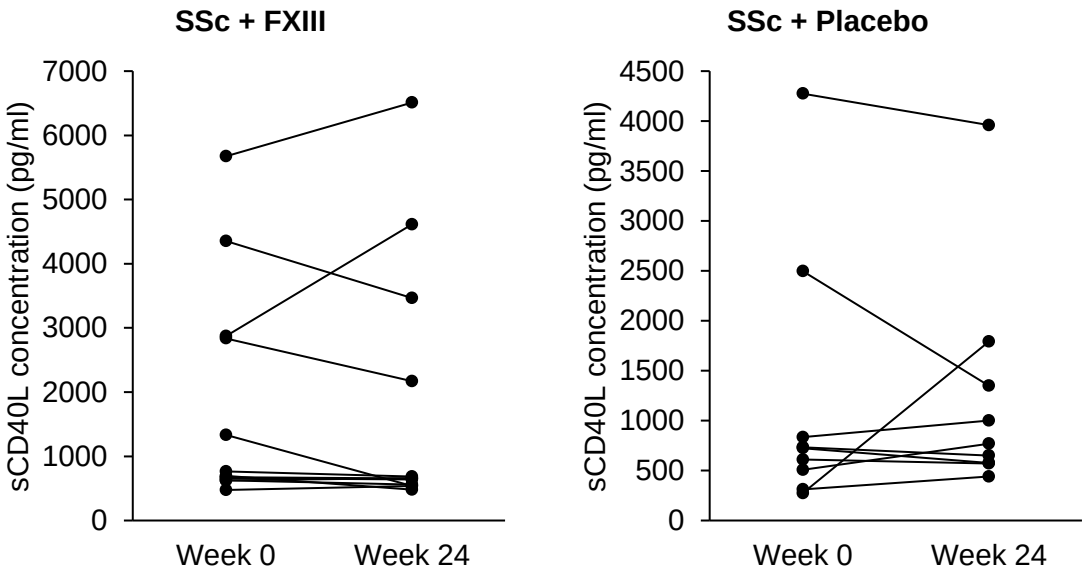
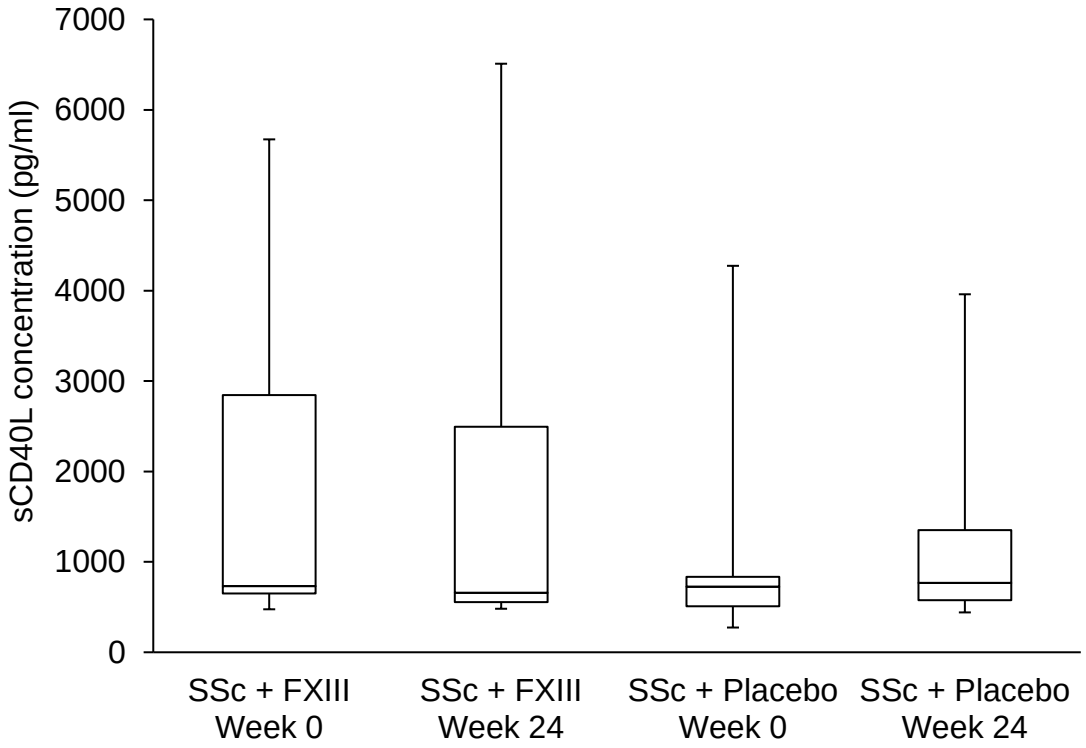


Figure 61 **Distribution of sCD40L concentrations at week 0 vs. week 24. Boxes represent median and IQR, whiskers represent maximum and minimum values.**



6.5.2.2 Tumour necrosis factor alpha

Figure 62 TNFα concentration at week 0: SSc vs. Healthy. Boxes represent median and IQR, whiskers represent maximum and minimum values.

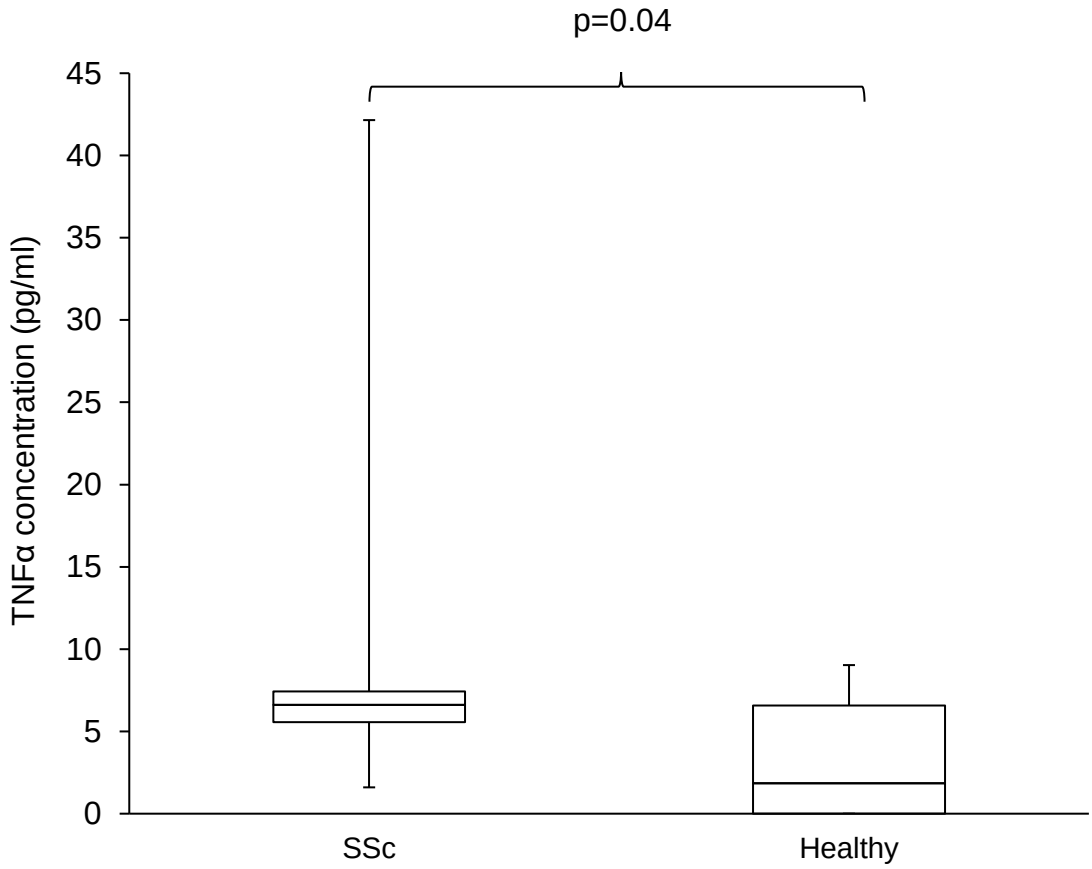


Table 6.7 Descriptive statistics for TNFα (pg/ml) from week 0 to week 24 in SSc + FXIII, SSc + placebo and healthy control groups

Treatment Group		n	Mean	SD	Min	Median	Max	p-value
SSc + FXIII	Week 0 baseline	12	6.54	1.76	1.60	6.86	8.57	
	Week 24	12	4.82	2.17	0.11	5.00	7.43	
	Change from baseline	12	-1.72	2.18	-6.72	-0.76	0.99	
	Adjusted mean change from baseline (95% CI)	12	-0.84 (-2.08 to 0.39)					0.02
SSc + Placebo	Week 0 baseline	6	11.87	13.59	3.59	6.46	42.15	
	Week 24	6	8.17	6.39	2.84	6.05	22.10	
	Change from baseline	6	-3.70	7.42	-20.05	-0.69	1.90	
	Adjusted mean change	6	-0.43 (-6.37 to 5.51)					0.17

	from baseline (95% CI)							
Difference between groups		Adjusted mean change from baseline (95% CI)		-0.41 (-6.07 to 5.24)				0.42
Healthy	Week 0	12	4.02	3.64	0.00	3.91	9.03	

Figure 63 Change in TNFα concentration from week 0 to week 24

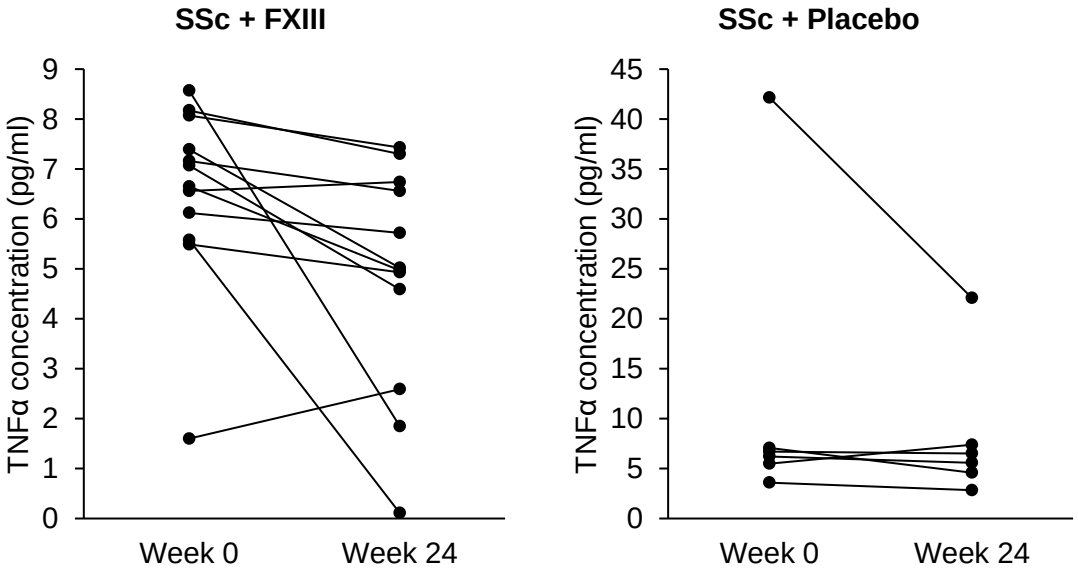
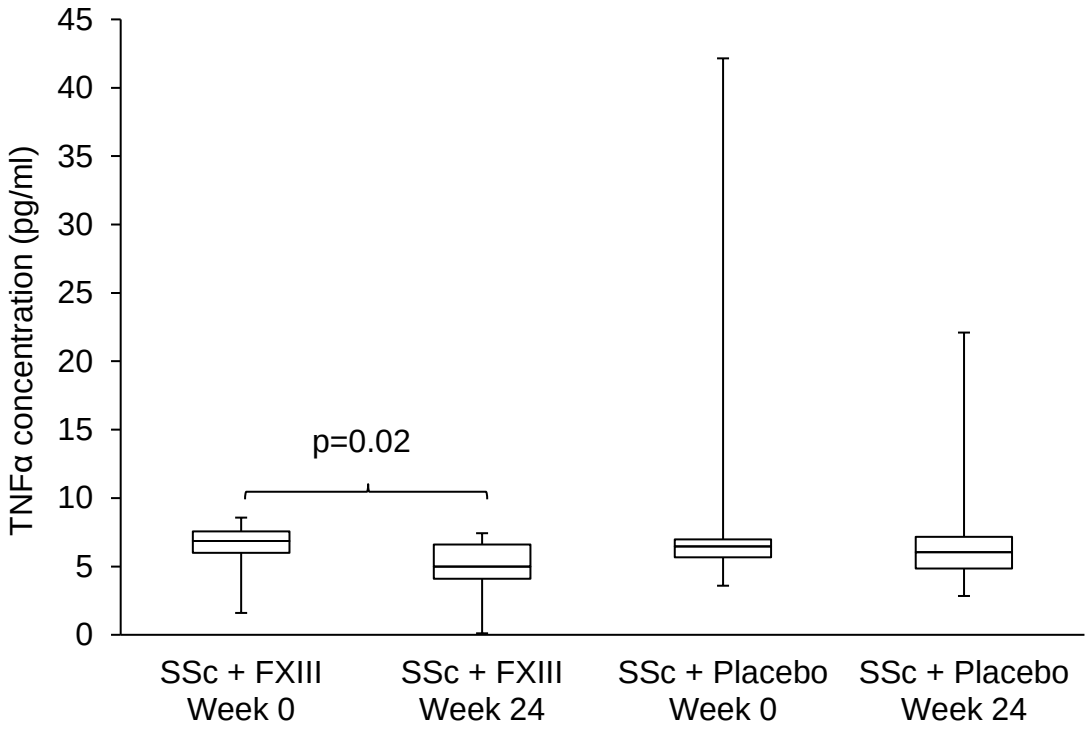


Figure 64 **Distribution of TNFα concentrations week 0 vs. week 24. Boxes represent median and IQR, whiskers represent maximum and minimum values.**



6.5.3 Multiplex assays

6.5.3.1 Angiopoietin-2

Figure 65 Angiopoietin-2 concentration at Week 0: SSc vs. Healthy. Boxes represent median and IQR, whiskers represent maximum and minimum values.

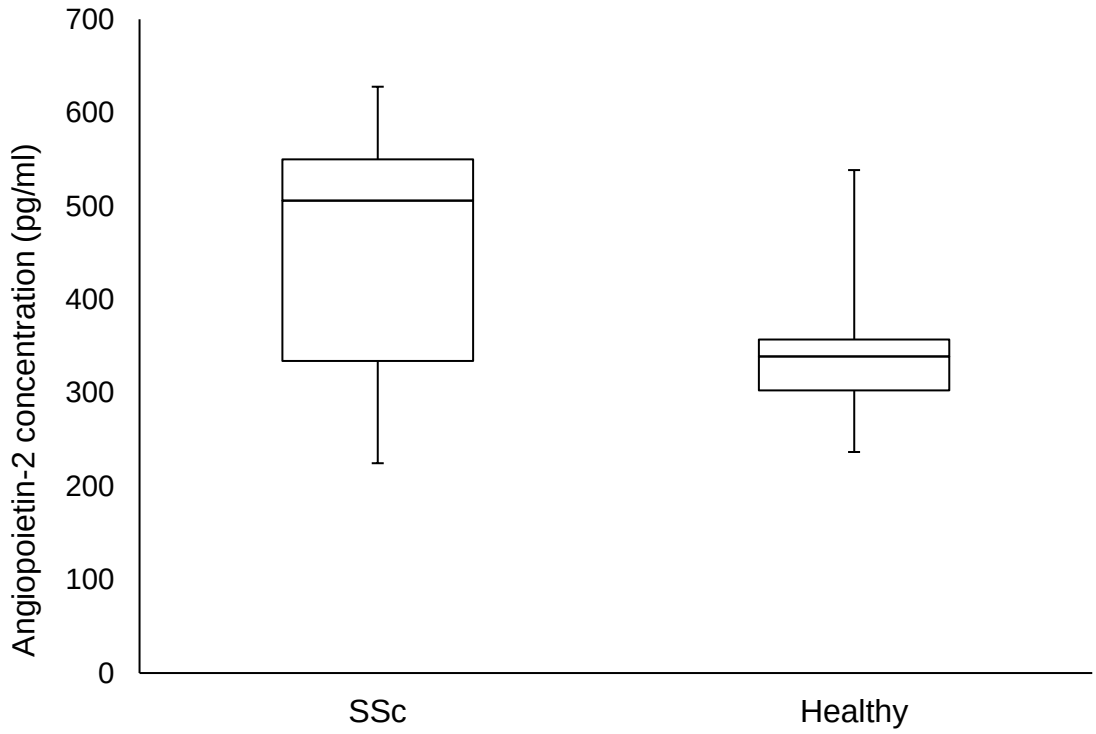


Table 6.8 Descriptive statistics for angiopoietin-2 (pg/ml) at Week 0 and Week 24 in SSc + FXIII, SSc + placebo and healthy control groups

Treatment Group		n	Mean	SD	Min	Median	Max	p-value
SSc + FXIII	Week 0 baseline	9	505.39	104.42	313.83	530.44	627.81	
	Week 24	9	446.18	84.00	312.83	422.40	585.16	
	Change from baseline	9	-59.21	84.90	-180.01	-79.07	119.03	
	Adjusted mean change from baseline (95% CI)	9	-81.49 (-136.95 to -26.02)					0.07
SSc + Placebo	Week 0 baseline	6	354.91	116.73	224.64	301.30	535.50	
	Week 24	6	380.49	141.40	174.60	370.08	556.77	
	Change from baseline	6	25.58	48.92	-50.04	29.20	98.41	
	Adjusted mean	6	25.58 (-13.56 to 64.72)					0.25

	change from baseline (95% CI)							
Difference between groups		Adjusted mean change from baseline (95% CI		-107.07 (-146.24 to -67.89)				0.38
Healthy	Week 0	5	354.78	100.68	236.66	338.92	538.54	

Figure 66 Change in angiopoietin-2 concentration from week 0 to week 24

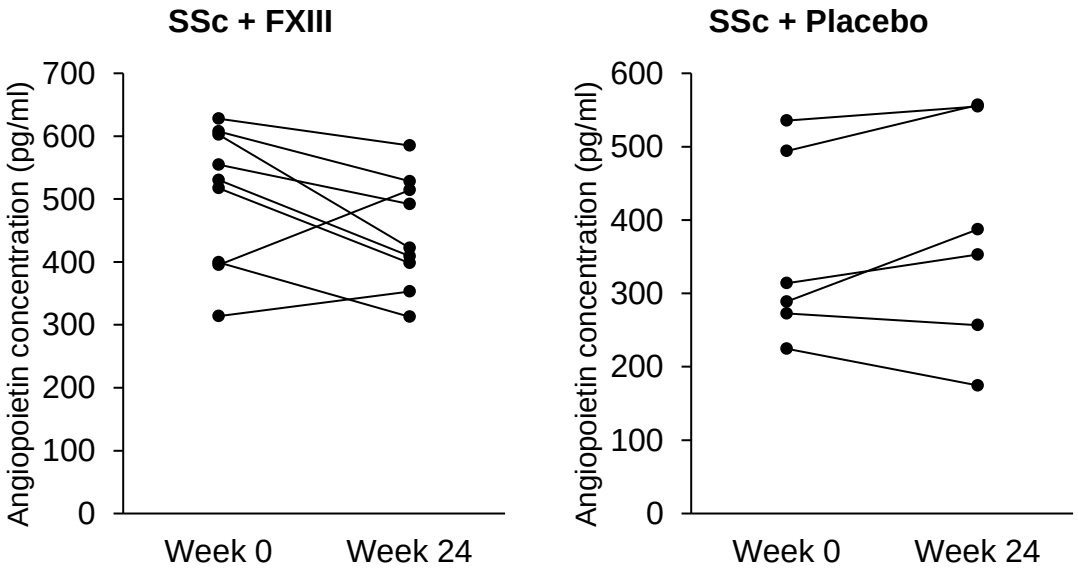
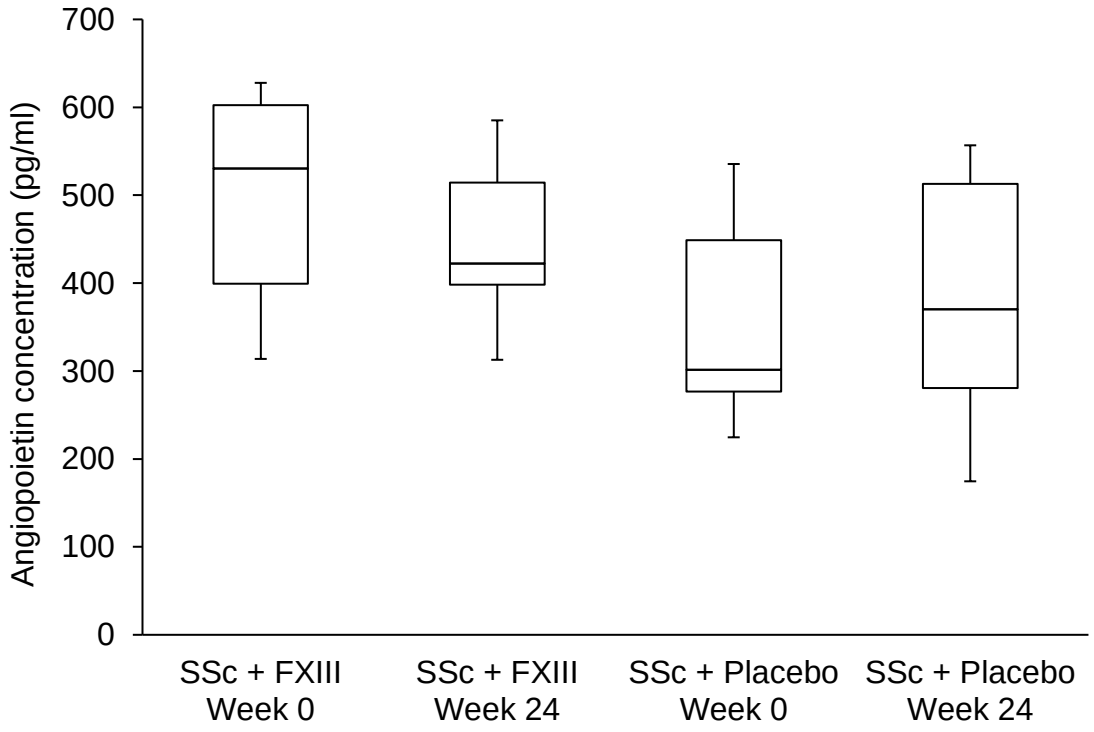


Figure 67 **Distribution of angiopoietin-2 concentrations week 0 vs. week 24. Boxes represent median and IQR, whiskers represent maximum and minimum values.**



6.5.3.2 EGF

Figure 68 EGF concentration at Week 0: SSc vs. Healthy. Boxes represent median and IQR, whiskers represent maximum and minimum values.

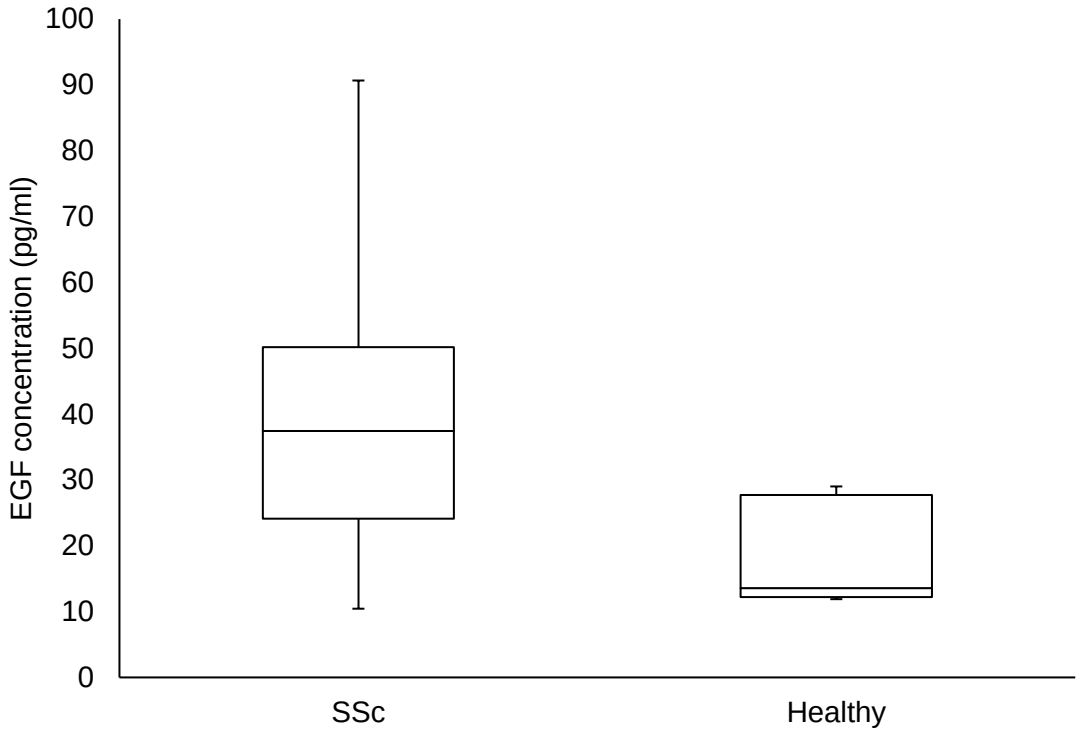


Table 6.9 Descriptive statistics for EGF (pg/ml) at Week 0 and Week 24 in SSc + FXIII, SSc + placebo and healthy control groups

Treatment Group		n	Mean	SD	Min	Median	Max	p-value
SSc + FXIII	Week 0 baseline	9	45.59	22.64	10.44	38.33	90.67	
	Week 24	9	27.70	12.44	9.62	26.54	57.67	
	Change from baseline		-17.89	17.26	-54.96	-10.00	-0.82	
	Adjusted mean change from baseline (95% CI)		-17.89 (-29.17 to -6.62)					**0.01**
SSc + Placebo	Week 0 baseline	6	37.96	27.54	11.09	30.50	90.67	
	Week 24	6	34.58	14.11	17.55	35.07	57.67	
	Change from baseline		-3.38	17.04	-33.00	-1.08	20.83	
	Adjusted mean change from baseline (95% CI)		-3.38 (-17.01 to 10.25)					0.60

Difference between groups		Adjusted mean change from baseline (95% CI)		-14.51 (-17.69 to -11.33)				0.38
Healthy	Week 0	5	18.88	7.77	11.89	13.59	29.01	

Figure 69 Change in EGF concentration from week 0 to week 24

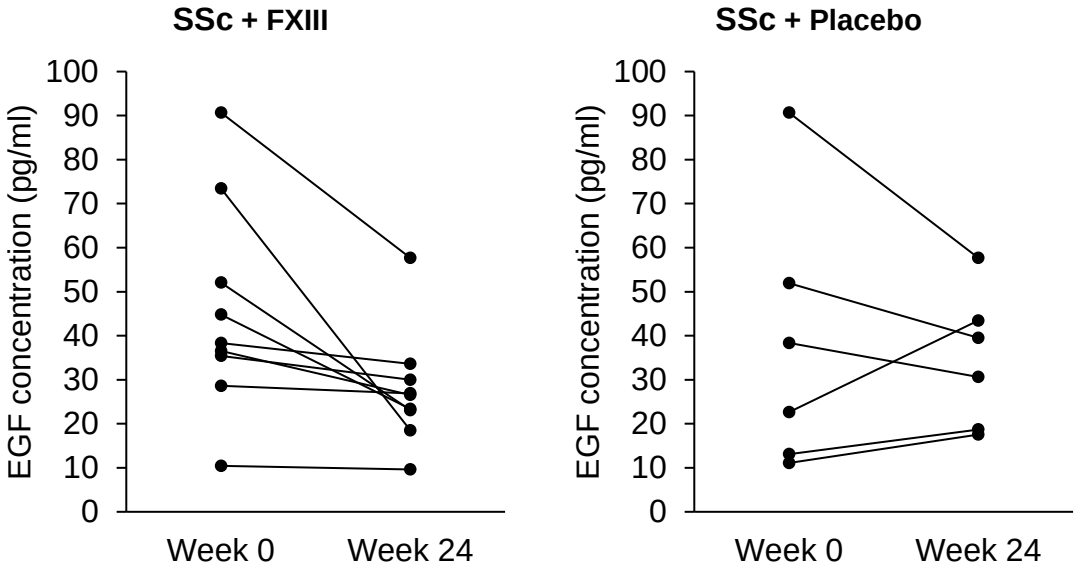
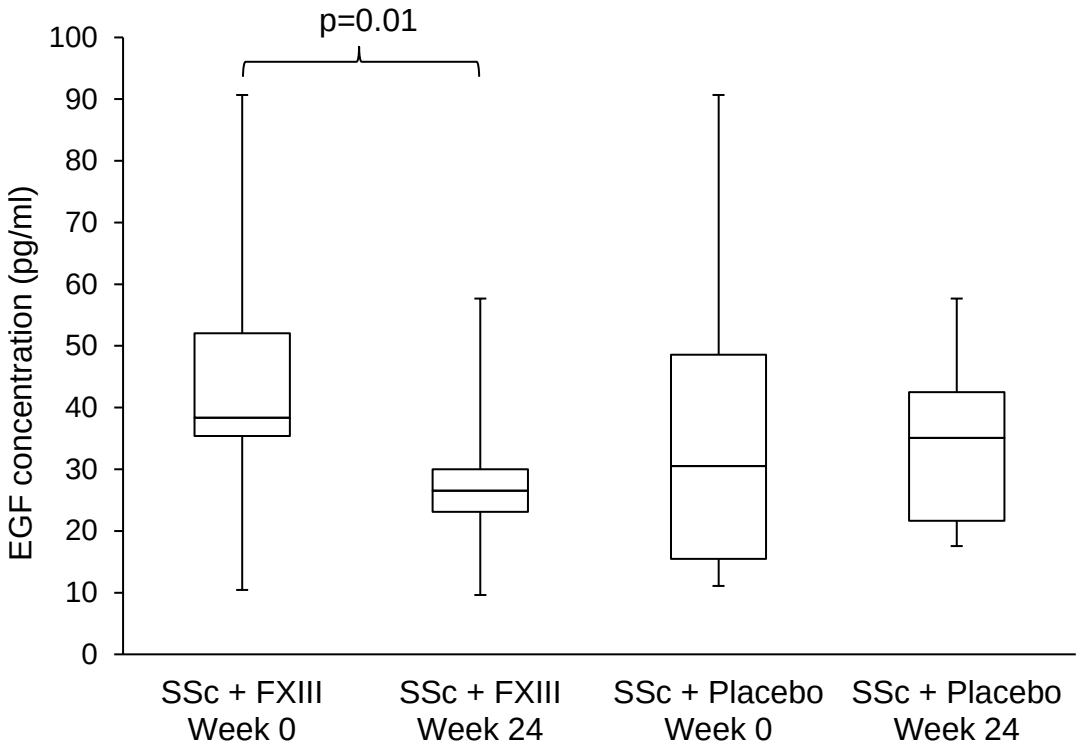


Figure 70 **Distribution of EGF concentrations week 0 vs. week 24. Boxes represent median and IQR, whiskers represent maximum and minimum values.**



6.5.3.3 Endoglin

Figure 71 Endoglin concentration at Week 0: SSc vs. Healthy. Boxes represent median and IQR, whiskers represent maximum and minimum values.

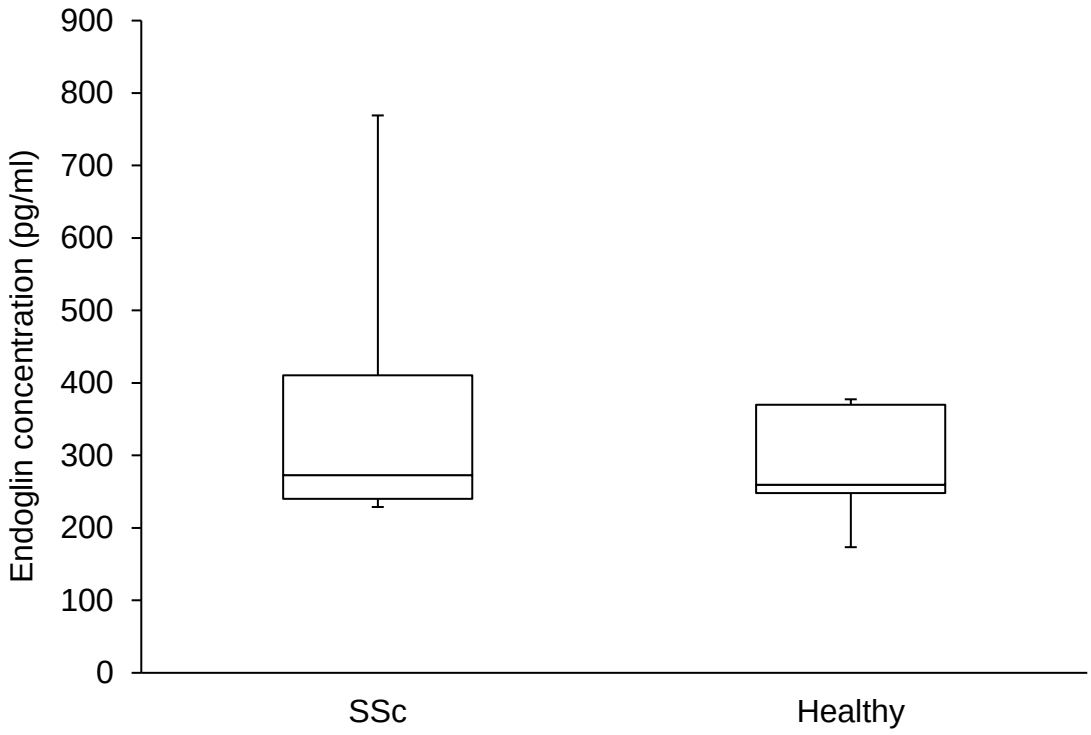


Table 6.10 Descriptive statistics for endoglin (pg/ml) at Week 0 and Week 24 in SSc + FXIII, SSc + placebo and healthy control groups

Treatment Group		n	Mean	SD	Min	Median	Max	p-value
SSc + FXIII	Week 0 baseline	9	324.27	110.70	228.82	287.14	536.96	
	Week 24	9	325.20	166.64	201.23	263.04	774.68	
	Change from baseline	9	0.92	189.99	-302.80	12.08	450.10	
	Adjusted mean change from baseline (95% CI)	9	-19.86 (-143.98 to 104.27)					0.86
SSc + Placebo	Week 0 baseline	6	407.30	192.25	233.06	348.67	769.13	
	Week 24	6	368.46	134.12	201.23	323.77	574.04	
	Change from baseline	6	-38.84	208.32	-302.80	-33.33	315.77	
	Adjusted mean change	6	-38.84 (-205.53 to 127.85)					0.75

	from baseline (95% CI)							
Difference between groups		Adjusted mean change from baseline (95% CI)		18.98 (-207.83 to 245.8)				0.32
Healthy	Week 0	5	285.70	77.76	173.38	259.59	377.44	

Figure 72 Change in endoglin concentration from week 0 to week 24

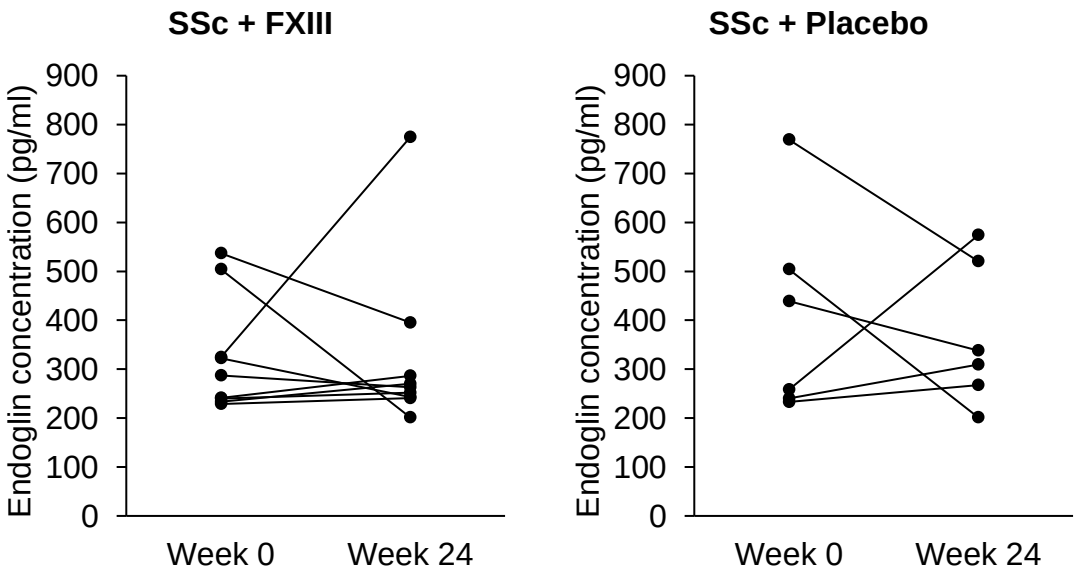
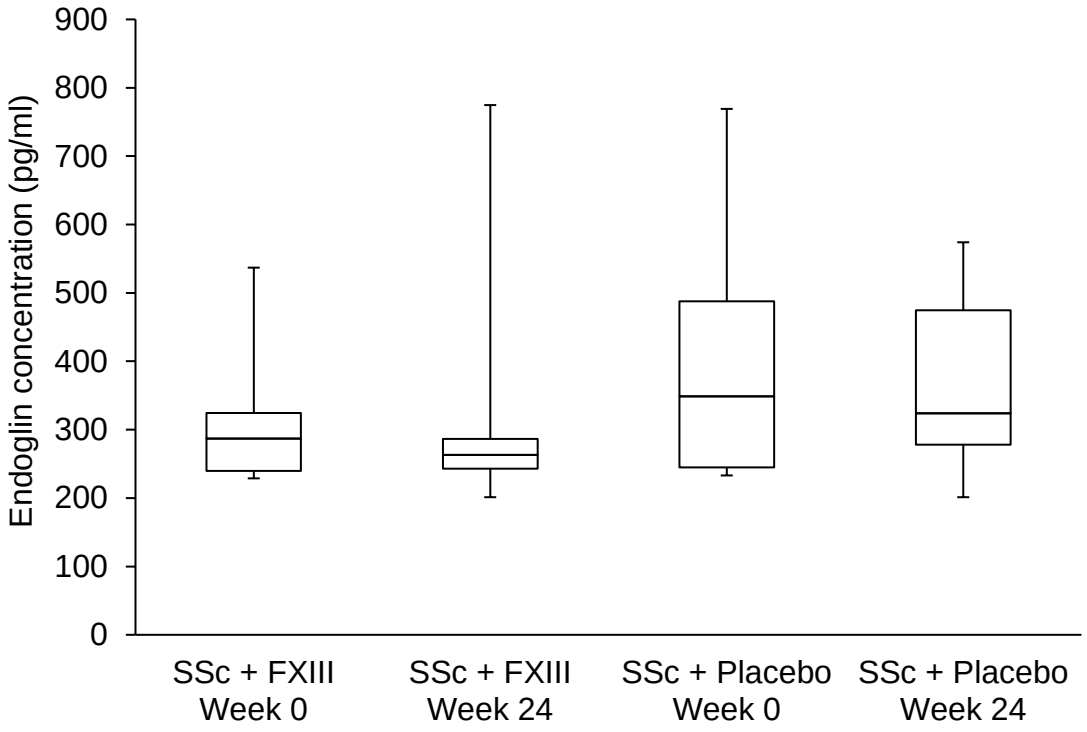


Figure 73 **Distribution of EGF concentrations week 0 vs. week 24. Boxes represent median and IQR, whiskers represent maximum and minimum values.**



6.5.3.4 sFASL

There was no significant difference in sFASL levels when SSc patients were compared to healthy controls (p=0.36).

Figure 74 sFASL concentration at Week 0: SSc vs. Healthy. Boxes represent median and IQR, whiskers represent maximum and minimum values.

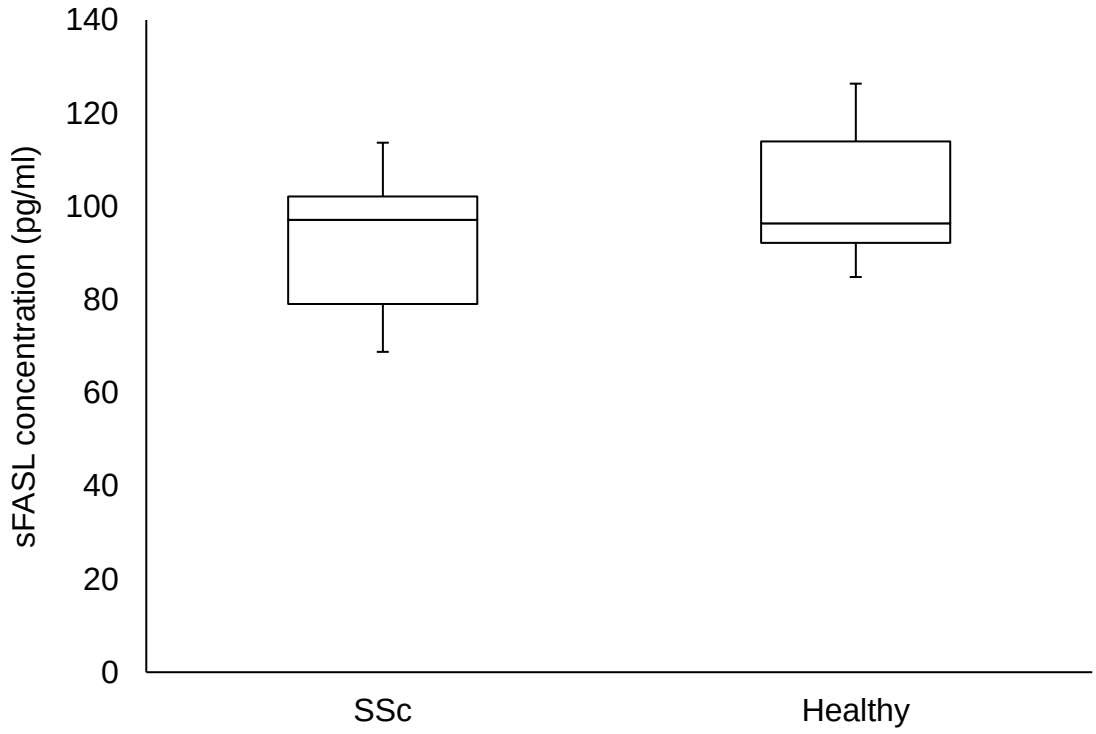


Table 6.11 Descriptive statistics for sFASL (pg/ml) at Week 0 and Week 24 in SSc + FXIII, SSc + placebo and healthy control groups

Treatment Group		n	Mean	SD	Min	Median	Max	p-value
SSc + FXIII	Week 0 baseline	9	92.89	13.72	68.76	96.95	113.66	
	Week 24	9	85.75	15.01	66.08	84.83	105.71	
	Change from baseline	9	-7.14	8.03	-21.10	-10.47	6.40	
	Adjusted mean change from baseline (95% CI)	9	-7.14 (-12.38 to -1.89)					**0.04**
SSc + Placebo	Week 0 baseline	6	88.13	13.34	71.42	87.58	104.56	
	Week 24	6	87.97	15.10	67.43	90.36	105.71	
	Change from baseline	6	-0.16	7.74	-10.47	-0.99	13.77	
	Adjusted mean change	6	-0.16 (-6.34 to 6.03)					0.89

	from baseline (95% CI)							
Difference between groups		Adjusted mean change from baseline (95% CI)		-6.98 (-8.11 to -5.85)				0.59
Healthy	Week 0	5	102.73	15.20	84.83	96.36	126.33	

Figure 75 Change in sFASL concentration from week 0 to week 24

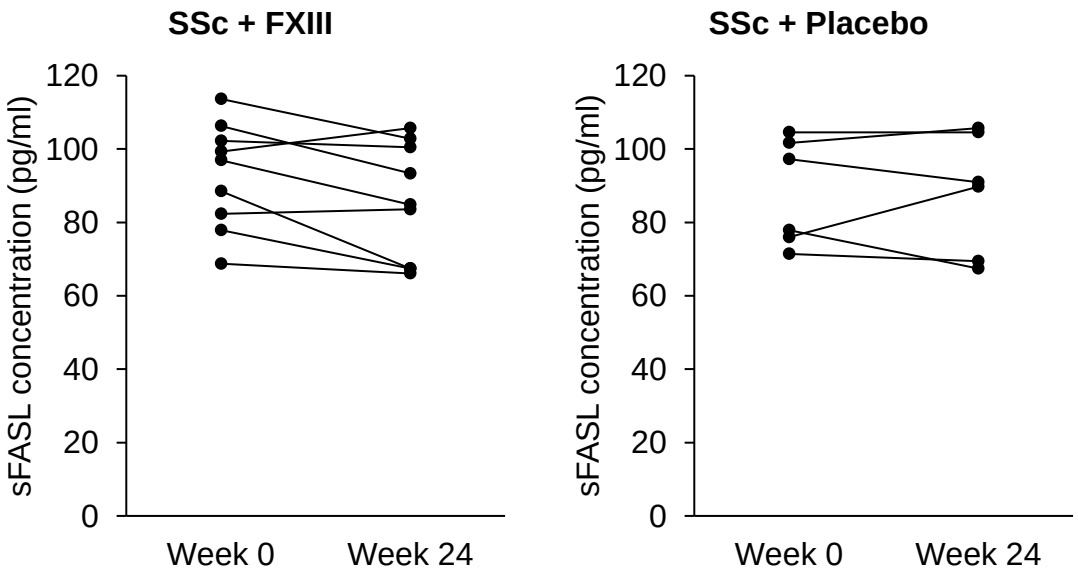
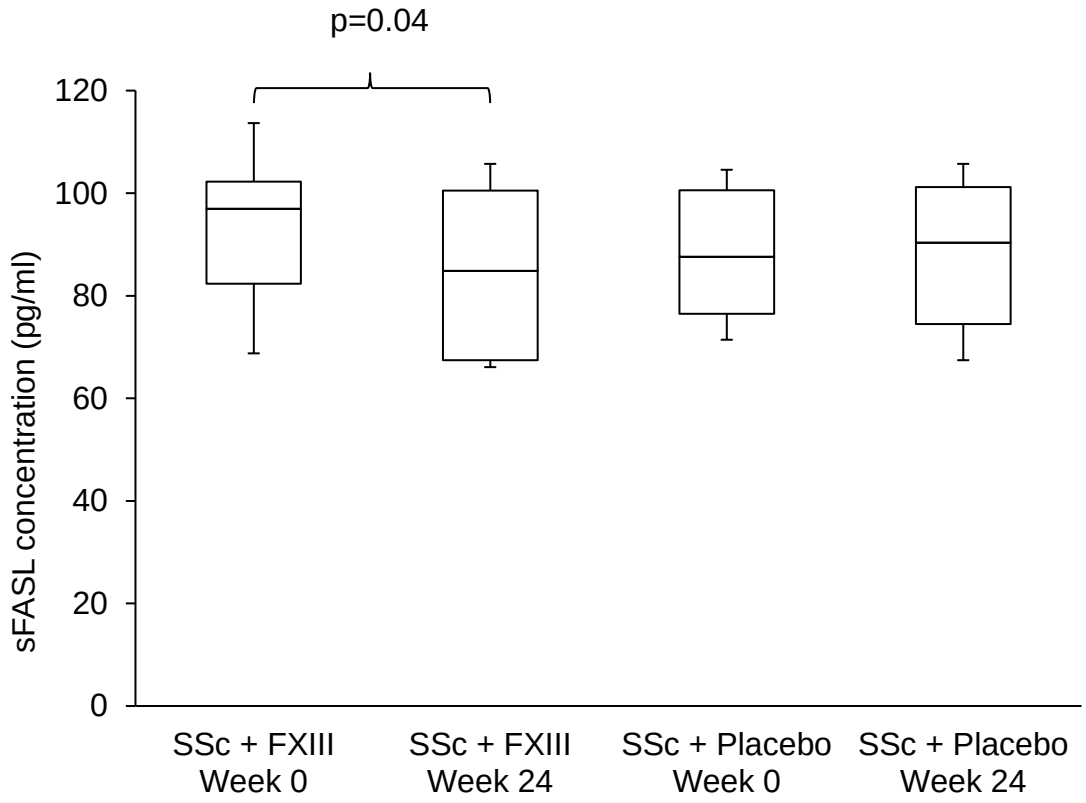


Figure 76 **Distribution of sFASL concentrations week 0 vs. week 24. Boxes represent median and IQR, whiskers represent maximum and minimum values.**



6.5.3.5 HB-EGF

Figure 77 HB-EGF concentration at week 0: SSc vs. Healthy. Boxes represent median and IQR, whiskers represent maximum and minimum values.

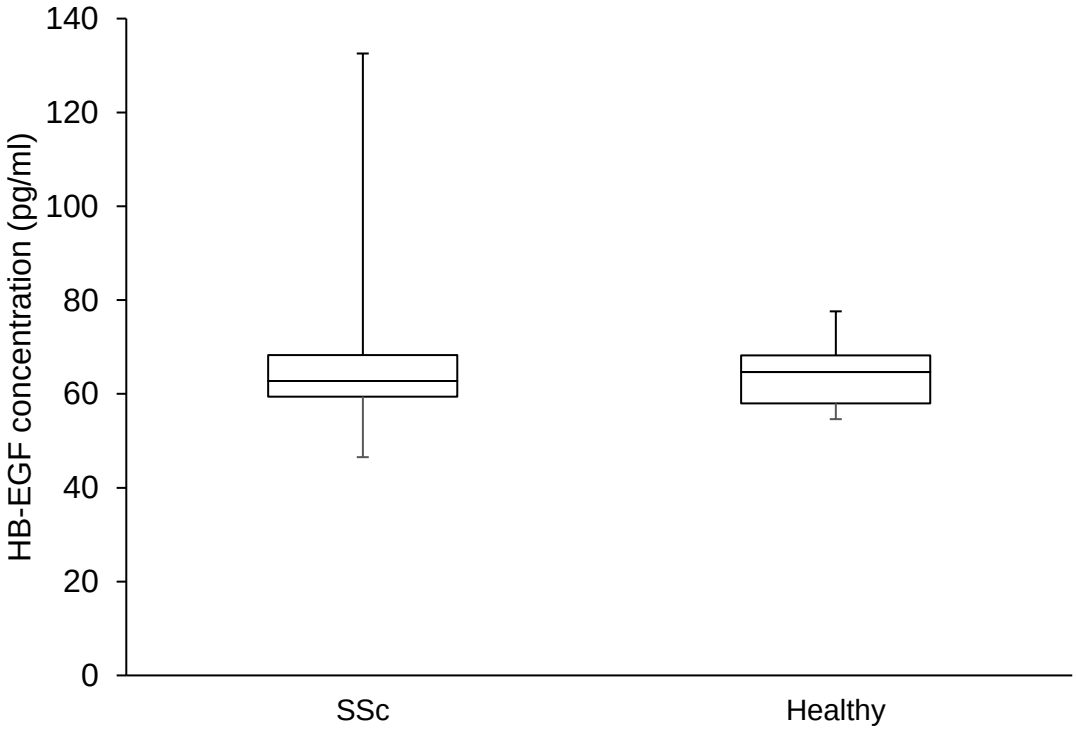


Table 6.12 Descriptive statistics for HB-EGF (pg/ml) at Week 0 and Week 24 in SSc + FXIII, SSc + placebo and healthy control groups

Treatment Group		n	Mean	SD	Min	Median	Max	p-value
SSc + FXIII	Week 0 baseline	9	64.85	5.96	52.29	65.17	73.81	
	Week 24	9	60.34	8.31	47.57	60.56	76.08	
	Change from baseline	9	-4.51	5.11	-13.53	-4.72	4.08	
	Adjusted mean change from baseline (95% CI)	9	-4.51 (-7.84 to -1.17)					**0.04**
SSc + Placebo	Week 0 baseline	6	66.83	29.87	46.52	55.59	132.56	
	Week 24	6	69.74	28.65	47.31	62.42	131.53	
	Change from baseline	6	2.91	7.31	-4.72	0.71	18.31	
	Adjusted mean change	6	-0.17 (-6.02 to 5.68)					0.60

	from baseline (95% CI)							
Difference between groups		Adjusted mean change from baseline (95% CI)		-4.34 (-1.94 to -6.74)				0.95
Healthy	Week 0	5	64.62	8.06	54.63	64.65	77.60	

Figure 78 Change in HB-EGF concentration from week 0 to week 24

Dashed line represents outlying value (see text above)

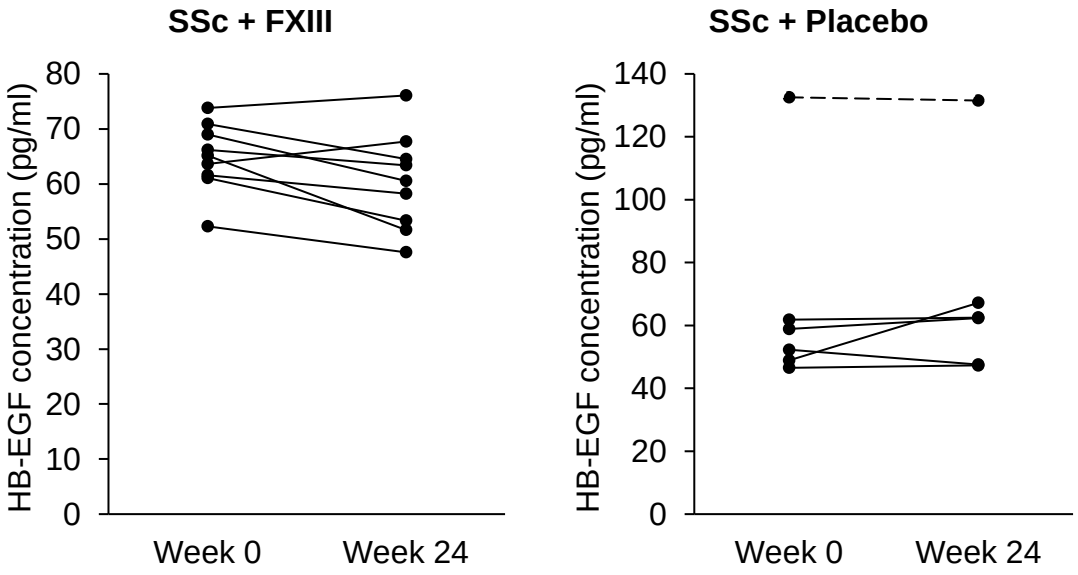
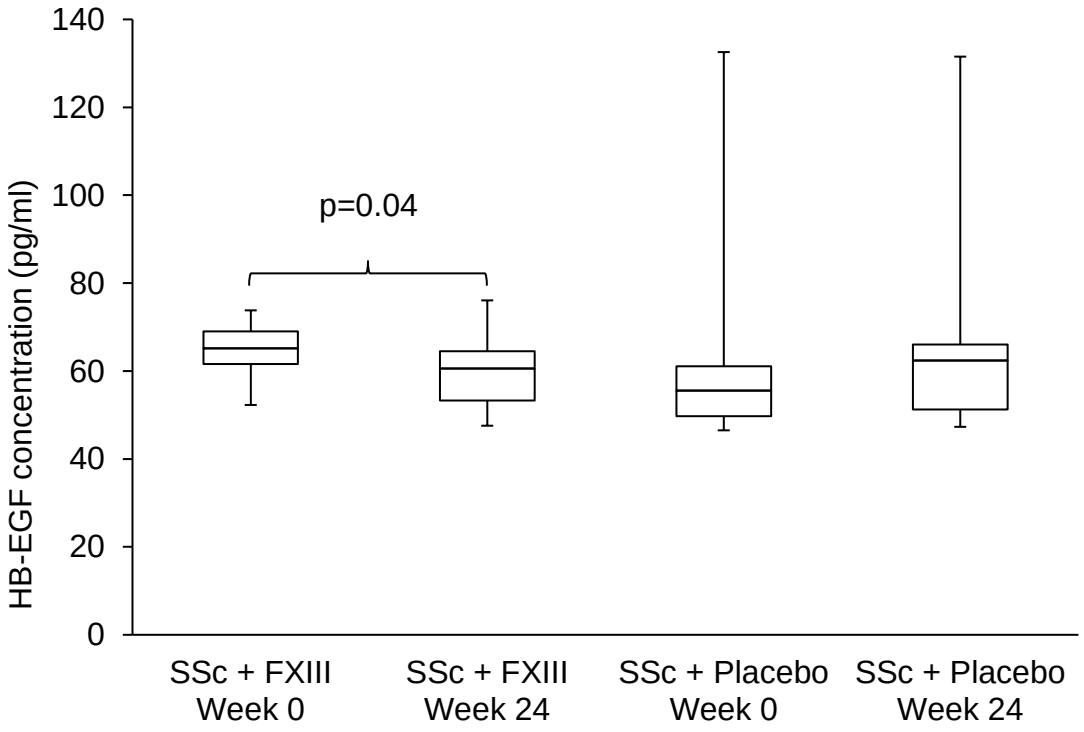


Figure 79 **Distribution of HB-EGF concentrations week 0 vs. week 24. Boxes represent median and IQR, whiskers represent maximum and minimum values.**



6.5.3.6 IGFBP-1

Figure 80 IGFBP-1 concentration at Week 0: SSc vs. Healthy. Boxes represent median and IQR, whiskers represent maximum and minimum values.

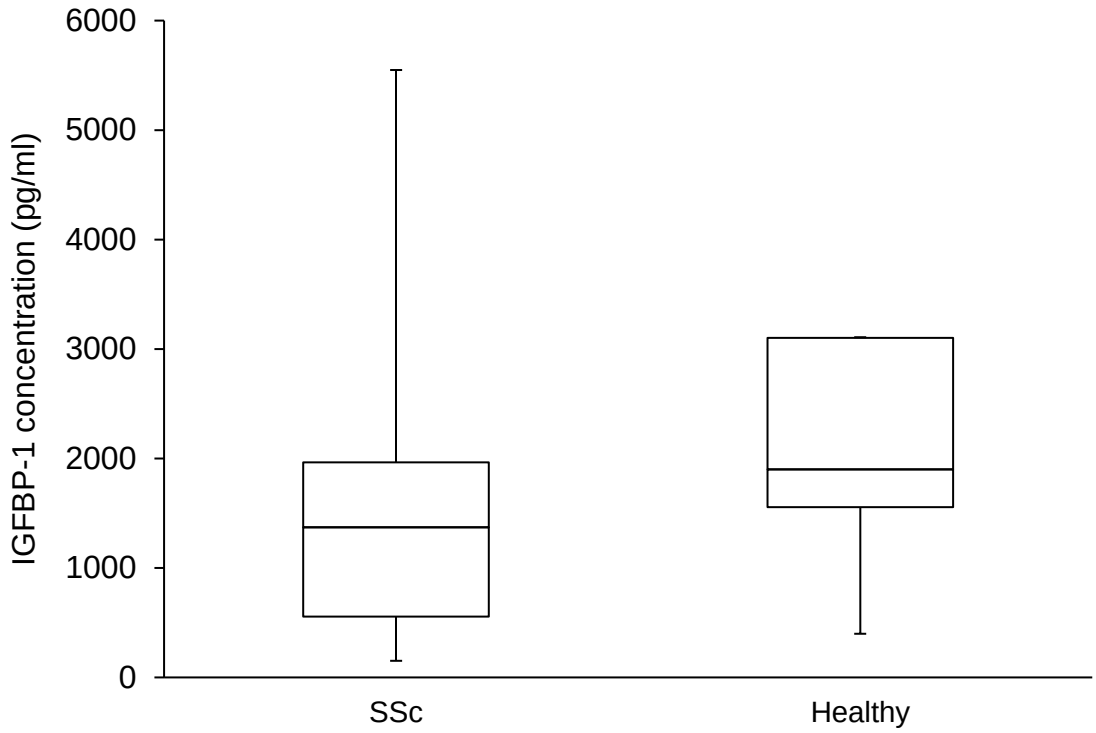


Table 6.13 Descriptive statistics for IGFBP-1 (pg/ml) at Week 0 and Week 24 in SSc + FXIII, SSc + placebo and healthy control groups

Treatment Group		n	Mean	SD	Min	Median	Max	p-value
SSc + FXIII	Week 0 baseline	9	2263.9	1875.0	441.5	1510.3	5548.5	
	Week 24	9	2106.5	1735.7	344.8	1564.1	5504.6	
	Change from baseline	9	-157.4	1714.3	-2859.6	-150.5	3994.3	
	Adjusted mean change from baseline (95% CI)	9	-364.5 (-1484.5 to 755.5)					0.31
SSc + Placebo	Week 0 baseline	6	834.0	509.9	151.7	701.4	1674.0	
	Week 24	6	1787.7	2332.2	195.2	928.8	6867.6	
	Change from baseline	6	953.7	2081.5	-229.2	-35.3	5567.5	
	Adjusted mean change from	6	31.0 (-1634.5 to 1696.5)					0.75

	baseline (95% CI)							
Difference between groups		Adjusted mean change from baseline (95% CI)		-395.47 (-2007.09 to 1216.15)				0.32
Healthy	Week 0	5	2012.8	1020.5	398.7	1901.0	3107.6	

Figure 81 Change in IGFBP-1 concentration from week 0 to week 24

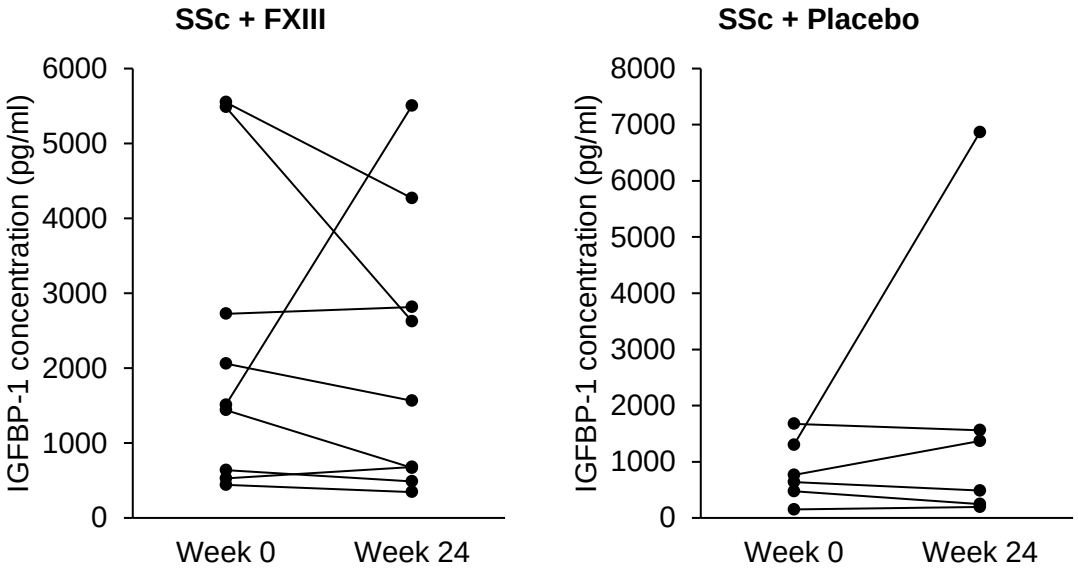
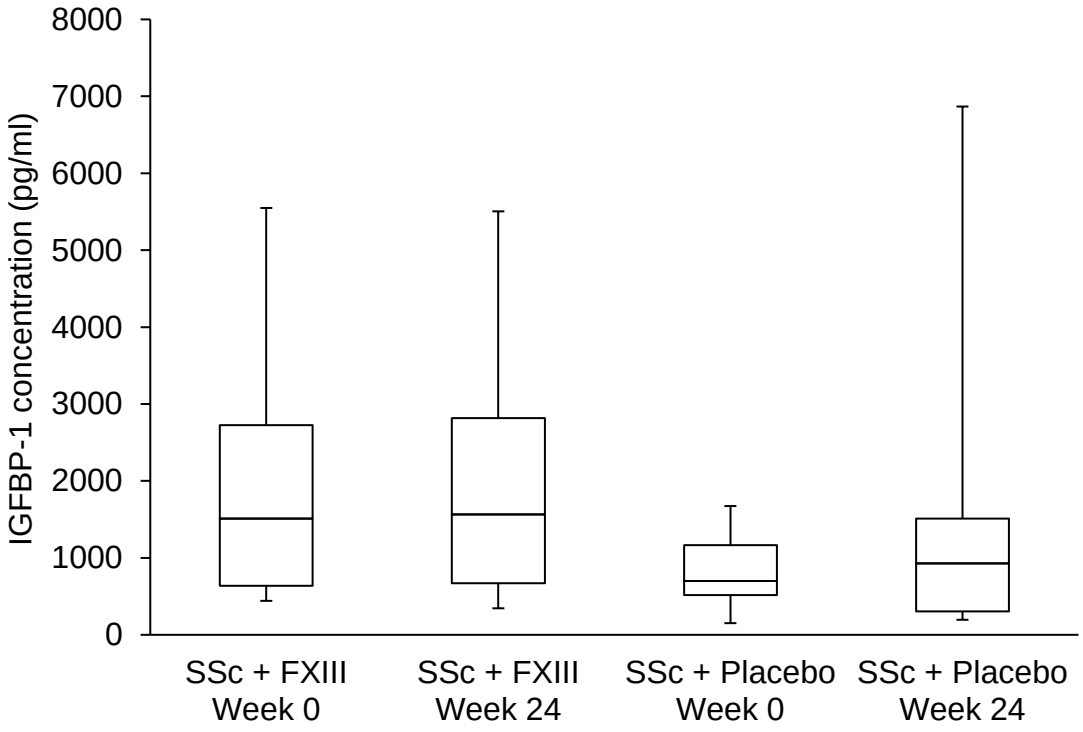


Figure 82 **Distribution of IGFBP-1 concentrations week 0 vs. week 24. Boxes represent median and IQR, whiskers represent maximum and minimum values.**



6.5.3.7 Interleukin-6

Figure 83 IL-6 concentration at week 0: SSc vs. Healthy. Boxes represent median and IQR, whiskers represent maximum and minimum values.

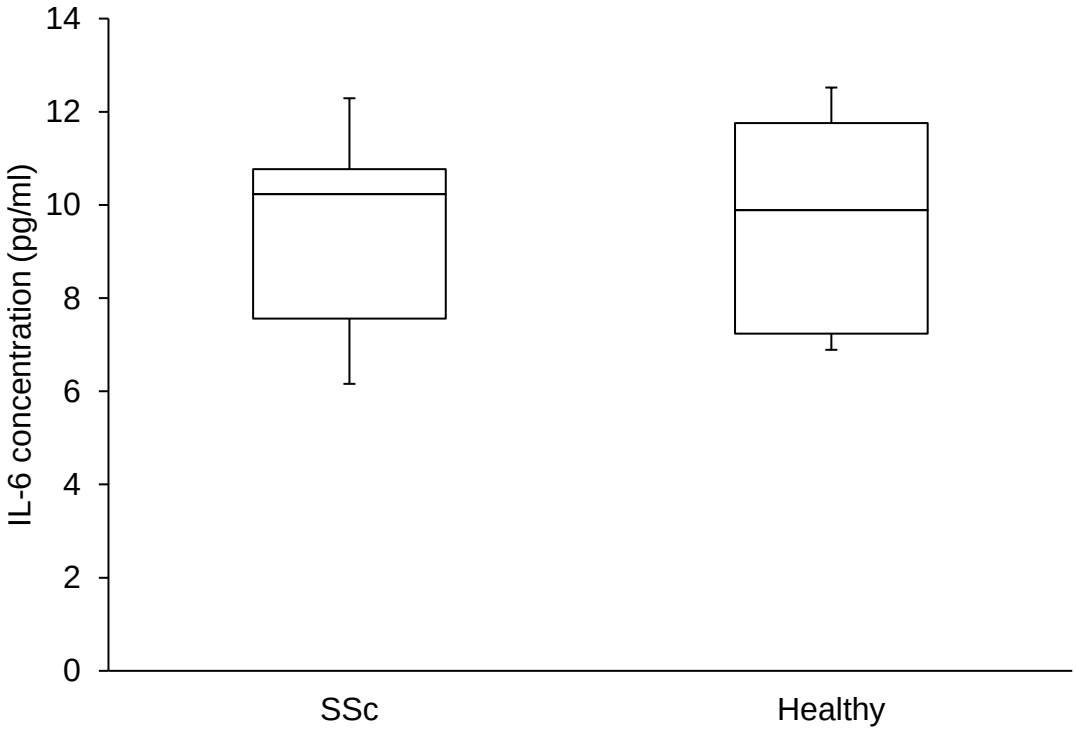


Table 6.14 Descriptive statistics for IL-6 (pg/ml) at Week 0 and Week 24 in SSc + FXIII, SSc + placebo and healthy control groups

Treatment Group		n	Mean	SD	Min	Median	Max	p-value
SSc + FXIII	Week 0 baseline	9	9.98	1.72	7.01	10.42	12.29	
	Week 24	9	9.42	2.75	4.52	9.47	15.29	
	Change from baseline	9	-0.56	2.04	-2.49	-1.07	4.52	
	Adjusted mean change from baseline (95% CI)	9	-1.19 (-2.52 to 0.14)					0.21
SSc + Placebo	Week 0 baseline	6	8.32	1.71	6.16	7.86	10.77	
	Week 24	6	8.25	2.25	4.52	8.40	11.15	
	Change from baseline	6	-0.06	1.25	-2.49	0.33	1.61	
	Adjusted mean change	6	0.13 (-0.88 to 1.13)					0.92

	from baseline (95% CI)							
Difference between groups		Adjusted mean change from baseline (95% CI)		-1.32 (-1.67 to -0.97)				0.52
Healthy	Week 0	5	9.66	2.29	6.89	9.89	12.52	

Figure 84 Change in IL-6 concentration from week 0 to week 24

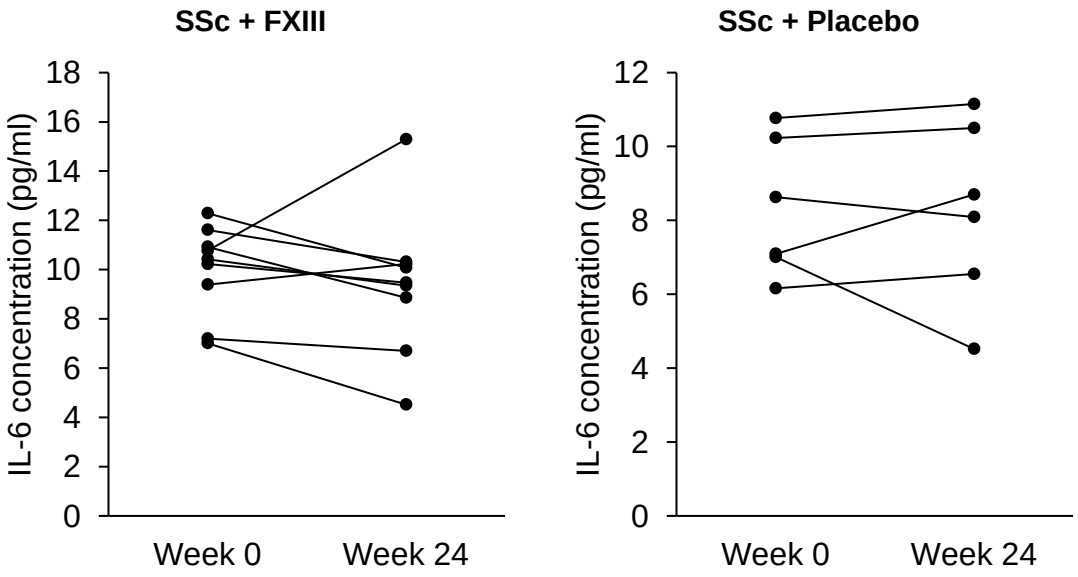
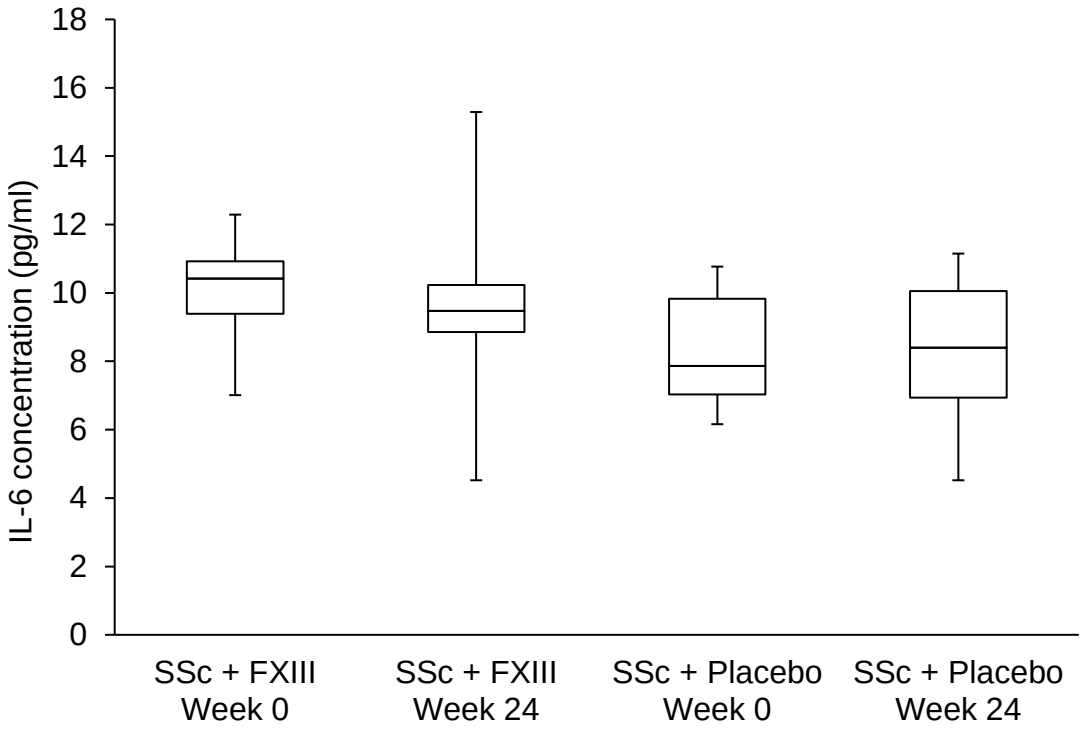


Figure 85 **Distribution of IL-6 concentrations week 0 vs. week 24. Boxes represent median and IQR, whiskers represent maximum and minimum values.**



6.5.3.8 Interleukin-8

Figure 86 IL-8 concentration at Week 0: SSc vs. Healthy. Boxes represent median and IQR, whiskers represent maximum and minimum values.

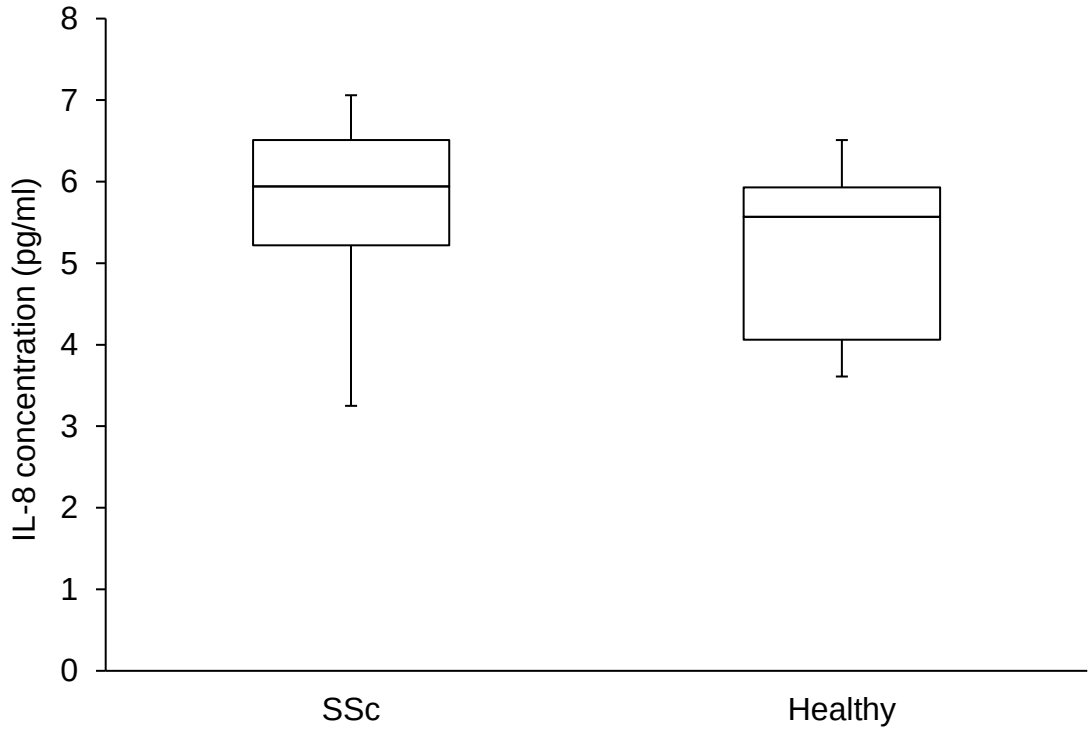


Table 6.15 Descriptive statistics for IL-8 (pg/ml) at Week 0 and Week 24 in SSc + FXIII, SSc + placebo and healthy control groups

Treatment Group		n	Mean	SD	Min	Median	Max	p-value
SSc + FXIII	Week 0 baseline	9	5.69	1.24	3.25	6.06	7.06	
	Week 24	9	5.81	1.11	3.39	6.06	7.43	
	Change from baseline	9	0.12	0.98	-0.94	-0.28	2.08	
	Adjusted mean change from baseline (95% CI)	9	0.12 (-0.52 to 0.76)					0.86
SSc + Placebo	Week 0 baseline	6	5.20	1.20	3.25	5.52	6.88	
	Week 24	6	5.91	0.79	5.27	5.52	7.47	
	Change from baseline	6	0.71	0.88	-0.60	0.52	2.08	
	Adjusted mean change	6	0.71 (0.01 to 1.41)					0.17

	from baseline (95% CI)							
Difference between groups		Adjusted mean change from baseline (95% CI)		-0.59 (-0.95 to -0.23)				0.95
Healthy	Week 0	5	5.14	1.11	3.61	5.57	6.51	

Figure 87 Change in IL-8 concentration from week 0 to week 24

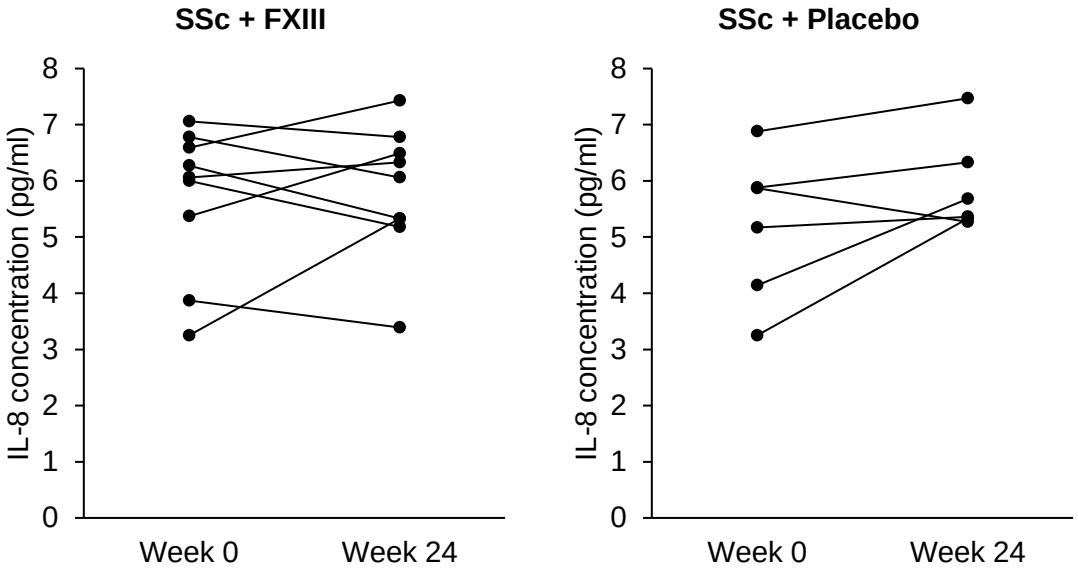
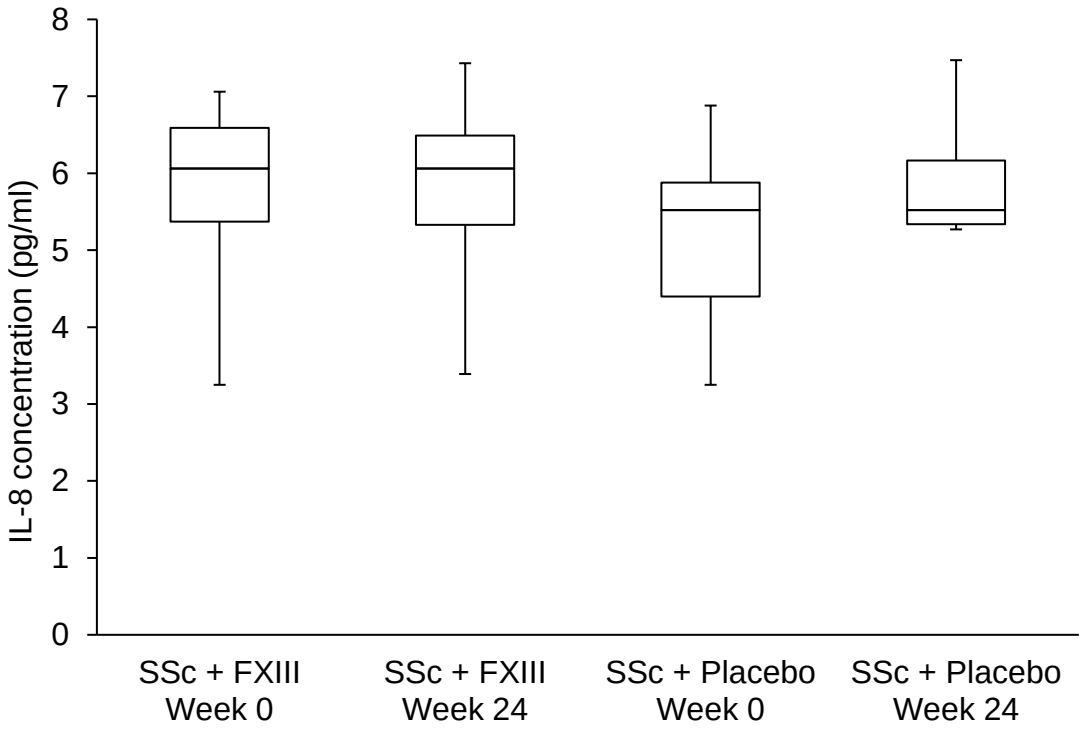


Figure 88 **Distribution of IL-8 concentrations week 0 vs. week 24. Boxes represent median and IQR, whiskers represent maximum and minimum values.**



6.5.3.9 Interleukin-18

Figure 89 IL-18 concentration at Week 0: SSc vs. Healthy. Boxes represent median and IQR, whiskers represent maximum and minimum values.

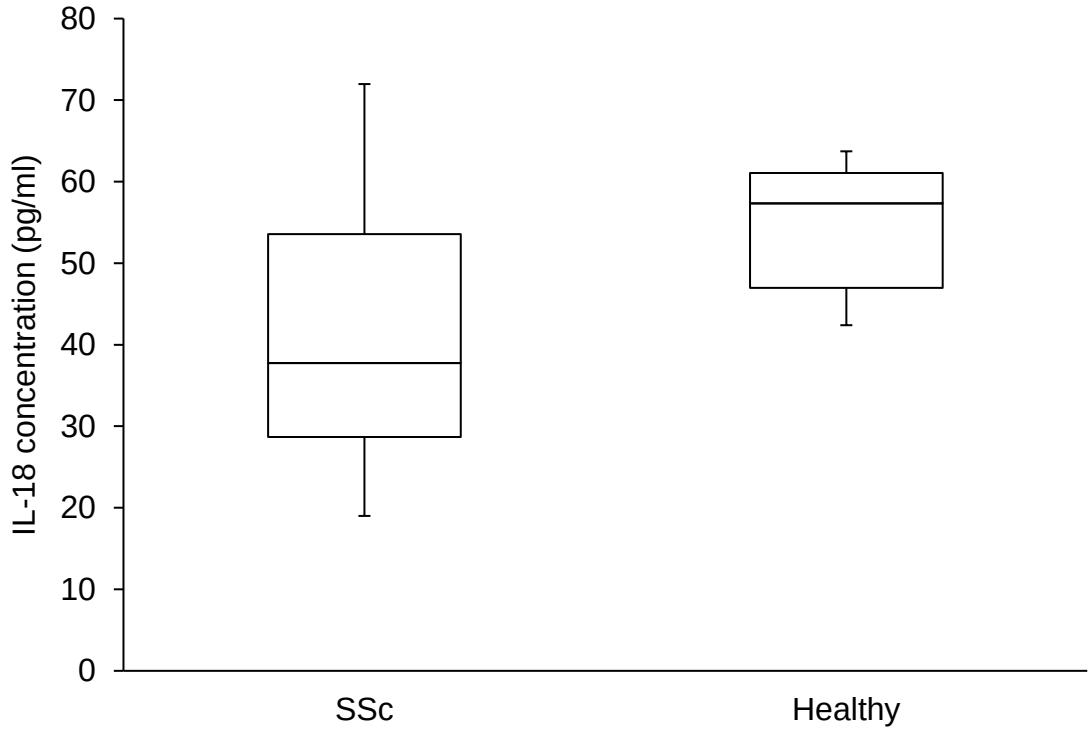


Table 6.16 Descriptive statistics for IL-18 (pg/ml) at Week 0 and Week 24 in SSc + FXIII, SSc + placebo and healthy control groups

Treatment Group		n	Mean	SD	Min	Median	Max	p-value
SSc + FXIII	Week 0 baseline	9	39.60	15.97	19.00	37.18	71.97	
	Week 24	9	41.16	14.96	21.84	37.40	70.24	
	Change from baseline	9	1.56	14.88	-16.39	-2.75	40.69	
	Adjusted mean change from baseline (95% CI)	9	-1.47 (-11.19 to 8.25)					0.52
SSc + Placebo	Week 0 baseline	6	48.85	18.27	24.01	49.06	71.97	
	Week 24	6	51.01	19.56	25.61	52.29	72.95	
	Change from baseline	6	2.16	4.76	-4.31	1.98	10.77	
	Adjusted mean change	6	2.16 (-1.65 to 5.97)					0.46

	from baseline (95% CI)							
Difference between groups		Adjusted mean change from baseline (95% CI)		-3.63 (-10.44 to 3.18)				0.44
Healthy	Week 0	5	54.30	8.24	42.39	57.35	63.72	

Figure 90 Change in IL-18 concentration from week 0 to week 24

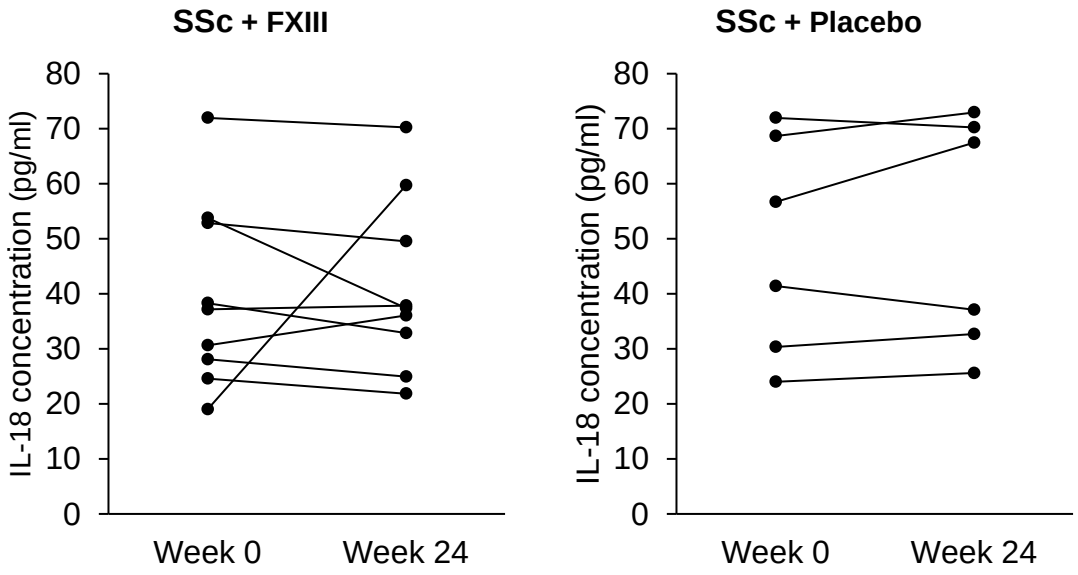
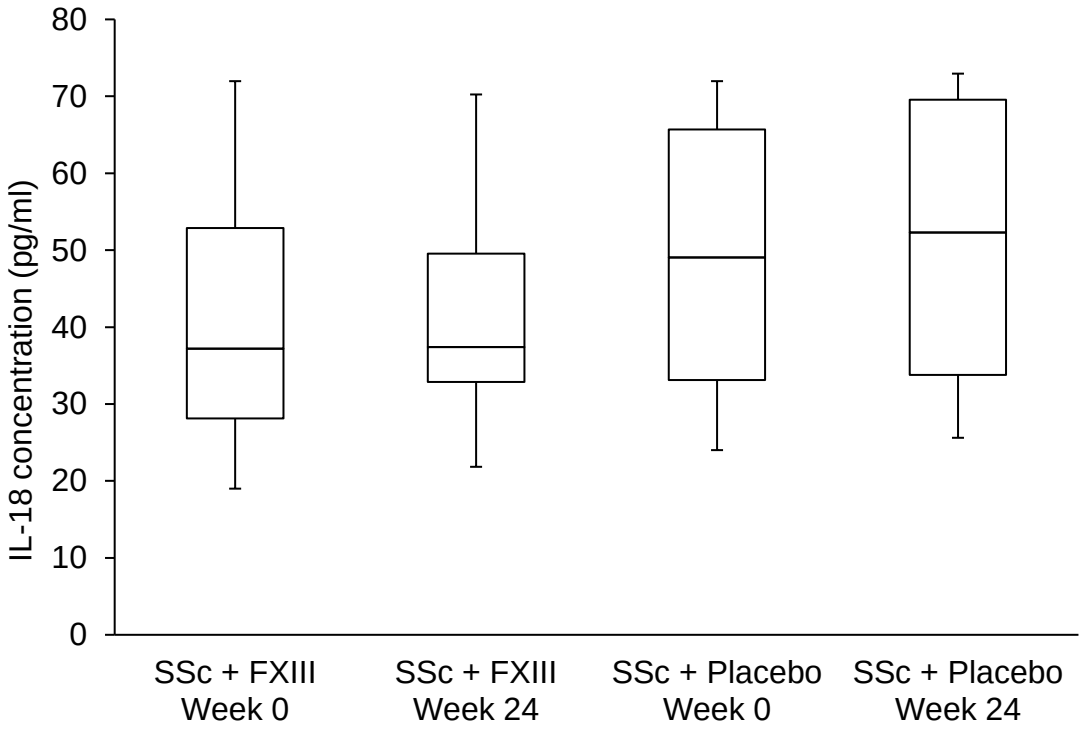


Figure 91 **Distribution of IL-18 concentrations week 0 vs. week 24. Boxes represent median and IQR, whiskers represent maximum and minimum values.**



6.5.3.10 PAI-1

Figure 92 PAI-1 concentration at Week 0: SSc vs. Healthy. Boxes represent median and IQR, whiskers represent maximum and minimum values.

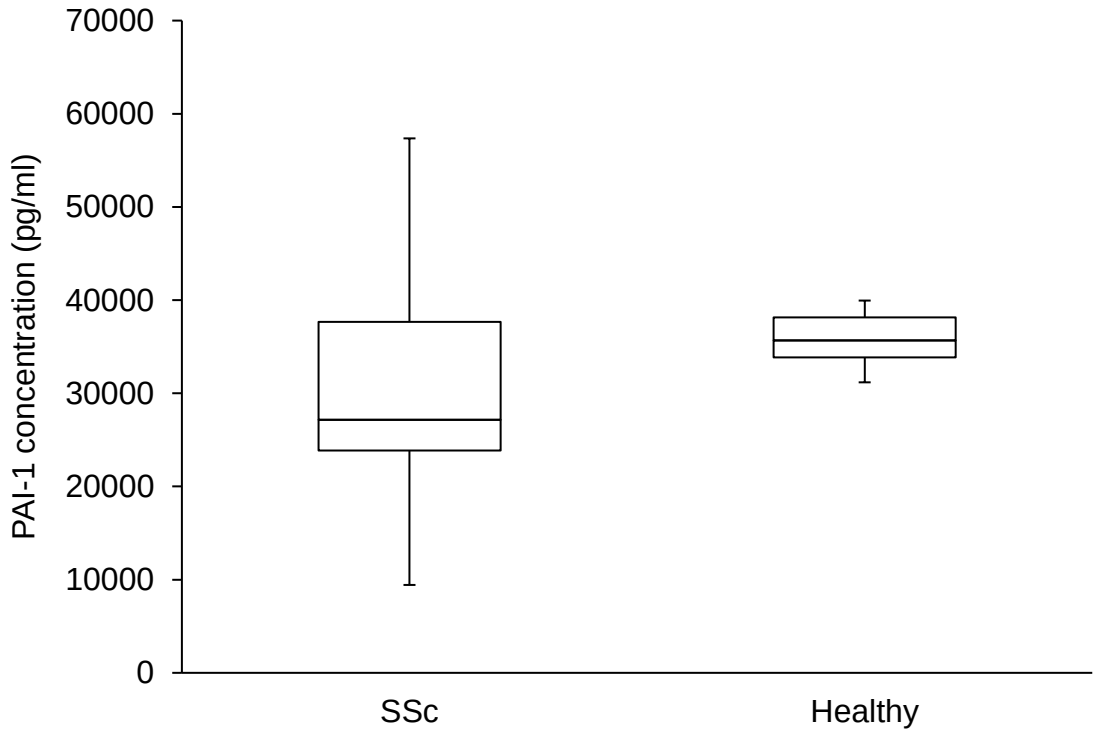


Table 6.17 Descriptive statistics for PAI-1 (pg/ml) at Week 0 and Week 24 in SSc + FXIII, SSc + placebo and healthy control groups

Treatment Group		n	Mean	SD	Min	Median	Max	p-value
SSc + FXIII	Week 0 baseline	9	28841.69	13231.33	9432.39	26373.53	57358.22	
	Week 24	9	34008.45	19161.50	9738.70	26298.55	76119.76	
	Change from baseline	9	5166.77	7045.16	-4406.31	3564.34	18761.54	
	Adjusted mean change from baseline (95% CI)	9	5166.77 (564.02 to 9769.52)					0.05
SSc + Placebo	Week 0 baseline	6	35165.30	9392.72	25178.92	33097.42	52377.11	
	Week 24	6	36970.31	8603.49	26851.82	36537.06	49785.58	
	Change from baseline	6	1805.02	5705.05	-8740.92	3043.61	10126.11	

	Adjusted mean change from baseline (95% CI)	6	2361.23 (-2203.68 to 6926.14)					0.35
Difference between groups		Adjusted mean change from baseline (95% CI)		2805.54 (-6482.69 to 12093.76)				0.26
Healthy	Week 0	5	35761.12	3095.69	31178.28	35657.17	39950.33	

Figure 93 Change in PAI concentration from week 0 to week 24

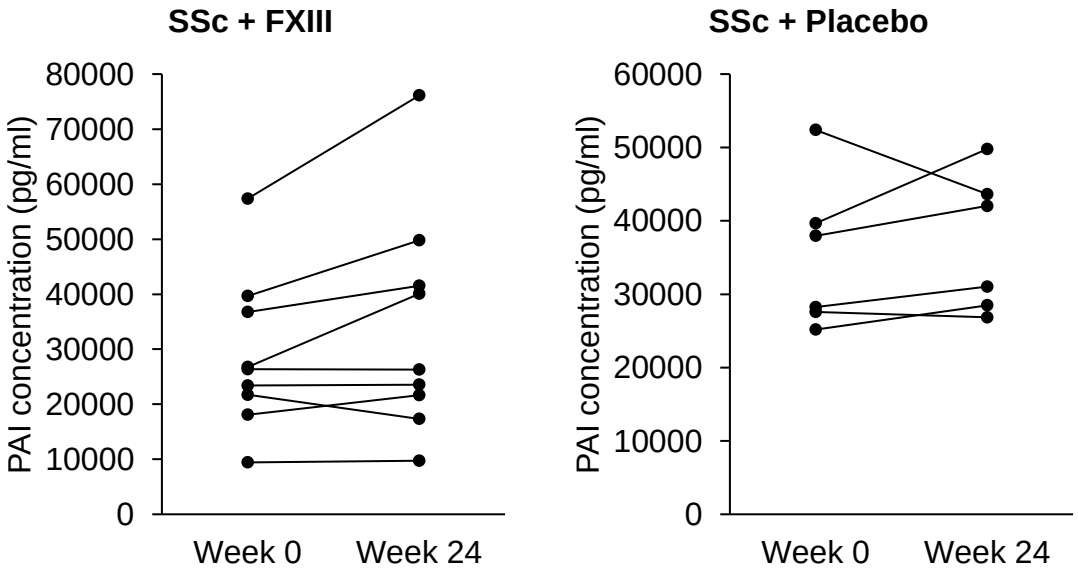
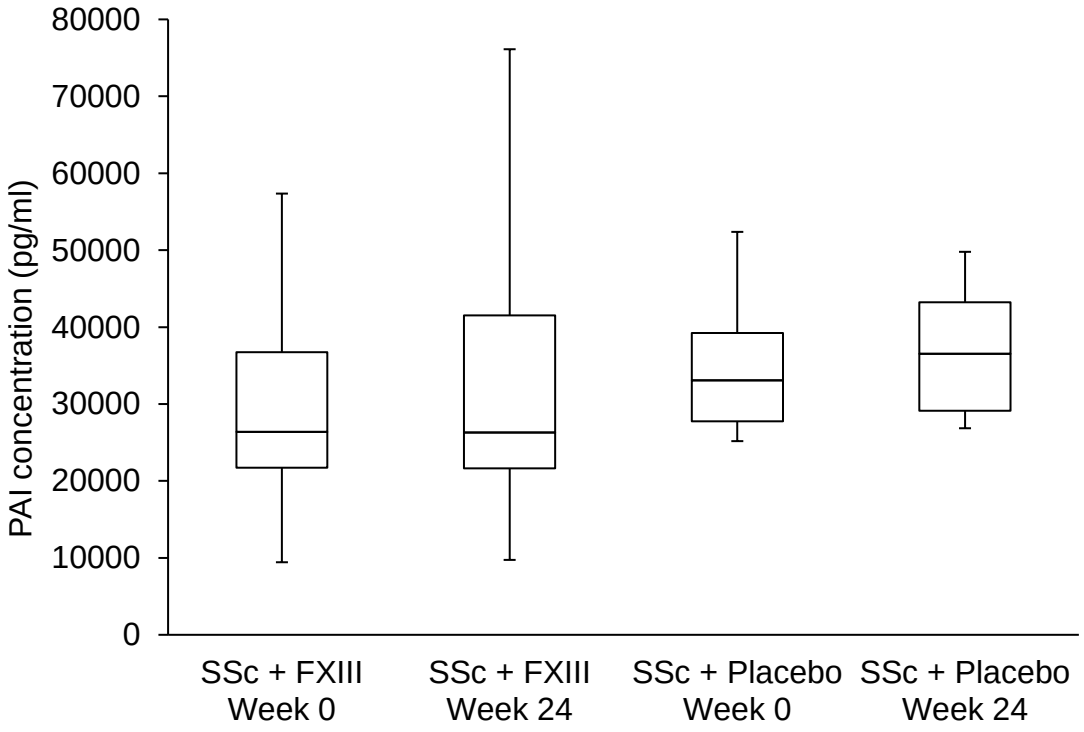


Figure 94 **Distribution of PAI concentrations week 0 vs. week 24. Boxes represent median and IQR, whiskers represent maximum and minimum values.**



6.5.3.11 Placental growth factor

Figure 95 **PLGF concentration at Week 0: SSc vs. Healthy. Boxes represent median and IQR, whiskers represent maximum and minimum values.**

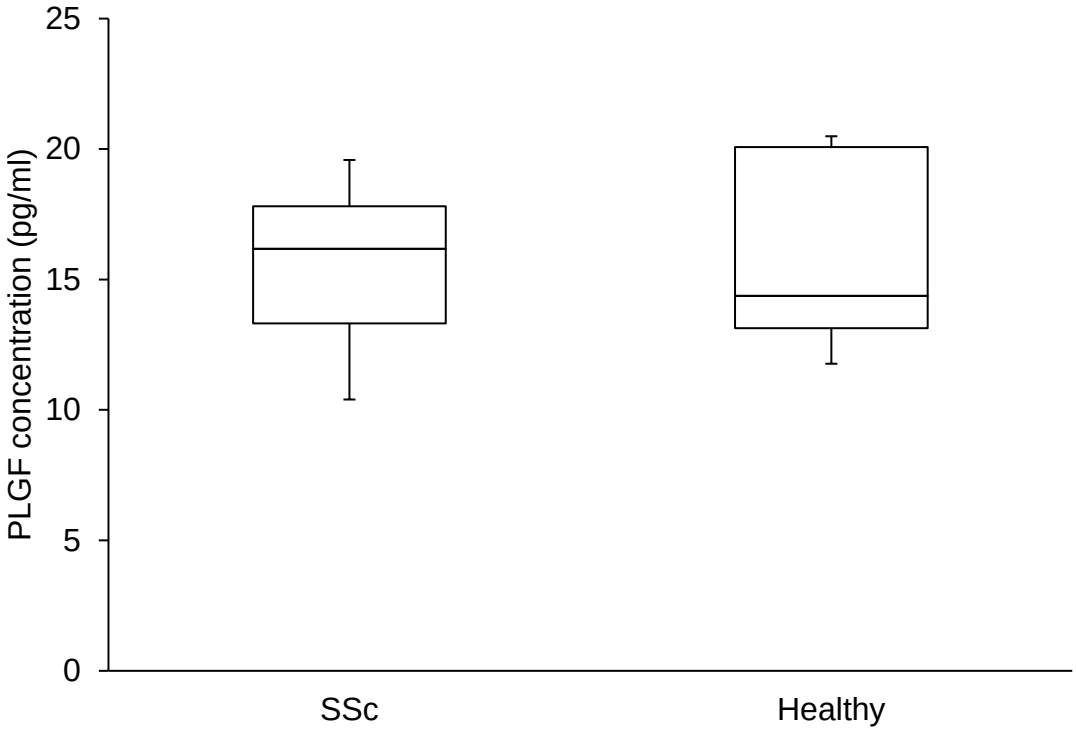


Table 6.18 Descriptive statistics for PLGF (pg/ml) at Week 0 and Week 24 in SSc + FXIII, SSc + placebo and healthy control groups

Treatment Group		n	Mean	SD	Min	Median	Max	p-value
SSc + FXIII	Week 0 baseline	9	15.65	2.54	11.97	15.92	19.58	
	Week 24	9	13.91	3.01	8.80	15.03	17.37	
	Change from baseline	9	-1.73	1.74	-4.55	-2.03	0.52	
	Adjusted mean change from baseline (95% CI)	9	-1.73 (-2.87 to -0.60)					0.05
SSc + Placebo	Week 0 baseline	6	14.88	3.00	10.40	14.97	18.93	
	Week 24	6	15.11	4.29	8.80	16.30	20.40	
	Change from baseline	6	0.23	2.35	-3.75	-0.24	3.21	
	Adjusted mean change	6	0.23 (-1.65 to 2.11)					0.75

	from baseline (95% CI)							
Difference between groups		Adjusted mean change from baseline (95% CI)		-1.96 (-2.2 to -1.73)				0.44
Healthy	Week 0	5	15.97	3.62	11.77	14.37	20.49	

Figure 96 Change in PLGF concentration from week 0 to week 24

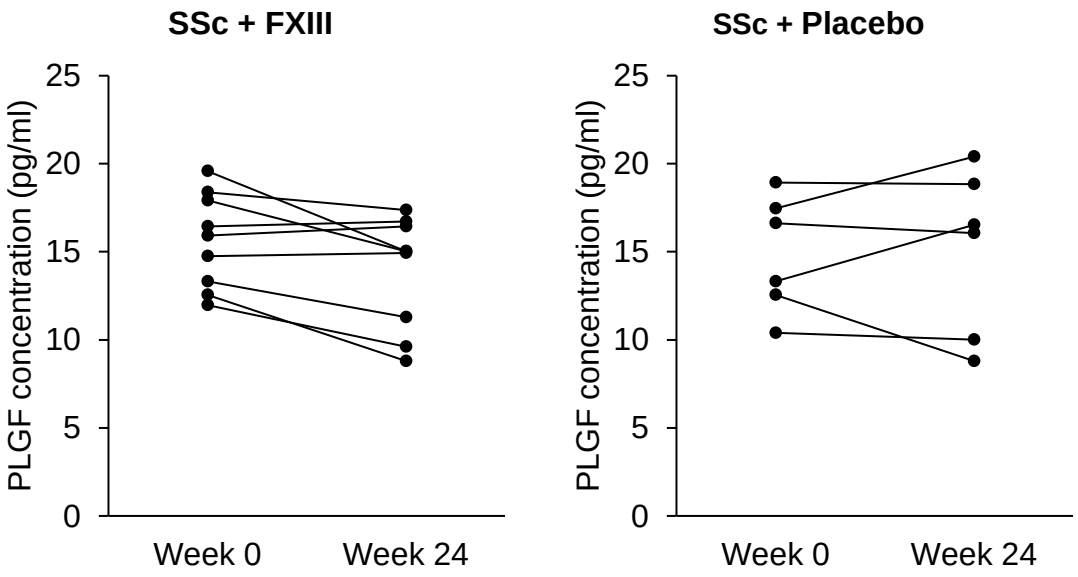
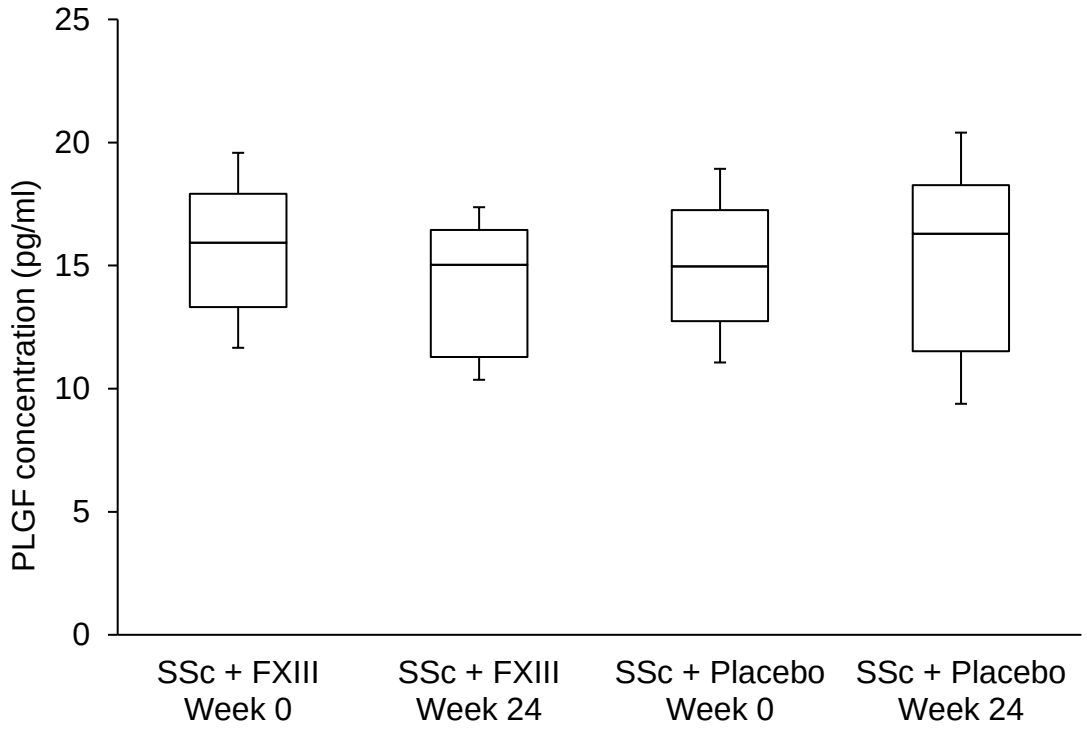


Figure 97 **Distribution of PLGF concentrations week 0 vs. week 24. Boxes represent median and IQR, whiskers represent maximum and minimum values.**



6.5.3.12 Transforming growth factor alpha

Figure 98 TGF-α concentration at Week 0: SSc vs. Healthy. Boxes represent median and IQR, whiskers represent maximum and minimum values.

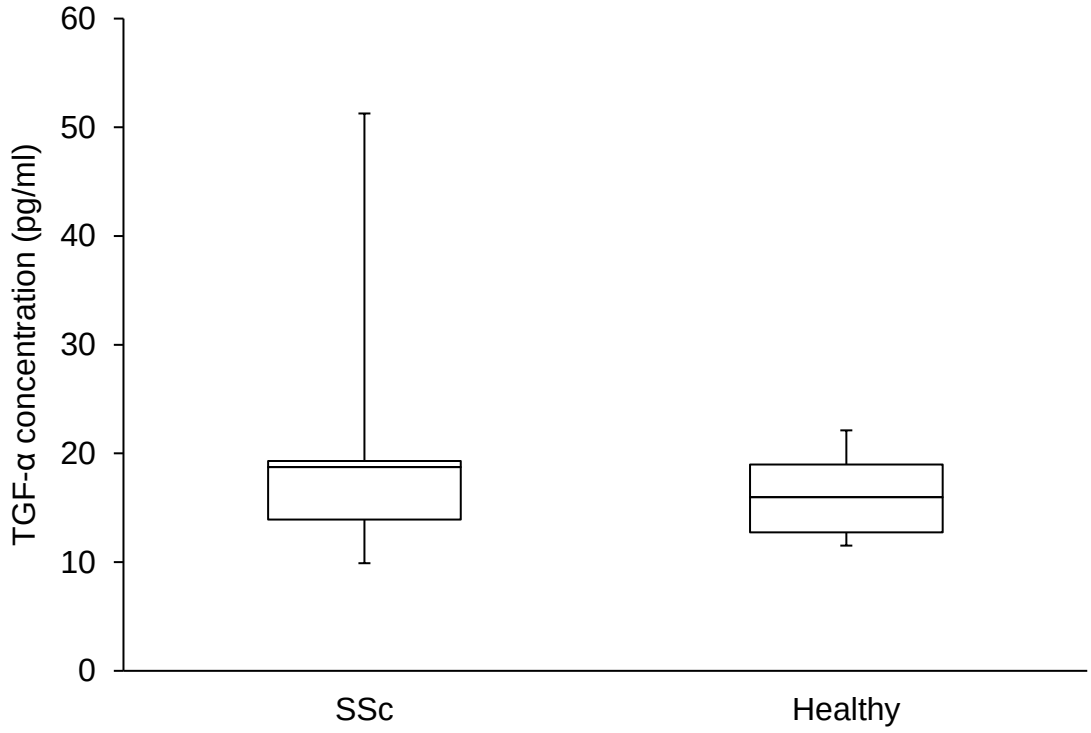


Table 6.19 Descriptive statistics for TGF-α (pg/ml) at Week 0 and Week 24 in SSc + FXIII, SSc + placebo and healthy control groups

Treatment Group		n	Mean	SD	Min	Median	Max	p-value
SSc + FXIII	Week 0 baseline	9	17.87	2.98	13.14	18.98	21.84	
	Week 24	9	15.59	3.70	8.28	16.26	20.62	
	Change from baseline		-2.28	2.44	-5.70	-1.48	1.23	
	Adjusted mean change from baseline (95% CI)		-2.28 (-3.87 to -0.68)					**0.04**
SSc + Placebo	Week 0 baseline	6	20.44	14.13	9.90	15.66	51.27	
	Week 24	6	20.31	14.37	8.28	16.67	51.34	
	Change from baseline		-0.13	2.93	-5.27	0.11	4.81	
	Adjusted mean change from baseline (95% CI)		-0.09 (-2.43 to 2.26)					0.92
Difference between groups		Adjusted mean	-2.19 (-2.84 to -1.55)					0.86

		change from baseline (95% CI)						
Healthy	Week 0	5	16.27	3.92	11.52	15.99	22.12	

Figure 99 Change in TGFα concentration from week 0 to week 24

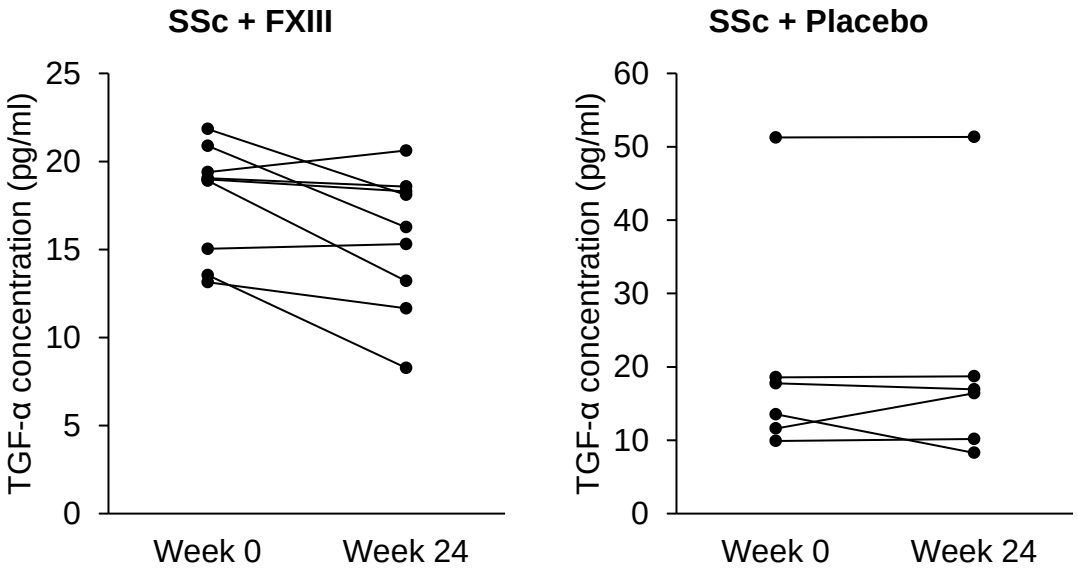
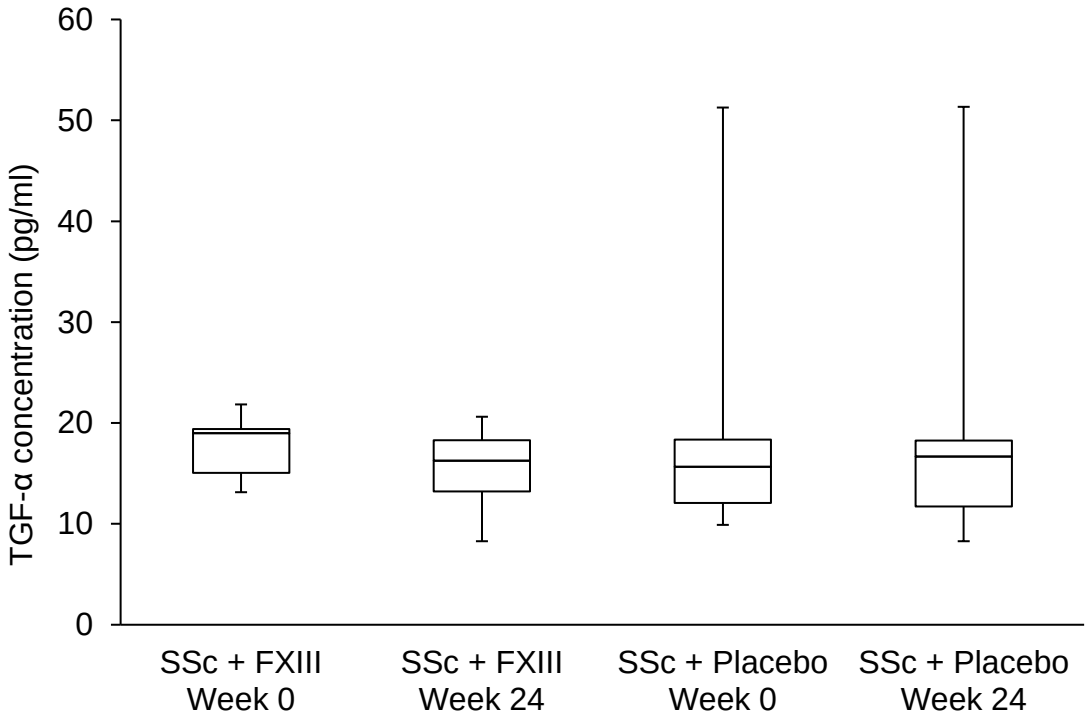


Figure 100 **Distribution of TGFα concentrations week 0 vs. week 24. Boxes represent median and IQR, whiskers represent maximum and minimum values.**



6.5.3.13 uPA

Figure 101 uPA concentration at Week 0: SSc vs. Healthy. Boxes represent median and IQR, whiskers represent maximum and minimum values.

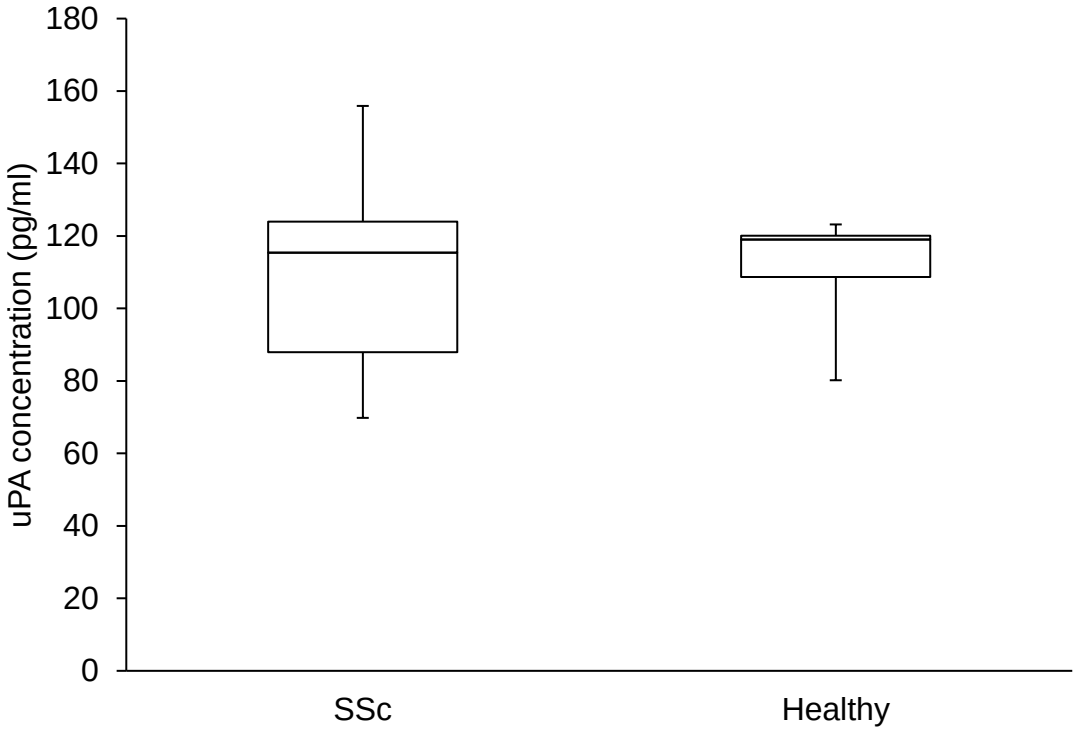


Table 6.20 Descriptive statistics for uPA (pg/ml) at Week 0 and Week 24 in SSc + FXIII, SSc + placebo and healthy control groups

Treatment Group		n	Mean	SD	Min	Median	Max	p-value
SSc + FXIII	Week 0 baseline	9	109.23	31.13	69.81	113.84	174.37	
	Week 24	9	104.40	31.23	64.10	101.42	167.64	
	Change from baseline		-4.83	17.74	-35.71	1.55	23.79	
	Adjusted mean change from baseline (95% CI)		3.47 (-8.12 to 15.06)					0.52
SSc + Placebo	Week 0 baseline	6	113.49	15.91	82.26	120.05	129.36	
	Week 24	6	120.06	44.11	81.23	111.77	213.77	
	Change from baseline		6.57	39.98	-35.71	-4.91	90.62	
	Adjusted mean change from baseline (95% CI)		-10.25 (-42.23 to 21.74)					0.75
Difference between groups		Adjusted mean	13.72 (-34.02 to 61.46)					0.52

		change from baseline (95% CI)						
Healthy	Week 0	5	118.60	27.33	80.19	119.01	165.06	

Figure 102 Change in uPA concentration from week 0 to week 24

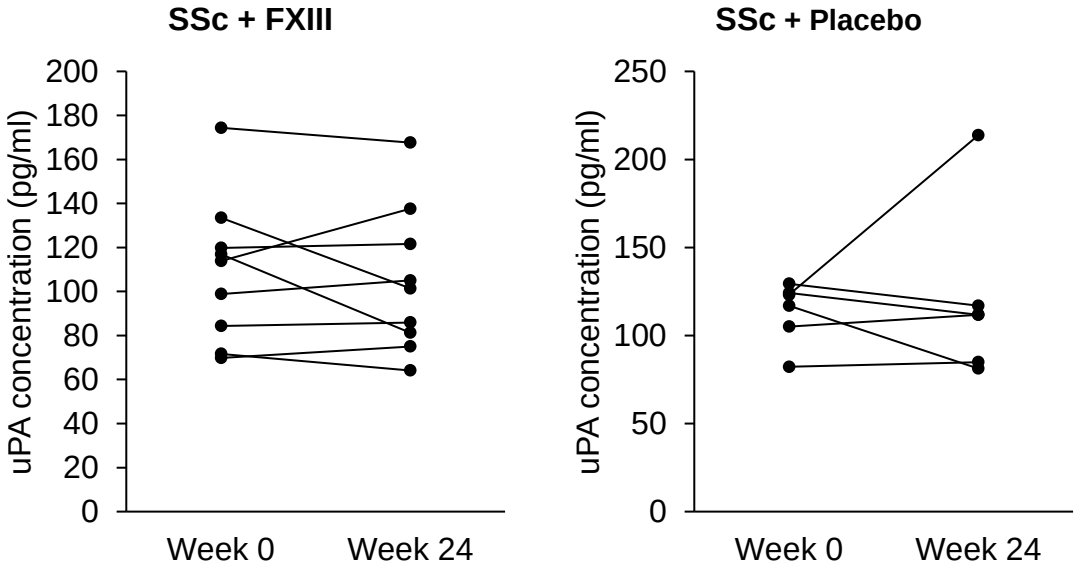
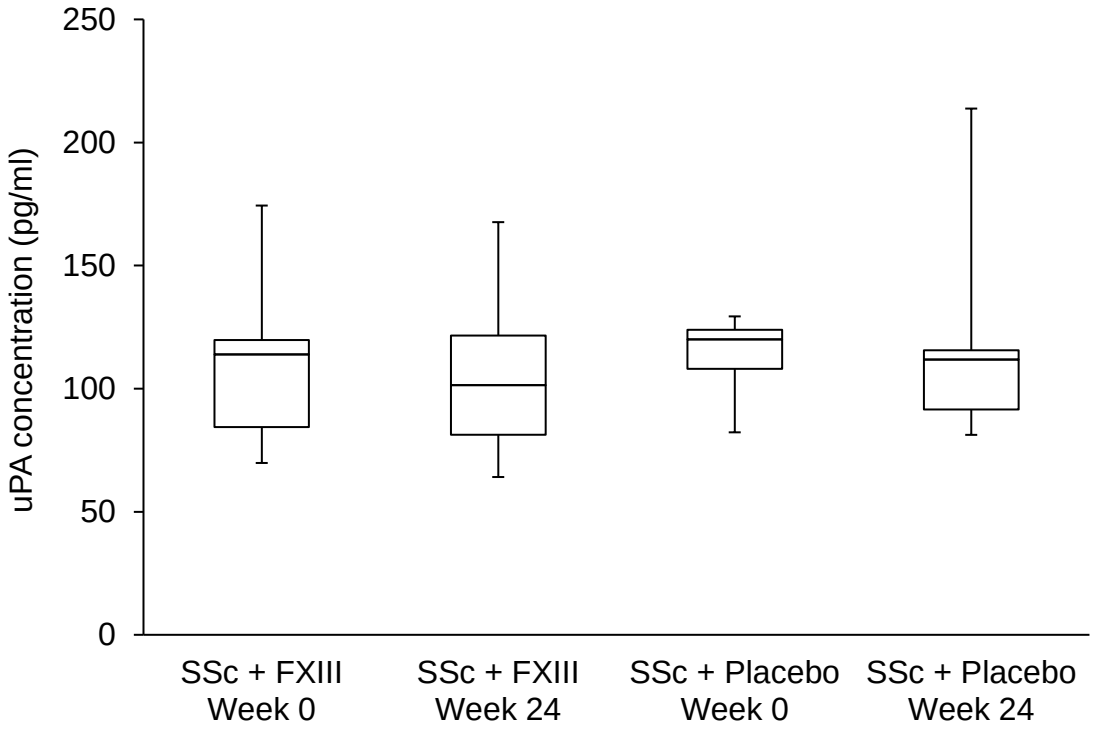


Figure 103 Distribution of uPA concentrations week 0 vs. week 24. Boxes represent median and IQR, whiskers represent maximum and minimum values.



6.5.3.14 VEGF-A

Figure 104 VEGF-A concentration at Week 0: SSc vs. Healthy. Boxes represent median and IQR, whiskers represent maximum and minimum values.

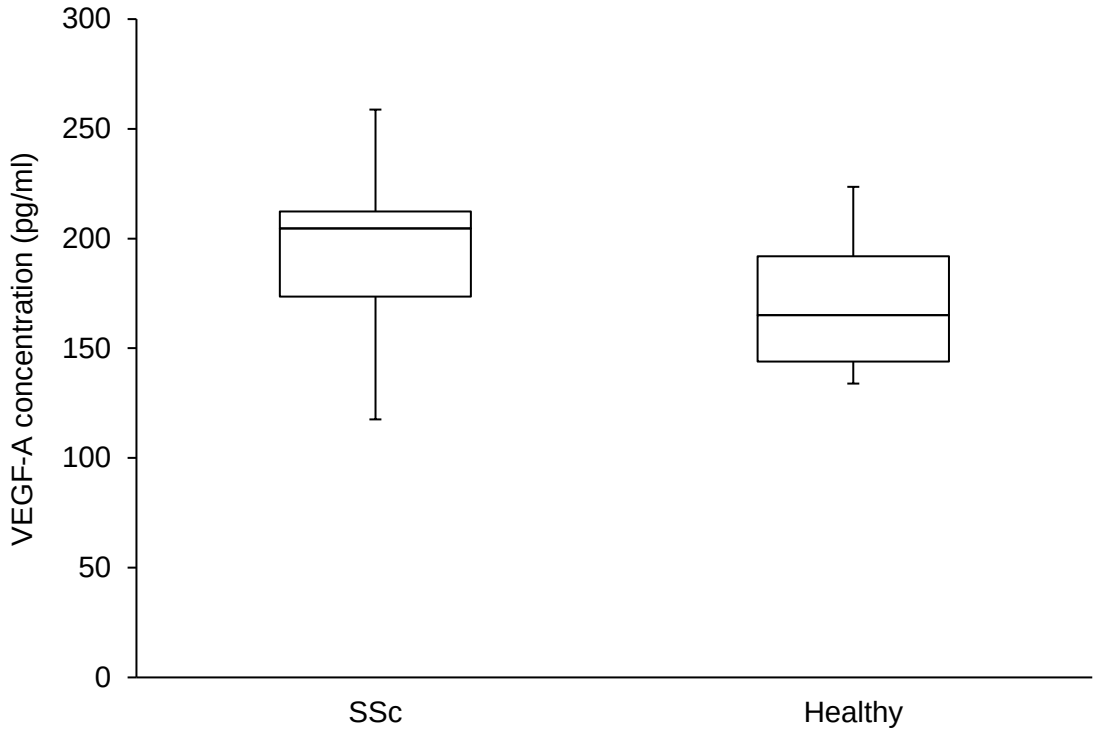


Table 6.21 Descriptive statistics for VEGF-A (pg/ml) at Week 0 and Week 24 in SSc + FXIII, SSc + placebo and healthy control groups

Treatment Group		n	Mean	SD	Min	Median	Max	p-value
SSc + FXIII	Week 0 baseline	9	201.30	14.57	170.82	204.80	220.12	
	Week 24	9	195.85	29.07	138.02	193.86	231.18	
	Change from baseline		-5.45	22.54	-38.50	-1.50	26.13	
	Adjusted mean change from baseline (95% CI)		-5.45 (-20.17 to 9.27)					0.52
SSc + Placebo	Week 0 baseline	6	187.36	53.75	117.58	180.26	258.76	
	Week 24	6	195.95	61.28	127.45	181.84	304.83	
	Change from baseline		8.60	18.36	-8.79	5.58	46.07	
	Adjusted mean change from baseline (95% CI)		1.10 (-13.59 to 15.80)					0.25
Difference between groups		Adjusted mean	-6.55 (-20.8 to 7.7)					0.77

		change from baseline (95% CI)						
Healthy	Week 0	5	171.65	32.68	133.90	165.02	223.56	

Figure 105 Change in VEGF-A concentration from week 0 to week 24

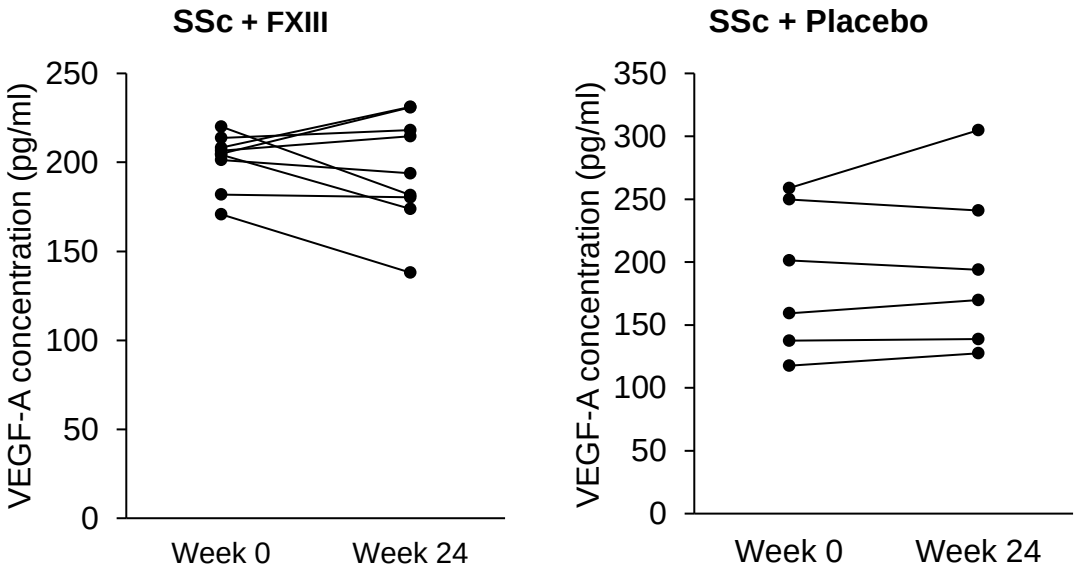
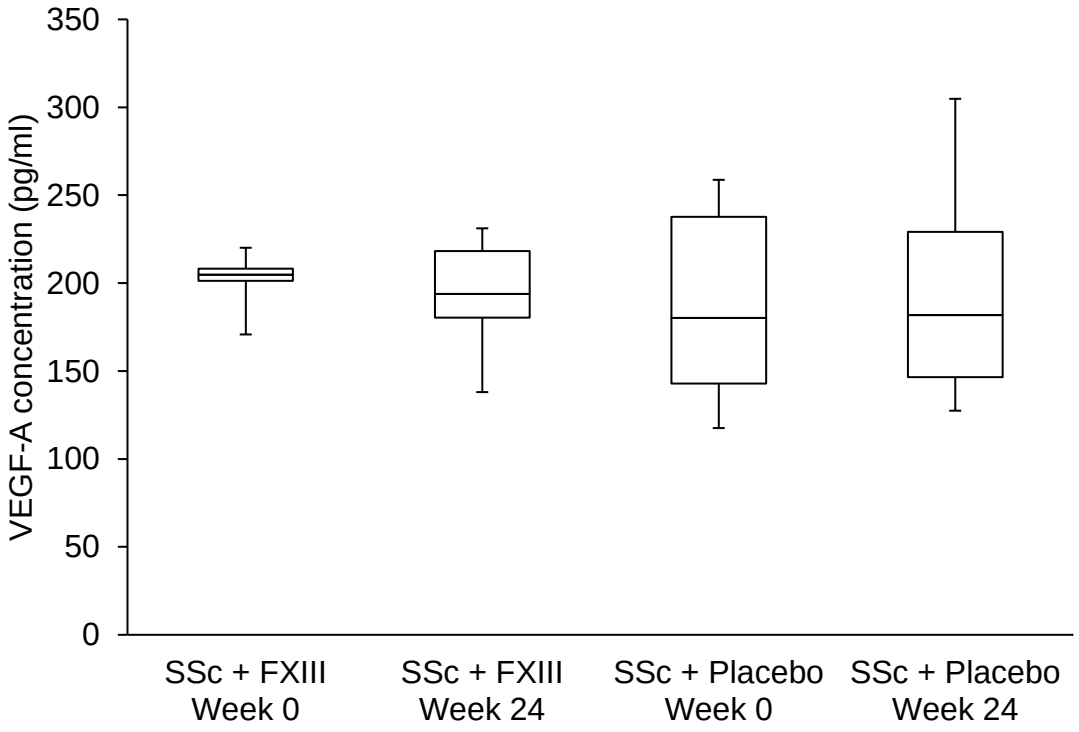


Figure 106 Distribution of VEGF-A concentrations week 0 vs. week 24. Boxes represent median and IQR, whiskers represent maximum and minimum values.



6.5.4 Summary of candidate biomarker results

Table 6.22 Summary of candidate biomarker studies and their significant findings

Candidate biomarker	Comparison	p-value
Angio-poietin-2	Week 0 SSc vs. healthy	0.23
	FXIII group: week 0 vs. week 24	0.07
	Placebo group: week 0 vs. week 24	0.25
	Difference between FXIII and placebo groups at week 24	0.38
sCD40L	Week 0 SSc vs. healthy	0.06
	FXIII group: week 0 vs. week 24	0.43
	Placebo group: week 0 vs. week 24	0.95
	Difference between FXIII and placebo groups at week 24	0.94
COMP	Week 0 SSc vs. healthy	**0.01**
	FXIII group: week 0 vs. week 24	0.53
	Placebo group: week 0 vs. week 24	0.21
	Difference between FXIII and placebo groups at week 24	0.39

EGF	Week 0 SSc vs. healthy	0.07
	FXIII group: week 0 vs. week 24	**0.01**
	Placebo group: week 0 vs. week 24	0.60
	Difference between FXIII and placebo groups at week 24	0.38
Endoglin	Week 0 SSc vs. healthy	0.85
	FXIII group: week 0 vs. week 24	0.86
	Placebo group: week 0 vs. week 24	0.75
	Difference between FXIII and placebo groups at week 24	0.32
sFASL	Week 0 SSc vs. healthy	0.36
	FXIII group: week 0 vs. week 24	**0.04**
	Placebo group: week 0 vs. week 24	0.89
	Difference between FXIII and placebo groups at week 24	0.59
Fibronectin	Week 0 SSc vs. healthy	0.07
	FXIII group: week 0 vs. week 24	0.08
	Placebo group: week 0 vs. week 24	0.59
	Difference between FXIII and placebo groups at week 24	**0.03**

HB-EGF	Week 0 SSc vs. healthy	0.85
	FXIII group: week 0 vs. week 24	**0.04**
	Placebo group: week 0 vs. week 24	0.60
	Difference between FXIII and placebo groups at week 24	0.95
IGFBP-1	Week 0 SSc vs. healthy	0.41
	FXIII group: week 0 vs. week 24	0.31
	Placebo group: week 0 vs. week 24	0.75
	Difference between FXIII and placebo groups at week 24	0.32
IL-6	Week 0 SSc vs. healthy	0.78
	FXIII group: week 0 vs. week 24	0.21
	Placebo group: week 0 vs. week 24	0.92
	Difference between FXIII and placebo groups at week 24	0.52
IL-8	Week 0 SSc vs. healthy	0.36
	FXIII group: week 0 vs. week 24	0.86
	Placebo group: week 0 vs. week 24	0.17
	Difference between FXIII and placebo groups at week 24	0.95

IL-18	Week 0 SSc vs. healthy	0.07
	FXIII group: week 0 vs. week 24	0.52
	Placebo group: week 0 vs. week 24	0.46
	Difference between FXIII and placebo groups at week 24	0.44
PAI	Week 0 SSc vs. healthy	0.17
	FXIII group: week 0 vs. week 24	0.05
	Placebo group: week 0 vs. week 24	0.35
	Difference between FXIII and placebo groups at week 24	0.26
PLGF	Week 0 SSc vs. healthy	0.85
	FXIII group: week 0 vs. week 24	0.05
	Placebo group: week 0 vs. week 24	0.75
	Difference between FXIII and placebo groups at week 24	0.44
TGF-α	Week 0 SSc vs. healthy	0.61
	FXIII group: week 0 vs. week 24	**0.04**
	Placebo group: week 0 vs. week 24	0.92
	Difference between FXIII and placebo groups at week 24	0.86

TNFα	Week 0 SSc vs. healthy	**0.04**
	FXIII group: week 0 vs. week 24	**0.02**
	Placebo group: week 0 vs. week 24	0.17
	Difference between FXIII and placebo groups at week 24	0.42
TSP-1	Week 0 SSc vs. healthy	**0.02**
	FXIII group: week 0 vs. week 24	**0.02**
	Placebo group: week 0 vs. week 24	0.68
	Difference between FXIII and placebo groups at week 24	0.57
uPA	Week 0 SSc vs. healthy	0.78
	FXIII group: week 0 vs. week 24	0.52
	Placebo group: week 0 vs. week 24	0.75
	Difference between FXIII and placebo groups at week 24	0.52
VEGF-A	Week 0 SSc vs. healthy	0.27
	FXIII group: week 0 vs. week 24	0.52
	Placebo group: week 0 vs. week 24	0.25
	Difference between FXIII and placebo groups at week 24	0.77

VEGF-C	Week 0 SSc vs. healthy	0.89
	FXIII group: week 0 vs. week 24	**0.04**
	Placebo group: week 0 vs. week 24	0.91
	Difference between FXIII and placebo groups at week 24	0.86
VEGF-D	Week 0 SSc vs. healthy	0.97
	FXIII group: week 0 vs. week 24	0.59
	Placebo group: week 0 vs. week 24	**0.02**
	Difference between FXIII and placebo groups at week 24	0.18

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