

# **The causes and treatment of kidney disease in scleroderma**

Dr Edward Phineas Stern

Centre for Rheumatology

Division of Medicine

University College London

*A thesis submitted to UCL in partial fulfilment of the requirements for the degree of Doctor of Philosophy.*

# **Declaration**

I, Edward Phineas Stern, confirm that the work presented in my thesis is my own.

Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

# Abstract

This project examines the pathogenic processes that lead to kidney disease in scleroderma and tests a novel therapy for scleroderma kidney disease in a clinical trial. I describe three programmes of experiment to identify possible pathogenic targets and new treatment strategies in scleroderma kidney disease.

The first divides a large cohort of patients according to their immunological and renal phenotypes, uses genome wide association to identify possible risk genes and then interrogates candidate genes further by staining renal disease tissue for the relevant gene products.

In the second stream of investigation, I describe a project to develop novel biomarkers of renal disease activity by measuring concentrations of candidate proteins in urine and serum of scleroderma patients and compare the measurements from matched control groups.

The final set of investigations is a randomised control clinical trial, testing the safety and efficacy of the highly selective endothelin antagonist zibotentan in renal outcomes for patients with scleroderma-associated chronic kidney disease and scleroderma renal crisis. Outcomes are assessed by traditional clinical measures of renal function as well as deploying novel disease activity biomarkers developed in parallel in my earlier experiments. In a parallel study I assess the pharmacokinetics of zibotentan in patients on haemodialysis.

# Impact statement

This thesis makes a significant contribution to understanding the biological processes that lead to kidney disease in scleroderma, a life-threatening rheumatic condition, and works towards developing new treatments for this condition.

In this project I use genetic analysis to improve our understanding of why some people with scleroderma get kidney disease and others don't. I also examine proteins in the urine and blood which offer clues as to how and why the kidney disease occurs. Drawing on the information gained in these first two studies I test a new treatment for kidney disease in scleroderma, by carrying out the first ever placebo-controlled trial of a drug for this specific condition.

For the academic community, these three studies are a starting point for further research, which we hope in time will allow us to greatly improve our understanding of kidney disease in scleroderma. From an industry perspective, this work provides evidence of potential avenues for future drug development, including phase 3 trials that could bring new drugs to market. But the most important outcome of this research will be for individuals with scleroderma, for whom we hope to develop better treatments that will improve their quality of life and increase their life expectancy.

# Research paper declaration forms

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**Stern EP, Denton CP. The Pathogenesis of Systemic Sclerosis. Rheumatic Disease Clinics of North America. 2015 May**

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**Stern EP, Guerra SG, Chinque H, Acquaah V, González-Serna D, Ponticos M, et al. Analysis of Anti-RNA Polymerase III Antibody-positive Systemic Sclerosis and Altered GPATCH2L and CTNND2 Expression in Scleroderma Renal Crisis. The Journal of Rheumatology. 2020 Nov 1;47(11):1668–77.**

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E Stern drafted manuscript and contributed to study design, genetic analysis, histopathology experiments and statistical analysis. S Guerra contributed to study design, genetic analysis, statistical analysis and manuscript revision, D Gonzalez-Serna and J Martin contributed to genetic analysis. H Chinque, V Acquaah, M Ponticos, and K Khan contributed to histopathology experiments. S Nihtyanova contributed to phenotyping and statistical analysis. V Ong, M Mayes and S Assassi contributed to patient recruitment and phenotyping. M Harber and Aine Burns contributed to study design and patient phenotyping. C Fonseca contributed to study design, genetic and statistical analysis. C Denton contributed to study design, analysis of all experiments and manuscript revision.

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E Stern drafted manuscript, contributed to study design, recruited all patients and conducted all patients' study visits, prepared all samples and conducted the multiplex immunoassays. R Unwin and A Burns contributed to study design. V Ong contributed to phenotyping and recruitment of patients. C Denton contributed to study design, phenotyping and recruitment of patients and manuscript revision.

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E Stern drafted manuscript, contributed to study design, recruited all patients, conducted patient study visits, prepared all samples, conducted biomarker immunoassays and contributed to statistical analysis. L Host conducted patient study visits, I Wanjiku and R Ochiel provided clinical trial support, data management and conducted patient study visits., J Escott and P Gilmour contributed to study design, provided IMP management and data oversight and contributed manuscript revisions. R Unwin and A Burns contributed to study design. H Cadiou provided trial monitoring and oversight. A O’Keeffe, contributed to study design, oversight, statistical analysis and contributed manuscript revisions. V Ong contributed to phenotyping and recruitment of patients. C Denton contributed to study design, phenotyping and recruitment of patients, statistical analysis and manuscript drafting and revision.

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Finally, my greatest debt is to the patients who participated in the research, and it is to them that I dedicate this work.

# Commonly used abbreviations

ACA	Anti centromere antibody
ANA	Anti nuclear antibodies
ARA	Anti RNA polymerase III antibody
ATA	Anti topoisomerase I antibody (Scl 70)
CKD	Chronic kidney disease
dcSSC	Diffuse systemic sclerosis
eGFR	Estimated glomerular filtration rate
ET-1	Endothelin 1
ETRA	Endothelin receptor A
ETRB	Endothelin receptor B
GWAS	Genome-wide association study
HD	Haemodialysis
HLA	Human leucocyte antigen
ICAM-1	Intercellular adhesion molecule 1
lcSSC	Limited systemic sclerosis
MCP-1	Monocyte chemoattractant protein 1
MHC	Major histocompatibility complex
ODU	Optical density unit
SRC	Scleroderma renal crisis
SSC	Systemic sclerosis
VCAM-1	Vascular adhesion molecule 1

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# Chapter 1: Introduction

The pathogenesis of scleroderma

Scleroderma (also referred to as systemic sclerosis, SSc) is a complex multisystem disorder with a wide range of clinical features. It is commonly divided by clinical phenotypes, most broadly the two skin subgroups: limited cutaneous (lcSSc) and diffuse cutaneous systemic disease (dcSSc). The vast majority of individuals with SSc have circulating antibodies against nuclear proteins (antinuclear antibodies or ANAs) and different clinical phenotypes are associated with the different antibodies.

However, in most cases the recognised antibodies are not directly disease-causing.

To date we have only limited evidence as to the causes of SSc and the molecular processes leading to its clinical features. By reviewing the current understanding of the pathogenesis of this disease, in this chapter I will provide a platform for further investigation of the causes and treatment of kidney complications of scleroderma in particular.

The overarching scleroderma phenotype includes abnormalities in the immune, connective tissue and vascular systems of those affected. I will summarise some key findings regarding dysregulation in these three systems and interplay between them in the scleroderma disease process. Evidence presented below suggests that scleroderma occurs when a susceptible individual is exposed to particular environmental factors, and I will also describe the molecular processes in this

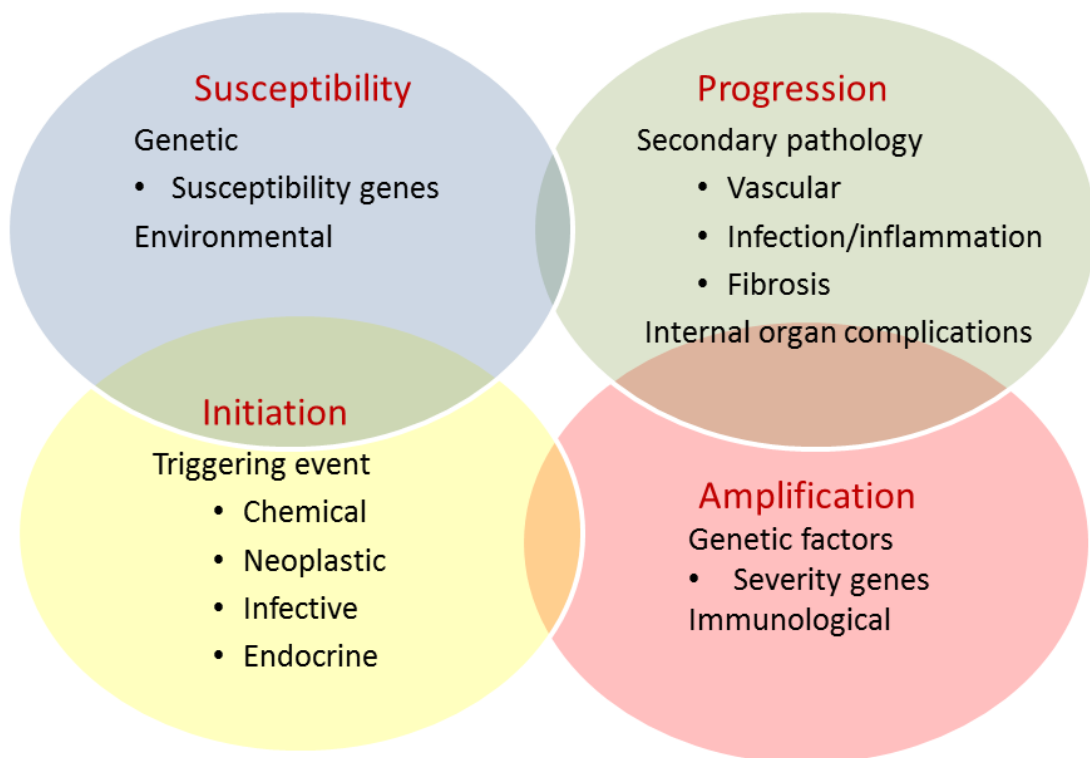


context. While reviewing these common processes in the disease I will attempt to account for the heterogeneity of clinical presentations in scleroderma.

#### Overview of aetiopathogenesis

The interplay between vascular damage, inflammation and connective tissue repair is a hallmark of scleroderma. This previously led to models of pathogenesis that were linear, suggesting that vascular injury was followed by immune or inflammatory activity, which in turn led to fibrosis and scarring. This paradigm does not account well for clinical observations of disease heterogeneity and the great variety in involvement of individual organ systems. A more integrated model sees all three systems as relevant as the disease initiates, progresses and potentially improves. So in this section I describe interlocking processes that reflect or contribute to initiation or triggering, amplification and later progression of SSc. The relationship between these stages in scleroderma is summarised in Figure 1.

Figure 1: Overlapping events in the pathogenesis of systemic sclerosis



Susceptibility

*Epidemiology*

Epidemiological studies have demonstrated a moderate increase in the prevalence of scleroderma in first-degree relatives of patients with the disease—1.6% versus a 0.026% risk in the general population(1) and there is evidence of clustering of cases in families that appear to have a more marked risk. In these clusters, relatives tend to have the same disease-associated autoantibody(1,2). These data imply genetic susceptibility to scleroderma overall as well as an inherited tendency to develop the various subgroups of disease.

*HLA associations*

Like other auto-immune diseases, scleroderma is associated with polymorphisms in the human leucocyte antigen (HLA) region of the major histocompatibility complex (MHC). Modest associations have been seen between given haplotypes and the disease overall(3,4). Stronger specific HLA associations have been demonstrated for each of the major autoantibody subgroups of scleroderma—for example, HLA DQB1\*0501 is associated with the anti-centromere antibody (ACA)(5), and DRB1\*1104 and DPB1\*1301 are independently associated with the anti-topoisomerase I antibody (ATA)(6,7).

*Candidate gene studies*

In addition to HLA associations, candidate gene studies have identified disease-associated polymorphisms in genes relevant to the three “compartments” of systemic sclerosis described above i.e. immune, connective tissue and vascular systems.

Associations identified in candidate genes related to vascular function include endothelial nitric oxide synthase (eNOS), angiotensin converting (ACE)(8) and the endothelin receptor B (ETRB)(9). Polymorphisms overrepresented in scleroderma related to genes involved in the connective tissue system have included CTGF(10), Fibrillin-1(11) and SPARC(12). However, there has not yet been a study that has successfully replicated any of these associations across multiple populations. On the other hand, studies examining associations with genes regulating the immune system have been successfully replicated. These include STAT4, which regulates differentiation of T-cells (13), BANK1, which regulates B-cell activation(14), and IRF5, which acts as an activator of type 1 interferon(15). Interestingly, these target genes include at least one which associates with a specific internal organ complication of scleroderma—STAT4 and interstitial lung disease (16).

#### *GWAS*

Genome-wide association studies (GWAS) in North America, Europe and East Asia, have provided statistical confirmation for some associations identified in candidate gene studies, including STAT4 (17) and IRF5(18). The most convincing replication data for a gene newly identified via GWAS is for CD247, a cell-surface marker mediating T-cell receptor signaling. CD247 has also been implicated in the pathogenesis of another connective tissue disease, Systemic Lupus Erythematosus (SLE)(17,19).

Other GWAS studies have been successful in identifying common variants associated with skin subgroup (limited versus diffuse) and immunological subgroup (ACA versus ATA) in scleroderma(20). In view of the easily observed associations between skin subgroups, circulating antibodies and organ complications, future GWAS analysis

could be enriched by looking only within a given clinical or immunological subgroup and interrogating the genetic risk for a given complication that occurs at high frequency within that subgroup(21)Genetic associations for scleroderma are summarized in Table 1.

Table 1: Genes with associations for increased scleroderma risk (excluding Human Leucocyte Antigen system locations).

<b><u>Pathogenic association</u></b>	<b><u>Gene</u></b>	<b><u>Study type</u></b>
<b>Vascular</b>	eNOS	Candidate gene
	ACE	Candidate gene
	ET-1	Candidate gene
	ETR A/B	Candidate gene
<b>Immune/inflammation</b>	STAT4	Candidate gene/GWAS†
	IRF5	Candidate gene/GWAS†
	CD247	GWAS†
	TNIP1	GWAS†
	BLK	Candidate gene†
	TNFSF4	Candidate gene†
	BANK1	Candidate gene†
	MIF	Candidate gene†
<b>Connective tissue</b>	CTGF	Candidate gene
	Fibrillin 1	Candidate gene
	SPARC	Candidate gene

†These genetic associations have been replicated in more than one population. Abbreviations: ACE, angiotensin converting enzyme; BANK1, B cell scaffold protein with ankyrin repeats 1; BLK, B lymphocyte kinase; CD247, cluster of differentiation 247; CTGF, connective tissue growth factor; eNOS, endothelial nitric oxide synthase; ET-1, endothelin 1; ETRA/B, endothelin receptor A/B; IRF5, interferon regulatory factor 5; MIF, macrophage migration inhibitory factor; SPARC, secreted protein acidic and rich in cysteine; STAT4, signal transducer and activator of transcription 4; TNFSF4, tumor necrosis factor superfamily 4; TNIP1, TNFAIP3 interacting protein 1.

Initiation

### *Chemical triggers*

A syndrome associating Raynaud's phenomenon, skin-thickening and acro-osteolysis in individuals who had exposure to vinyl chloride was recognised as early as the 1960s(21). This syndrome has marked similarities to scleroderma. It is interesting to note that HLA antigens predicting development and severity of vinyl chloride disease mirror those which are predictive in scleroderma (22,23). This helps to illustrate that scleroderma is a condition occurring in a susceptible individual but that it requires *initiation* by exposure factors (often more than one). Other chemical agents have been observed to cause sclerodermatous reactions including taxane chemotherapy(24), contaminated rapeseed oil(25) and tryptophan(26).

Developing this concept further, several environmental factors have been investigated as risk factors, in those with a clinical diagnosis of scleroderma itself. The best-evidenced association is between scleroderma and occupational exposure to solvents (27,28). The exposure risk does not seem to be dose-dependent and is more marked in male individuals(29), both of which support my hypothesis that exposure risks are acquired in the context of a degree of genetic susceptibility which varies in individuals. Other strong occupational risk factors for scleroderma have been identified, including crystalline silica and white spirit, both of which also show a marked gender difference in the degree of risk associated with exposure (30).

### *Endocrine triggers*

Numerous investigators have tried to account for the marked prevalence of scleroderma and other autoimmune diseases in females (ratio is around 5:1 for SSc). One hypothesis is that endocrine triggers contribute to the initiation or progression of scleroderma. It has been observed that the female sex hormone oestradiol

potentiates the pro-fibrotic effects of some cell-to-cell mediators in scleroderma, including interferon-gamma, interleukin-1 and tumour necrosis factor alpha (31).

#### *Infective triggers*

As has often been proposed in analogous auto-immune conditions, it is presumed that scleroderma might be triggered in some cases by a host immune response to an infective agent that acts as a “molecular mimic” for the host’s own proteins. By way of example, there are close resemblances in the amino acid sequence of the protein that binds ATA antibodies and an antigen that appears on a number of mammalian retroviruses(32).

More detailed analysis has focussed on the hypothesis that latent cytomegalomvirus (CMV) infection, localising to the vascular endothelium, might act as a disease trigger in scleroderma. To support this hypothesis, potentially pathogenic auto-antigens seen in SSc bind to the UL94 protein on CMV as well as to the endothelial cell surface in humans, where once bound they cause endothelial cell apoptosis, which is traditionally considered an early event in scleroderma—see “Progression” below (33).

#### *Neoplastic triggers*

The incidence of scleroderma in relation to a trigger has been defined on a molecular level in the subgroup of patients with SSc and cancer. There is a relatively high prevalence of malignancy in patients with scleroderma and the anti RNA polymerase III (ARA) antibody. In this subgroup, the clinical onset of connective tissue disease and the diagnosis of cancer often come close together in time (34,35). Joseph and colleagues describe mutations in the RNA polymerase III polypeptide A gene (POL3RA) in cancer cells extracted from patients in this subgroup, but not in control



tissues from the same patients or in the cancer cells of patients from other scleroderma subgroups. They hypothesise that specific cellular immunity triggered by a neoplastic mutation could lose its specificity in the humoral response (a concept known as “epitope spreading”) and this could subsequently result in disease-causing autoimmunity. Supporting this concept, they describe a population of CD4 T cells in the peripheral blood in some of these patients that reacted to the mutated POL3RA in the patient’s cancer cells (but not to their own wild-type POL3RA protein) and then demonstrated that antibody response in these patients could not discriminate between wild type and mutant POL3RA (36).

This study gives a useful outline of the pathobiology whereby an autoimmune condition like scleroderma could be triggered in a susceptible individual by exposure of the immune system to a cross-reactive antigen (in this case a somatic mutation in cancer tissue).

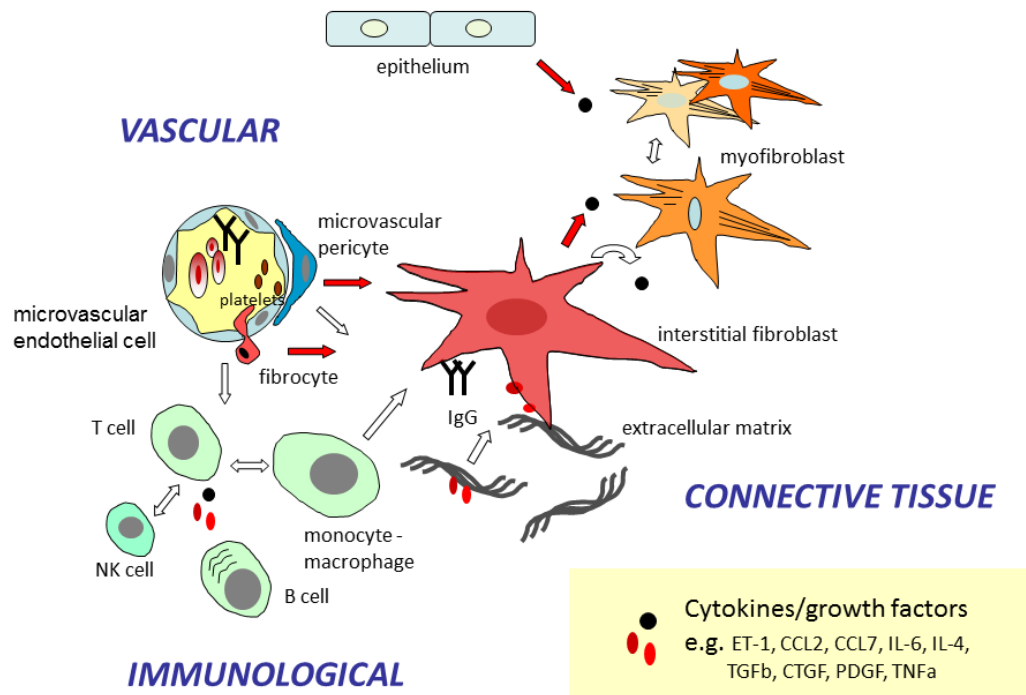
#### Progression

The traditionally proposed model of disease progression in scleroderma is sequential, with vasculopathy and/or immune activation usually described as happening first and then resulting in the activation of fibroblasts and subsequently excess extracellular matrix production and scarring (37). However, there is no definitive evidence as to the order in which these events occur. It seems likely the disease state will only be tolerated if there is *simultaneous* dysregulation of all three relevant compartments (ie immune system, vascular endothelium and connective tissue repair system). It is in the context of the disease susceptibility and initiation discussed above that the key clinical features of scleroderma—hardening of the skin,

fibrosis of the internal organs and systemic vascular dysfunction—are allowed to develop and are subsequently amplified. The interplay of skin, vascular and immune cell-types via important signalling molecules which are implicated in the disease process is summarised in Figure 2 and described further below.

Figure 2: Cell-to-cell interaction in multiple systems in scleroderma pathogenesis.

Abbreviations: CCL2, chemokine ligand 2; CCL7, chemokine ligand 7; CTGF, connective tissue growth factor; ET1, endothelin 1; IL6, interleukin 6; PDGF, platelet-derived growth factor; TGFb, transforming growth factor beta; TNFa, tumor necrosis factor alpha. *Cell types are not illustrative of source of specific mediators, e.g. ET-1 is unlikely to be of epithelial origin.*



Raynaud's phenomenon and the role of vasoconstrictors

Abnormalities in the cell behaviour and therefore the physiological activity of the vascular endothelium play a role of particular importance in the pathophysiology of scleroderma (38). Circulating mediators of vascular tone, which include the endothelins and nitric oxide, have a key role(39).

Endothelin-1 (ET-1), is best recognised as a marked vasoconstrictor but additionally acts as a mediator of fibrosis (40). ET-1 has been proposed as an important effector molecule in scleroderma. ET-1 levels respond dynamically to changes in temperature in scleroderma patients with Raynaud's phenomenon but not in healthy individuals (41). ET-1 was shown to promote collagen synthesis in the same patients, the degree of effect correlating with the ET-1 levels. Serum ET-1 concentration correlates with the degree of scleroderma disease severity and with the development and progression of internal organ complications of the disease including the three with the highest associated mortality: pulmonary arterial hypertension (PAH), interstitial lung disease (ILD) and scleroderma renal crisis (SRC)(42–44). The A and B subtypes of endothelin receptor (ETRA/ETRB) are both overexpressed in vascular and other renal tissues in SRC(45). The pathogenesis of SRC is discussed further below.

It is possible ET-1 plays a linking and promoting role between abnormal vascular tone and pathological fibrosis in scleroderma: staining intensity of ET-1 and its receptors were increased in the vasculature and superficial dermis of areas of skin which were either not-yet-involved or showed only early skin fibrosis, but not in skin which had progressed to late fibrosis (46).

Pharmacological antagonists of both ETRA and ETRB have been demonstrated in patient care as effective clinical treatments for what are clinically apparently diverse

complications of scleroderma (digital ulcers and pulmonary arterial hypertension), providing additional evidence that the endothelin system plays a significant part in the disease process.

In normal human biology, endothelin activity is regulated by negative feedback directly from its own ETRB receptor, but also by nitric oxide synthase (NOS) and superoxide anions. Data as to the specific defects in these homeostatic mechanisms that allow the scleroderma disease state to persist are contradictory (47–49).

#### *Endothelial damage in scleroderma*

Anti-endothelial cell antibodies have been observed in the serum of some scleroderma patients and *in vitro* the sera of those patients induced endothelial cell apoptosis (50–52). Apoptosis was effectively blocked by anti-tumour necrosis factor (TNF) antibodies in some cases. The complement membrane attack complex (MAC) can be identified in the microvasculature in sclerodermatous tissues (53). After endothelial damage has occurred, high pressure shear stress and reperfusion injury both appear to have a role in progression of vascular dysfunction in scleroderma (54,55). Angiogenesis and the ability of the vasculature to repair are both dysfunctional in scleroderma, allowing the chronic disease state to evolve (56,57).

#### *Adhesion molecules*

Adhesion molecules act as mediators between vasculature and extracellular matrix and regulate migration of immune cells. These molecules seem to play a significant role in the pathology of scleroderma (58,59). Concentrations of intercellular adhesion molecule-1 (ICAM-1) and endothelial leukocyte adhesion molecule-1 (E-selectin) are both raised on the endothelial cell surface in scleroderma patients (60).

Further to this, the soluble forms of ICAM-1, E-selectin and vascular cell adhesion molecule 1 (VCAM-1) have been shown to be increased in concentration in serum from scleroderma patients (48,61,62). Increased concentrations of these three soluble adhesion molecules are also associated specifically with scleroderma renal crisis (63).

#### *Immunological activity in SSc*

As described earlier, the best evidence for genetic risk in scleroderma is related either to HLA alleles which act as general risks for automimmunity or to variants in genes that govern immune cell activity. The triggering events we have reviewed so far are presumably mediated to some degree via the immune system. The genetic findings suggest that the immune dysfunction will prove to have a primary importance in the pathogenesis of scleroderma. However, it has been a widespread clinical observation that medical immunosuppression has only a modest effect on patient outcome in scleroderma, particularly when we compare with the effectiveness of immunosuppressive medication on analogous systemic rheumatic conditions including Rheumatoid Arthritis and SLE. For this reason, we have to presume that immune dysfunction in the scleroderma disease state is taking place in concert with dysfunction in the vascular and connective tissue systems rather than as a reversible *cause* of those dysfunctions.

#### *The role of autoantibodies*

A large majority of scleroderma patients (85% or more) have autoantibodies, which can be identified in their serum and in most cases these are highly specific for the disease and mutually exclusive within patients. The antigen targets for the typically observed antibodies all have their origin within the nucleus, including: the

centromere (ACA), topoisomerase 1 (ATA, formerly known as Scl-70), RNA polymerase (ARA), ribonucleoproteins (fibrillarin or U3RNP) and the exosome complex (Pm-Scl).

Some of the environmental or neoplastic risks described above are believed to directly provoke antibody production in a minority of cases. In most scleroderma patients the provoking event for acquired antibody positivity is not clear, but in at least some cases, antibody production may be an event secondary to vasculopathy. Several of those scleroderma-associated autoantigens outlined can be fragmented by reactive oxygen species (ROS) in the context of ischaemia-reperfusion injury(64). This in turn generates the hypothesis that the fragmentation process could result in immunogenic peptides and subsequently the breaking of “self-tolerance”.

There is little available evidence to suggest that the IgG antibodies detected in clinical diagnostics for scleroderma are themselves directly pathogenic. It is more likely they reflect T-cell activity against their antigen targets. Nevertheless, other circulating antibodies, typically directed against targets outside the nucleus, may be directly implicated in the disease process. As well as anti-endothelial antibodies, which I described as potential pathogenic mediators above, anti-fibroblast antibodies can be detected in serum from scleroderma patients but not controls. These antibodies could activate fibroblasts in vitro (65). Anti-angiotensin receptor and anti-endothelin receptor antibodies are also much more common in scleroderma patients than controls and the concentrations of both these antibodies correlate positively with the degree of disease severity in those individuals (66). A further study found antibodies against the platelet derived growth factor receptor (PDGFR) in scleroderma patients compared with controls. Anti-PDGFR antibodies

were shown to generate ROS, stimulate the differentiation of myofibroblasts and subsequently increase expression of type 1 collagen (67). There is no evidence in the studies described above as to how these apparently pathogenic antibodies are acquired or allowed to persist in scleroderma.

#### *Cellular immunity*

With the increase in cell adhesion molecule activity described above, migration and activation of CD4 positive T-cells is promoted in scleroderma (68) and these cells are observed in high concentration in the lymphocytic infiltrates seen in both skin and lung tissue(69,70). Differentiation towards the Th17 subclass of T-cells appears to be important in scleroderma and these cells are pro-fibrotic compared with other subclasses(71). It has been hypothesised that regulatory T-cells (Tregs), which normally play a role in the maintenance of “self-tolerance” may be reduced in number in scleroderma or be defective in their action (72,73). Altered function of toll-like receptors may be responsible for the monocyte infiltration that has also been observed in the skin and lung tissue of patients (74,75).

#### *Cytokines and cell-signalling in SSc*

The important cell-to-cell signalling events in scleroderma are not only those which are stimulated or released by immune cells. Endothelial cells and fibroblasts both have a contribution to the signalling cascades. Unpicking abnormalities in the communication between these three different cell groups is a key part of understanding the scleroderma disease process.

This is a multi-directional interaction. For example, increased expression of ICAM-1, occurs in soluble form, as described above, but also on the surface of both fibroblasts and endothelial cells. This increases binding of lymphocytes to fibroblasts



and promotes migration of lymphocytes across the endothelial barrier. Increased ICAM-1 concentrations can be stimulated by interferon-gamma and tumour necrosis factor, both originating from lymphocytes (31,76).

In analysis of this “cross-talk” between the immune, vascular and connective tissue systems, interest has focussed on mechanisms which promote epithelial-to-mesenchymal transformation and thereby provide the enlarged population of myofibroblasts typically seen in scleroderma.

#### *Transforming Growth Factor-beta (TGF- $\beta$ )*

TGF- $\beta$  is the best-recognised mediator of extracellular matrix production and therefore has been widely investigated as a pathogenic mediator in scleroderma. It is seen in high concentration in tissues affected by the condition (77,78) but circulating TGF- $\beta$  levels are actually lower than controls in serum from scleroderma patients, and the concentrations correlate inversely with disease activity(79). A possible explanation is that there is an increase in the binding and/or the sequestration of the molecule in scleroderma. TGF- $\beta$  receptors are upregulated in scleroderma skin (80). Cell culture experiment have failed to either induce or maintain a scleroderma-like cellular phenotype via manipulation of the TGF- $\beta$  system, however (81).

TGF- $\beta$  presumably exerts its pro-fibrotic effects in scleroderma at least in part via other signalling molecules described above including ET-1 and PDGF. In culture, both the expression of PDGFR and the mitogenic response to PDGF are much more marked in scleroderma fibroblasts than control cells (82).

#### *Connective tissue growth factor (CTGF or CCN2)*

Expression of CTGF is increased in many fibrotic diseases and it is an important effector in the TGF- $\beta$  pathway (83). Scleroderma fibroblasts generate high

concentrations of CTGF and the N-Terminal fragment of this molecule was highly expressed in blister fluid and serum of patients, correlating with extent of disease (84). It seems likely CTGF is an essential co-factor for TGF- $\beta$  to activate or sustain ECM production in both normal health and disease (85).

#### *Interleukins and chemokines*

Interleukin-6 (IL-6) can be secreted by fibroblasts as well as lymphocytes and stimulates synthesis of collagen (86). Serum concentrations are elevated in patients with scleroderma and correlate with extent of skin disease (87).

Interleukin-4 (IL-4, typically secreted by Th2 cells) is seen in higher concentrations in serum and disease tissues in scleroderma(88,89), SSc fibroblasts overexpress its receptor and stimulation of the IL-4 receptor in SSc fibroblasts promotes production of collagen (90).

The secretion of Interleukin 1- $\alpha$  by skin keratinocytes is raised in SSc scleroderma. Culture of scleroderma keratinocytes together with normal skin fibroblasts causes activation of fibroblasts as well as their differentiation into myofibroblasts, and increased CTGF production, all of which are mediated via IL-1 $\alpha$ (91).

CXCL4 (or platelet-activating factor 4) is an angiogenic factor which downregulates expression of interferon- $\gamma$  (an anti-fibrotic) and promotes production of the pro-fibrotic cytokines IL-4 and IL-13 (92). Plasma CXCL4 concentration is raised in scleroderma and correlates positively with disease activity (93).

Monocyte chemotactic proteins 1 and 3 (MCP-1 and MCP-3 also referred to as CCL2/CCL7) are both overexpressed in scleroderma(90,94). MCP-1 may have a direct role in pathogenesis but is better understood presently as a biomarker of disease activity.

## *Fibroblasts*

The skin and internal organ fibrosis that gives scleroderma its name requires a population of activated fibroblasts which in turn produce excess collagen. In cell culture, scleroderma fibroblasts from skin and lung show these features compared with control cells and maintain the disease-causing phenotype through generations (95). In patients, cells with this phenotype are found predominantly in perivascular tissues and areas of inflammatory infiltrate, while normal fibroblasts persist elsewhere (96,97). The key abnormality of increased matrix production could be dependent primarily on an excess of the pro-fibrotic signals I have outlined above, but equally a lack of inhibitory signals may be responsible. Such inhibition acts as a negative feedback loop in normal health when fibroblasts come into contact with collagen and fibronectin and may be defective in scleroderma.

How far fibroblasts require TGF- $\beta$  to sustain ECM production has been examined in animal models with deletion of the TGF- $\beta$  receptor gene. Transgenic mice were unable to achieve normal wound healing(98) but were resistant to an experimental model of lung fibrosis(99). Unlike these models, the human scleroderma fibroblast, appears to have some autonomy in ECM production, with no absolute requirement for external signals (100).

It is possible that immune abnormality in scleroderma results in the selection of a population of fibroblasts that can sustain collagen overproduction indefinitely.

Another hypothesis is that there is epigenetic modulation of fibroblast activity: hypermethylation of the promoter region for the FLI 1 gene (an inhibitor of collagen synthesis) produces fibroblasts capable of excess collagen production and impervious to negative feedback mechanisms (101).

Another possibility is that abnormalities in the ECM contents allow abnormal fibroblast activity. Microfibrils of fibrillin-1 in scleroderma appear to be less stable than in controls and this cellular environment might be partially responsible for the altered fibroblast behaviour(102,103).

#### Amplification

Up to here I have outlined a number of interrelated factors that make an individual susceptible to systemic sclerosis, the events and exposures that could result in triggering the disease in a prone individual and the dysfunctional activity which allows the disease to progress on a molecular level, eventually producing the typical scleroderma phenotype.

Happening in concert, these processes of simultaneous multisystem dysregulation allow amplification of scleroderma over time and lead to the internal organ complications that are responsible for the high levels of morbidity and mortality. But susceptibility factors, including the genetic ones described, may also at least partially determine the severity and phenotype of each patient's disease and their risk of developing specific complications of the disease. I will explore this further below in the context of renal complications of scleroderma.

## Renal disease in systemic sclerosis

Like other organ systems affected by scleroderma, the renal tract is subject to chronic, progressive parenchymal fibrosis and vasculopathy which can be acute and chronic. In keeping with this, prevalence of chronic kidney disease (CKD), as defined by urinary abnormalities or a reduced estimated glomerular filtration rate (eGFR), was as high as 50% in one series of scleroderma patients(104). Inflammatory disease of the kidney, including ANCA-associated vasculitis(105,106) and interstitial nephritis(107) are also seen in the context of SSc, but the most clinically significant renal manifestation in scleroderma is scleroderma renal crisis (SRC) and therefore I will cover that topic in some detail.

### Definition of scleroderma renal crisis

Scleroderma renal crisis can be defined as new onset of accelerated arterial hypertension and rapidly progressive excretory renal failure in the context of scleroderma. Neither the hypertension nor a rise in serum creatinine in isolation can be presumed to be diagnostic of SRC. Given the complexity of this multisystem disorder, many other potential causes of acute kidney injury (AKI) have to be considered in the scleroderma population. In particular, a rise in creatinine secondary to circulatory dysfunction (sometimes described as “pre-renal AKI”) should always be suspected as should medication related causes. Variation in the criteria used to define SRC in different case series may account for discrepancies in the outcomes of the patients described.

Consensus criteria for the definition of SRC are summarised in Table 2 below but these are currently being developed further in a prospective case collection study,

which will build on a completed Delphi exercise that has already canvassed international expert opinion on the definition of SRC. When the completed consensus criteria are published, these will allow more consistent comparison between study cohorts in future (108–110).

Table 2. Proposed criteria for the diagnosis of scleroderma renal crisis

<b><u>Diagnostic criteria (essential)</u></b>	
New onset of BP >150/85 mmHg or Increase of $\geq 20$ mmHg from usual BP	} obtained at least twice over 24 hrs
A decline in renal function, defined as an increase in serum creatinine of $\geq 10\%$ (confirmed with repeat testing where possible)	
<b><u>Supportive evidence (desirable)</u></b>	
Evidence of MAHA (microangiopathic haemolytic anaemia) on blood film	
Acute hypertensive findings on fundoscopy	
Microscopic haematuria demonstrated on urinalysis or urine microscopy	
Oliguria or anuria	
Typical features of thrombotic microangiopathy on renal biopsy examination, including intimal proliferation and onion skin appearance in small and medium sized vessels, fibrinoid necrosis, glomerular shrinkage.	
Acute onset of pulmonary oedema	

## Pathogenesis of Scleroderma Renal Crisis

As discussed above, the pathology of SRC is typified by a triad of autoimmunity, vasculopathy and fibrosis. Dysfunction in all three of these three systems has been observed in the kidney in scleroderma patients(63). That notwithstanding, SRC is primarily a disease of impaired arterial blood flow within the kidney.

Endothelial injury, followed by increased permeability of the vessel wall and proliferation of the damaged intima is an early event in this process. This results in narrowing of the lumen, especially in interlobular and arcuate arteries. Subsequently there is a reduction in blood supply to the renal cortex.

Endothelial cell activation may play a role in addition to injury to these cells.

Increased circulating concentrations of ET-1(44), and VCAM-1 (63) have been observed in association with SRC. Immunohistochemical examination of SRC renal biopsies has revealed increased tissue concentration of ET-1 as well as its receptors ETRA/ETRB (45,111).

A consequence of hypoperfusion of the renal cortex is hyperplasia of the juxtaglomerular apparatus, which is the principal site for renin production (112). At the time of SRC diagnosis, patients have marked elevation in circulating renin concentrations. As there is a dramatic clinical response to therapeutic inhibition of the renin-angiotensin system in SRC (and previously to nephrectomy, removing renal renin production completely), it seems logical that overproduction in renin would have a key role in the evolution of the disease state(113). However, prior to the acute onset of SRC, renin levels are typically observed to be normal and raised renin in scleroderma patients does not predict future SRC(114), so while the susceptible



patients do appear to have underlying renal vasculopathy it is not clear what precipitates the acute crisis.

There is no evidence regarding the role played by a dysfunctional immune system in SRC, but a marked contrast has been observed between the frequency of SRC in patients with different disease defining antibodies, ranging from ACA (<1% risk of SRC) to ARA (33%). This suggests that developing a better understanding of the role these antibodies play in the pathophysiology of scleroderma in general may improve our understanding of the pathogenesis of SRC (115).

#### Epidemiology

SRC incidence is varied between populations and may be falling over time(116).

Most historical estimates suggested it appears in around 10% of the SSc population overall and up to 25% of patients within the diffuse skin subgroup (dcSSc). At least 75% of SRC cases occur within 4 years of the first symptom attributable to scleroderma(114). In one SRC case series, it was the presenting symptom of SSc for 22% (117). Males are more often affected than females and African-Americans are at least three times as likely to be affected as Caucasians but data are limited in black or other minority ethnic group risk outside of those American cohort studies (118,119).

#### Predicting renal crisis

Multiple predisposing factors have been observed in SRC cohort studies. These risk factors are summarized in Table 3 below. As discussed above, patients with diffuse cutaneous scleroderma (dcSSc) are at the greatest risk: this subgroup is defined by

skin thickening on trunk or the proximal part of the limbs. dcSSC patients account for (121)75-80% of SRC cases (120).

Patients who will in due course be categorised as dcSSc but don't yet have typical skin changes make up a further 15-20% of SRC cases. Identifying early those who will end up being categorised according to skin as "diffuse" is therefore of particular importance in predicting those at risk of SRC. Such patients have usually had scleroderma symptoms for less than one year. They often have arthralgias puffy or swollen limbs, and carpal tunnel syndrome(121). Palpable tendon friction rubs occur at some point in 65% of patients with dcSSc(122) but fewer than 5% of patients with limited disease, so may be an early clue to the eventual disease subgrouping.

As touched on earlier, autoantibodies can also help us to identify patients at increased risk of SRC. Anti-RNA polymerase III (ARA) is a scleroderma specific antibody seen almost exclusively in dcSSc and 24% to 33% of patients with this antibody develop SRC(123,124). In published cohorts with a low frequency of this antibody (those from Asia or Southern Europe, where its prevalence ranges from 6 to 9% of scleroderma patients) there is a correspondingly low incidence of SRC, relative to the United States and the United Kingdom, where ARA frequency is more than 20%(123). SRC occurs in 10% of those with ATA, the most prevalent antibody associated with dcSSC(125). ACA, typically seen in lcSSc, acts as a relative protective factor for SRC (126).

Identification and clinical management of patients at high risk of renal crisis

#### *Early identification*

For patients with early dcSSc (within 4 years of diagnosis) expert consensus recommends home BP monitoring twice weekly(127). Patients are given individualized blood pressure targets and are instructed to seek medical review if their blood pressure is above these targets. However, significant arterial hypertension is not usually observed prior to SRC in affected individuals. Normal blood pressures have been documented as little as 24 hours prior to the onset of SRC (118).

#### *Corticosteroid exposure*

An association between the use of medical corticosteroids and the likelihood of SRC has been observed since the earliest full descriptions of the disease (128,129). Incidence of SRC may be as much as twice as high among those patients given medium or high dose steroids (130). This is a challenging statistic to interpret. SRC is commonest in patients with early, aggressive disease and these individuals are more likely to be treated with high steroid doses. Nevertheless, a case-control study comparing patients with SRC to other patients at high risk (according to those factors described above) also found those who had received >15mg prednisolone were three times as likely to develop with SRC in the following six months(131).

#### *ACE inhibitors prior to renal crisis*

ACE-inhibitors (ACEi) are now standard therapy for SRC as will be discussed below. However, there are no data to support ACEi use for prophylaxis against SRC, even in those at high risk (117,132) and indeed this may be harmful(133). A prospective study of 75 patients with SRC found those who had been on ACEi prior to the

diagnosis of SRC had more than 2 times the risk of death in the year following SRC(134). There are several competing hypotheses for these observations. One possibility is that partial ACE-inhibition can mask the onset of hypertension and result in late diagnosis of SRC. Another is that individuals who developed SRC despite taking an ACEi are in a resistant group with an intrinsically poor prognosis that is not modifiable. A final possibility is confounding by indication: i.e. hypertension or left ventricular systolic dysfunction for which ACEi are commonly prescribed are at least in part responsible for the excess mortality in these patients.

Table 3. Risk factors for scleroderma renal crisis

<b>Associated with increased risk of SRC</b>	<b>No associated risk for SRC</b>
Disease symptoms < 4 years	Previous acute or chronic hypertension
Diffuse cutaneous skin subtype	Pre-existing proteinuria
Rapid progression of skin thickening	Pre-existing chronic kidney disease
Anti-RNA polymerase III antibody (ARA)	Pathological abnormalities of renal blood vessels
New onset of cardiac complications  Pericardial effusion  Left ventricular failure	Anti-topoisomerase (ATA)  Anti-centromere antibodies (ACA)
New onset of anaemia	
Recent corticosteroid exposure  >15mg prednisolone equivalent	

Diagnosis of scleroderma renal crisis

*Clinical presentation*

As in acute accelerated hypertension of other causes, SRC patients may complain of central nervous system symptoms including headache, visual change, confusion or even seizures in those most severely affected. In keeping with other forms of AKI, it is rare for affected individuals to present with oliguria or uraemic symptoms. The majority will be non-specifically unwell, complaining of increased fatigue, headache or occasionally dyspnoea.

90% of patients have blood pressure (BP) levels greater than 150/90 mm Hg at presentation with SRC and 30% have diastolic recordings over 120 mm Hg. About 10% of cases have normal blood pressure but an increase of 20 mm Hg in either the systolic or diastolic pressure would be potentially diagnostic, even if the measurement remains in the “normal” range. A category of “normotensive renal crisis” has been proposed, requiring the support other features, including unexplained AKI and microangiopathic haemolytic anaemia (MAHA). Some individuals present with symptoms and signs secondary to acute hypertension, including congestive cardiac failure, pericardial effusion, or arrhythmias. Fundoscopy will demonstrate acute hypertensive retinopathy in many.

Laboratory findings

In a majority of cases there is both protein and blood detectable on urinalysis. Proteinuria is typically mild or moderate (equivalent to <2g of urinary albumin per day) and microscopy shows granular casts in some (135). Serum creatinine is at least 150% of baseline value in most cases (ie Stage 1 Acute Kidney Injury in the consensus

guidelines for AKI care(136). Many scleroderma patients have low muscle mass, so creatinine can often reach this threshold without rising above the normal range values quoted by clinical laboratories. Creatinine will usually rise rapidly in the following days and will continue to rise even where blood pressure is adequately controlled.

Serological evidence of MAHA is apparent in approximately 50% of SRC cases e.g. one case series found a reduced platelet count in 50% of and red cell fragments in 52%(117). The presence of MAHA along with accelerated hypertension, or in some cases a finding of thrombotic microangiopathy on renal biopsy, can cause diagnostic confusion, raising the possibility of thrombotic thrombocytopenia purpura (TTP) or atypical haemolytic uraemic syndrome (aHUS). There are case reports of TTP in individuals with scleroderma (137–140). Fever and hemorrhagic manifestations were important clinical findings that helped differentiate these cases from SRC. Levels of the enzyme ADAMTS13, normally are reduced in TTP(141), were normal in a small study of patients with SRC (K.S. Torok et al. “Scleroderma renal crisis and thrombotic thrombocytopenic purpura – are they related”, American College of Rheumatology abstract 2008). However, in most institutions, results of the ADAMTS13 assay will not be available early enough to affect the immediate clinical management. Given the remaining diagnostic uncertainty in this field, if a diagnosis of TTP or HUS is suspected in a scleroderma patient, expert consensus has recommended that an ACE-inhibitor should be used in conjunction with plasmapheresis or anti-complement monoclonal antibody therapy(139,142).

## Imaging

Renal ultrasound does not show any specific features in SRC. Echocardiography will sometimes demonstrate pericardial effusions and reduced left ventricular ejection fraction, both common findings secondary to increased afterload on the heart.

## Renal Pathology

The primary clinical benefit of renal biopsy is to exclude other renal pathology that can mimic SRC (e.g. ANCA-associated vasculitis) in the acute phase. There is some evidence that biopsy findings can be informative about longterm prognosis. Data on renal pathology findings in SRC are limited by the low volume of biopsies performed, usually because of safety concerns with regard to renal biopsy in patients with high BP and low platelets.

Pathological findings in SRC are generally the same as are seen in other causes of accelerated hypertension(107). Fibrinoid necrosis is often widespread in both arterial walls and the subintima of interlobular and arcuate arteries. The resulting intimal thickening leads to narrowing or obliteration of the lumen and what is traditionally referred to as an “onion skin” appearance (Fig. 1 below). Adventitial and peri-adventitial fibrosis, which indicate chronic vasculopathy, are common in patients with SRC, but more rarely seen in accelerated hypertension without scleroderma. Glomeruli may be collapsed due to ischaemia, with wrinkling of the basement membrane. Unlike in other renal diseases the extent of interstitial scarring does not predict longterm renal outcome but extensive vascular injury (defined as

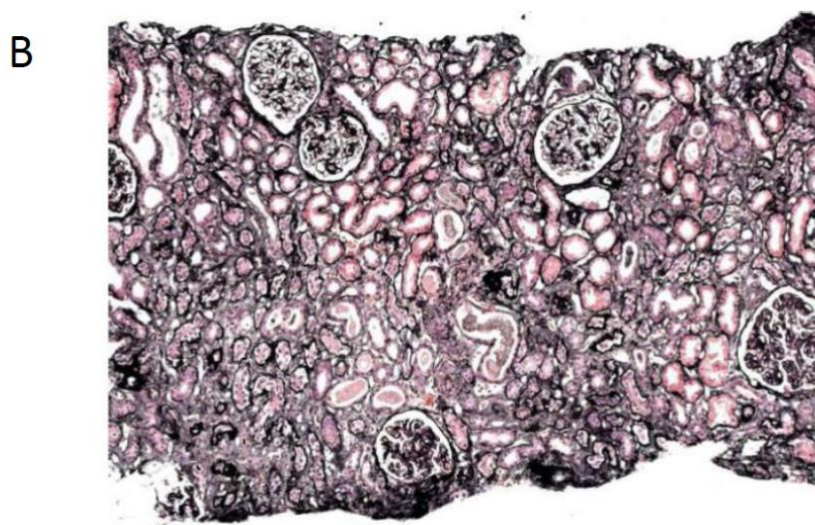
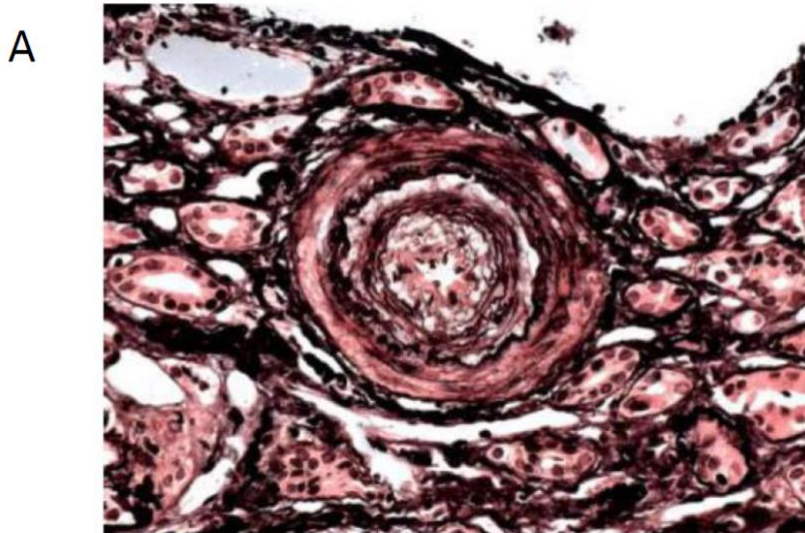


the area of fibrinoid necrosis and the percentage of thrombosed vessels) does predict poor renal outcome(107,117).

Figure 3: Typical renal histopathology in scleroderma renal crisis.

A. A medium-sized vessel (interlobular artery) in SRC demonstrates evidence of both acute injury (loose, concentric intimal thickening) and chronic vasculopathy (hypertrophied smooth muscle inside the internal elastic lamina).

B. Overview of the renal cortex in SRC. Glomeruli show wrinkling and shrinkage associated with acute ischaemia. Acute tubular necrosis predominates with only early evidence of tubular atrophy and dropout.



## Treatment of Scleroderma renal crisis

### *General considerations*

Extra-renal organ manifestations of SRC are managed supportively, which may include management of pulmonary oedema or encephalopathy using standard approaches such as supplementary oxygen, ventilatory support, and sedation or anti-seizure medications according to clinical circumstances.

### *ACE-inhibitors*

Immediately on diagnosis of SRC, an ACEi is typically introduced or the dose increased if the patient is already taking one(143). A short acting ACE-inhibitor (e.g. captopril) may be preferable in a haemodynamically unstable patient but there is no evidence that it is preferable in general to a once-daily medication. ACEi resistance is more typical than oversensitivity and typical practice is to initiate a long-acting drug as soon as possible and escalate the dose daily to maximum (127). While in typical hospital care, ACEi medications are discontinued in patients with acute kidney injury, there is no evidence in SRC that renal function can be spared or improved by minimizing ACEi dose.

### *Other antihypertensives*

Given dramatic survival benefits seen from antagonism of one part of the renin-angiotensin system (RAS) in SRC, there has been extensive interest in other approaches to improve outcome via blockade of this axis. Reports suggest angiotensin receptor blockers (ARBs) alone are less effective than ACEi in treating SRC(144,145) but conversely they may be safer as an agent to use for hypertension prior to the onset of SRC(133). There is no evidence available on the role of direct renin inhibitors. The combination of ACEi and ARB is generally avoided because it

offers only modest additional blood pressure control and an excess of adverse events(146). Beta blocking medication is contraindicated in SRC due to effects on peripheral circulation and intravenous antihypertensives are not usually indicated, although nitrate infusion is sometimes used when pulmonary oedema complicates SRC.

#### *Endothelin receptor antagonists (ERAs)*

Endothelin receptor antagonists have been demonstrated to have significant outcome benefit in both PAH and digital ulceration in patients with scleroderma (see above). This has raised the question of their utility in renal crisis, an analogous vasculopathic complication of scleroderma (147). As discussed above, high circulating levels of ET-1 and upregulation of the endothelin receptor have been demonstrated in SRC. Polymorphism in the endothelin ligand receptor axis has been associated with SRC(10) whereas polymorphism in the ACE axis has not(148), so it is possible that activation of the endothelin system is a key event in the evolution of the condition. In an open label pilot study, the non-selective ERA Bosentan was given to six patients with SRC. Although not significant, there was a trend towards lower rates of dialysis and better recovery of renal function in these patients(45). A further study of bosentan showed no evidence of benefit (149). However, neither of these studies used a randomised controlled design.

#### *Other drug treatments*

The prostacyclin analogue Iloprost is an additional way to reduce the systemic vascular resistance in SRC and has been shown specifically to increase blood flow within the kidney in patients with scleroderma(150). The role of immunosuppression in the treatment of SRC has not been defined.

## Renal Replacement therapy

In case series of patients treated with ACEi, around 60% of SRC patients progress to needing renal replacement therapy (RRT) compared to >90% prior to the availability of ACEi(117,151). The most frequently used form of RRT in the acute phase is intermittent haemodialysis (HD), but continuous haemofiltration is occasionally required for patients with marked haemodynamic instability. In the general chronic RRT population, peritoneal dialysis (PD) is associated with better preservation of residual renal function than HD(152–154) and this may be a particular consideration in the SRC group, given the potential for late recovery of renal function discussed below. PD in this patient group is not uncommon—in a case series which included all patients with scleroderma who received RRT in Australia or New Zealand between 1963 and 2005, 50% of patients had PD(155). Despite this, there are currently no data directly comparing outcomes of PD and HD after renal crisis.

Recovery of renal function can continue for months or years after SRC so ACEi therapy is typically continued indefinitely in all patients, regardless of whether they are on dialysis. In the more recent case series described above just under 50% of patients initiated on RRT later recovered independent renal function and were able to discontinue dialysis. Time to dialysis independence is typically longer than in other forms of acute kidney injury with one study showing the median time as 11 months (range 1-34 months)(117). Renal recovery is often slow and continuous both in those who do and do not require RRT and in the study above eGFR continued to improve for at least 3 years after SRC diagnosis.

Because of this possibility of late recovery of renal function, renal transplantation is typically deferred in patients on dialysis due to SRC for at least 12 months.

Calcineurin inhibitors, typically used in renal transplant immunosuppression, are renal vasoconstrictors and therefore associated with increased risk of SRC(156,157).

Although SRC recurrence is rare, case reports document both early and late recurrence after renal transplantation(145,158,159). Overall, as in other causes of end stage kidney disease, SRC patients treated with renal transplant had improved survival in scleroderma compared to those who remained on dialysis(160,161). But as in the general population this finding of a survival advantage is distorted by selection bias given that not all patients are deemed fit for transplantation.

Prognosis

*Mortality*

Before the introduction of ACEi, survival to 12 months in SRC was a rare event(118,120,151). From the early 1980s, when ACEi use became widespread, survival improved rapidly. Those who survive renal crisis either without the need for RRT or having had only temporary RRT now have a 5 year survival of 90%. However, the other subgroup of 40-50% of patients continues to do badly with a number of early deaths and permanent requirement for RRT in those who survive. Overall statistics must be interpreted in the light of these two distinct cohorts, but reviews examining SRC outcome since the 1980s have shown overall 5-year survival ranging between 50 and 70%(117,132,134,162,163). Sadly, there is no clear trend towards improvement in these outcomes over the past 40 years (164). Risk factors

for mortality in these studies included male sex, older age and lower blood pressure at the time of diagnosis as well as the development of congestive cardiac failure.

#### *Renal prognosis*

For those who have at least partial renal recovery in the acute phase, renal prognosis is good. In one representative study of 145 patients, 55 of 145 did not require any RRT in the acute phase. Peak mean serum creatinine among these patients was 336 umol/L. Seven years after diagnosis of SRC their mean creatinine was 159 umol/L. None went on to require RRT at a later stage. 34 patients in the series had temporary dialysis. Their mean serum creatinine 6 years after SRC was only slightly worse than the former group (194 umol/L). 2 of these 34 patients progressed to endstage kidney disease requiring RRT within the follow-up period(151).

#### *Survival on renal replacement therapy*

One series quoted above showed a median survival time of only 2.4 years for scleroderma patients on dialysis compared with 6.0 years for other patients(155). In a study of US national registry data, looking at dialysis patients with scleroderma between 1992 and 1997, two-year survival in the scleroderma group was 49% compared with 64% in the cohort overall(165). Similarly, a review of scleroderma renal transplant cases in the US from 1987 to 1996 showed that graft and patient survival times were both worse after SRC than in renal transplant patients without systemic diseases(166). More recently, review of transplantation outcomes in Europe between 2002 and 2013 showed no difference in graft or patient survival

when comparing scleroderma patients with the general renal transplant population(161).

#### Summary

The pathobiology of scleroderma renal crisis (SRC) reflects the complex interplay of cell types and cell-to-cell mediators in this multisystem disease.

There has been the marked improvement in outcome for most patients with SRC since the routine use of ACEi medications, but mortality and morbidity remain high.

Our ability to identify a subset of patients at high risk of developing the condition—those with early diffuse cutaneous systemic sclerosis and those with RNA polymerase III antibodies (ARA) in particular—offers potential avenues for subgroup enrichment both in pathobiology investigations and testing new therapies in SRC.



**Recent important developments in**  
**this field include increasing evidence**  
**of the role of corticosteroids as a risk**  
**factor for SRC and the potential harm**  
**of ACEi prophylaxis in patients prior**  
**to SRC. Potential new treatments for**  
**patients with renal crisis include**  
**endothelin receptor antagonists**  
**which will be discussed further later**  
**in this thesis. Chapter 2: Genetic risk**  
**for scleroderma renal crisis**

As described in the previous chapter, the distribution of the various internal organ complications of scleroderma differs between distinct skin-based clinical subsets (lcSSc versus dcSSc) and varies according to the different antinuclear antibody reactivities that are a hallmark of SSc (167). Typical antibodies include anti-centromere (ACA), anti-topoisomerase-1 (ATA) and anti-RNA polymerase III (ARA). The frequency of major complications of SSc differs between these clinical and immunological subgroups. For example, ATA positive patients are more prone to interstitial lung disease (ILD) (168) and ACA associates with pulmonary arterial hypertension (PAH) (169). An especially strong association has been demonstrated between the presence of ARA in sera of patients and the occurrence of scleroderma renal crisis (SRC)(117,170).

Given the observations that SRC occurs at different frequency in specific subgroups of SSc, that only a minority of patients develop this complication even within the highest risk groups, and finally that a large majority of cases occurs early in the course of disease, it would be reasonable to hypothesise a genetic predisposition to SRC that could be independent of the inherited risk factors for scleroderma as a whole. There are limited data available regarding the genetic contribution to SRC and like many autoimmune disease and disease complications they centre on MHC antigens; a single study reports an association between MHC class I haplotypes HLA-DRB1\*04:07 and HLA-DRB1\*13:04, and the risk of SRC but there has been no subsequent validation of this finding (171).

In this chapter I aim to improve our understanding of SRC by assessing genetic difference among patients within the subgroup who are positive for ARA. Scleroderma is a rare disease. By selecting a population already known to be at significantly increased risk I aim to have an enriched subgroup of patients, and thereby to amplify any evidence of genetic difference within scleroderma patients, making it easier to identify risks and protective factors for SRC. Examining only individuals in the antibody group at highest risk, comparing cases that develop SRC with those that appear to be protected from development of SRC during long-term follow-up is a novel experimental strategy. I hypothesise that starting at a baseline of clinical (dcSSc) and serological (ARA) homogeneity will make genetic susceptibility factors easier to unravel compared to a population of unselected scleroderma patients. The further hypothesis is that these susceptibility factors might include common variant single nucleotide polymorphisms (SNPs) detectable by conventional genome wide association study (GWAS).

## Patients and Methods

The scleroderma patients identified for this study were under the care of the UCL Centre for Rheumatology and Connective Tissue Diseases at the Royal Free Hospital, a national referral centre for scleroderma. The patients all met either the 1980 American College of Rheumatology (ACR) or 2013 ACR/EULAR (European League Against Rheumatism) classification criteria for scleroderma (172).

To improve our baseline understanding of the timing and frequency of SRC in contemporary scleroderma, I reviewed retrospective clinical and laboratory follow-up data for 2254 patients who had been seen in the centre. Among these there were 134 episodes of SRC each in distinct patients (see Results below). Using these findings, a cohort was assembled for genetic analysis. Based on the data summarised in Figure 1 I assumed ARA-positive patients who reached 60 months of follow-up without SRC were effectively “SRC negative”. A group of 99 ARA-positive patients with at least five years’ follow-up data was then assembled. This was a cohort with two approximately matched halves: 48 individuals who were “SRC positive” and 51 who were still SRC negative after 60 months of follow-up (see **Table 1**). All enrolled patients had given prior consent for DNA analysis and the study was approved by the local Research Ethics Committee. Blood sampling and ELISA assays for autoantibodies were performed by the clinical team and laboratories of the Royal Free Hospital. DNA extraction was performed according to standard local laboratory protocol. Overall, the gender and ethnic background of our cohort of ARA positive patients did not differ from that of the whole Royal Free cohort of dcSSc cases. To further amplify predictive genetic difference, 4 ARA-positive SRC patients of non-European ancestry in the overall cohort were not included in the genetic study.

Genotyping of the Royal Free cohort was performed using the Illumina HumanOmniExpress bead array chip at the UCL genomics centre. Quality control checks were performed for Hardy-Weinberg equilibrium and genotyping rate in

PLINK v1.07(173). After filtering of single nucleotide polymorphisms (SNPs), a case-control logistic regression was performed in PLINK. This compared SNP frequency between patients with and without SRC, with the aim of determining significant genetic difference between the two groups. Further statistical analysis was performed in R v3.4.1(174).

The 9 SNPs with highest statistical association ( $p < 4.5 \times 10^{-5}$ ) in this analysis were then selected for testing in a validation cohort including subjects from 18 specialist scleroderma centres in the United States. All subjects in this cohort were also of white European ancestry. Genotyping was undertaken using TaqMan SNP genotyping assays for each of the selected polymorphisms (175).

Based on the findings of the Royal Free and validation cohorts, two genes were selected for further validation with histological analysis.

Eight historical SRC biopsy samples were identified from Royal Free patients not included in the genetic analysis. These were compared with 8 control samples of normal kidneys donated to the UCL Centre for Nephrology by the NHS Blood and Transplant service from unused deceased donor organs. Renal tissues were assessed using polyclonal Anti-CTNND2 and anti-GPATCH2L IgG antibodies (Abcam, Cambridge, MA). The distribution of CTNND2 and GPATCH2L staining was assessed in four separate renal “compartments” (glomeruli, tubules, interstitium and vasculature) and myself and my PhD supervisor assigned blinded scores for each compartment, which were then aggregated. For each compartment two scores were assigned—one for the proportion of tissue stained positive (0-4) and a second score for the intensity of staining (0-3). The two scores were multiplied together to give a

total score of up to 12 for each compartment in each of 16 biopsy samples. For the CTNND2 analysis, localisation within the glomerulus was attempted using immunofluorescence. Nuclei were identified by counterstaining with 4',6-diamidino-2-phenylindole (DAPI) and endothelial cells by counterstaining with anti-Von Willebrand Factor (VWF) antibodies.

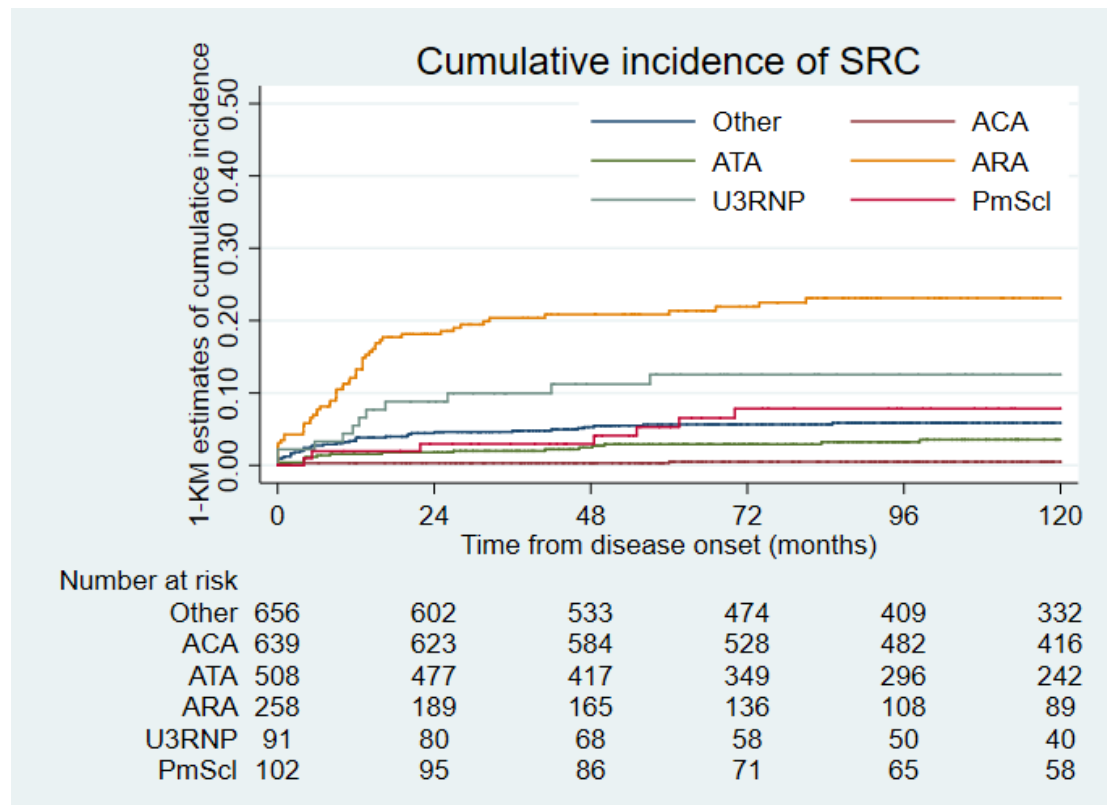
## Results

### Definition of serological risk for SRC

From the cohort of 2254 scleroderma patients with data available on circulating antibodies, 390 (17.3%) were male. 811 (36%) had dcSSc and the rest lcSSc. 258 (11.5%) of the patients were positive for ARA, 639 (28.4%) for ACA, 508 (22.5%) for ATA, 91 (4%) for U3RNP and 102 (4.5%) for PmScl. 869 (38.6%) had interstitial lung disease, 216 (9.6%) had pulmonary arterial hypertension, 89 (4%) had cardiac complications of scleroderma and 134 (5.9%) had a history of renal crisis. Among these 134 SRC cases, 92 (68.7%) had occurred in the first 18 months after diagnosis and 122 (91%) within the first 5 years. Cumulative incidence of renal crisis was calculated at 12, 24, 36 and 60 months as 3.3%, 4.3%, 4.8% and 5.6% respectively. Among the 258 ARA positive patients 59 (22.9%) had a history of SRC. Cumulative incidence of SRC at 12, 24, 36 and 60 months within the subgroup was 12.5%, 18%, 20.4% and 21.4% respectively. Figure 1 illustrates the cumulative incidence in each of the five antibody subgroups. The very high frequency of SRC in ARA positive patients and the trend towards even earlier occurrence in this antibody subgroup supported the hypothesis that this would be an appropriate cohort for examining the genetic susceptibility to SRC via common variant SNPs.

Figure 1: Association of SRC with scleroderma autoantibodies

Kaplan-Meier plot illustrates cumulative incidence of SRC divided into six groups according to circulating autoantibody. The total number of individuals at risk is documented at 24-month intervals up to 120 months of follow-up.





Genetic analysis cohort

99 ARA positive patients with follow-up of at least 60 months were available for analysis (48 SRC positive and 51 SRC negative). Their clinical characteristics are described in Table 1A. Overall the two groups were well matched with regards to age, gender and ethnicity.

GWAS analysis

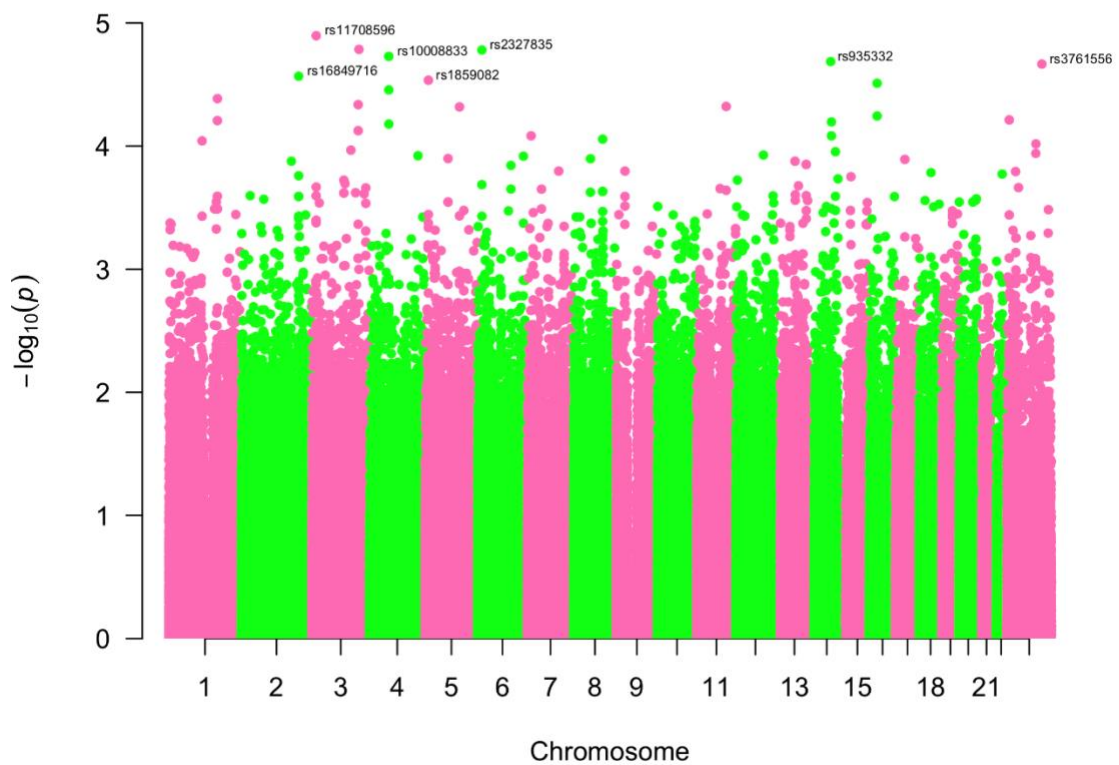
Hardy-Weinberg equilibrium was calculated in PLINK ( $p < 0.001$ ) and genotyping rate was  $> 90\%$ . 2309 SNPs were subsequently removed for missingness and 77122 SNPs failed MAF filters ( $MAF < 0.01$ ).

After these quality control checks, 641,489 SNPs remained for analysis comparing SRC positive and negative groups. Results of this Genome-Wide Association Study (GWAS) are shown by Manhattan plot in Figure 2. SNPs with GWAS  $p$  value  $< 3 \times 10^{-5}$  are annotated on the figure with their reference SNP identification number (*rs*).

Unlike the majority of previous GWAS analyses in scleroderma described in my introduction and including the only previously documented association with SRC, there is no marked association seen with the MHC on chromosome 6.

Figure 2: Single nucleotide polymorphism associations with SRC in ARA positive patients with scleroderma

Manhattan plot of genome-wide association analysis for the occurrence of scleroderma renal crisis among ARA-positive patients in the Royal Free cohort. X-axis groups dots according to chromosomal position. Y-axis marks the negative log value of the regression p-value for each single nucleotide polymorphism (SNP). SNPs with a regression p value  $< 3 \times 10^{-5}$  are annotated on the figure with their rsID.



## Genetic validation

The 9 SNPs with highest statistical association (GWAS  $p < 4.5 \times 10^{-5}$ ) were put forward for validation study in a further 256 ARA-positive subjects from a distinct cohort. The clinical characteristics of these individuals are described in Table 1B. The 9 SNPs identified in the Royal Free cohort and put forward for validation, together with their validation findings, are documented in Table 2.

From these 9 SNPs, only one was demonstrated to have a significant association in the validation cohort at the nominal threshold  $p < 0.05$ . This SNP (rs935332) is in the region of GPATCH2L on chromosome 14. GPATCH2L is a gene of unknown function, but polymorphisms in this gene region were shown to be associated with significant risk for hypertension in GWAS analysis of participants in the Framingham Heart Study (176) and in a UK-wide biobank study (177).

Table 1:

1. Demographics and clinical features of the Royal Free cohort (UK)

	<b>SRC (n=48)</b>	<b>No SRC during follow up (n=51)</b>
Male	8 (17%)	8 (16%)
Age at onset of SSc	50.3 (22-70)	50.1 (20-76)
Age at SRC	50.6 (28-70)	
Caucasian	48 (100%)	48 (100%)
dcSSc	37 (77%)	51 (100%)
Pulmonary arterial hypertension	3 (6%)	1 (2%)
Interstitial lung disease	12 (25%)	10 (20%)

2. Demographics and clinical features of the validation cohort (USA)

	<b>SRC (n=40)</b>	<b>No SRC during follow up (n=216)</b>
Male	7 (18%)	34 (16%)
Age at SRC	57 (44-82)	
Caucasian	40 (100%)	216 (100%)
dcSSc	31 (78%)	164 (76%)

Table 2: SNPs with strongest statistical associated with SRC in the Royal Free cohort (UK) and their degree of association in the validation cohort (USA)

<b>Chromosome</b>	<b>SNP</b>	<b>Gene</b>	<b>P value (UK cohort)</b>	<b>P value (USA cohort)</b>
1	rs2093658	POU2F1	4.12E-05	0.787
2	rs16849716	HECW2	2.71E-05	0.962
3	Rs11708596	C3orf20	1.27E-05	0.223
3	rs2118096	Near to OTOL1	1.64E-05	0.598
3	rs7643629	IQCJ-SCHIP1	4.61E-05	0.728
4	rs10008833	near to EPHA5	1.87E-05	0.078
5	rs1859082	CTNND2	2.92E-05	0.9714
6	rs2327835	LOC101928354	1.66E-05	0.258
14	rs935332	GPATCH2L	2.06E-05	0.025

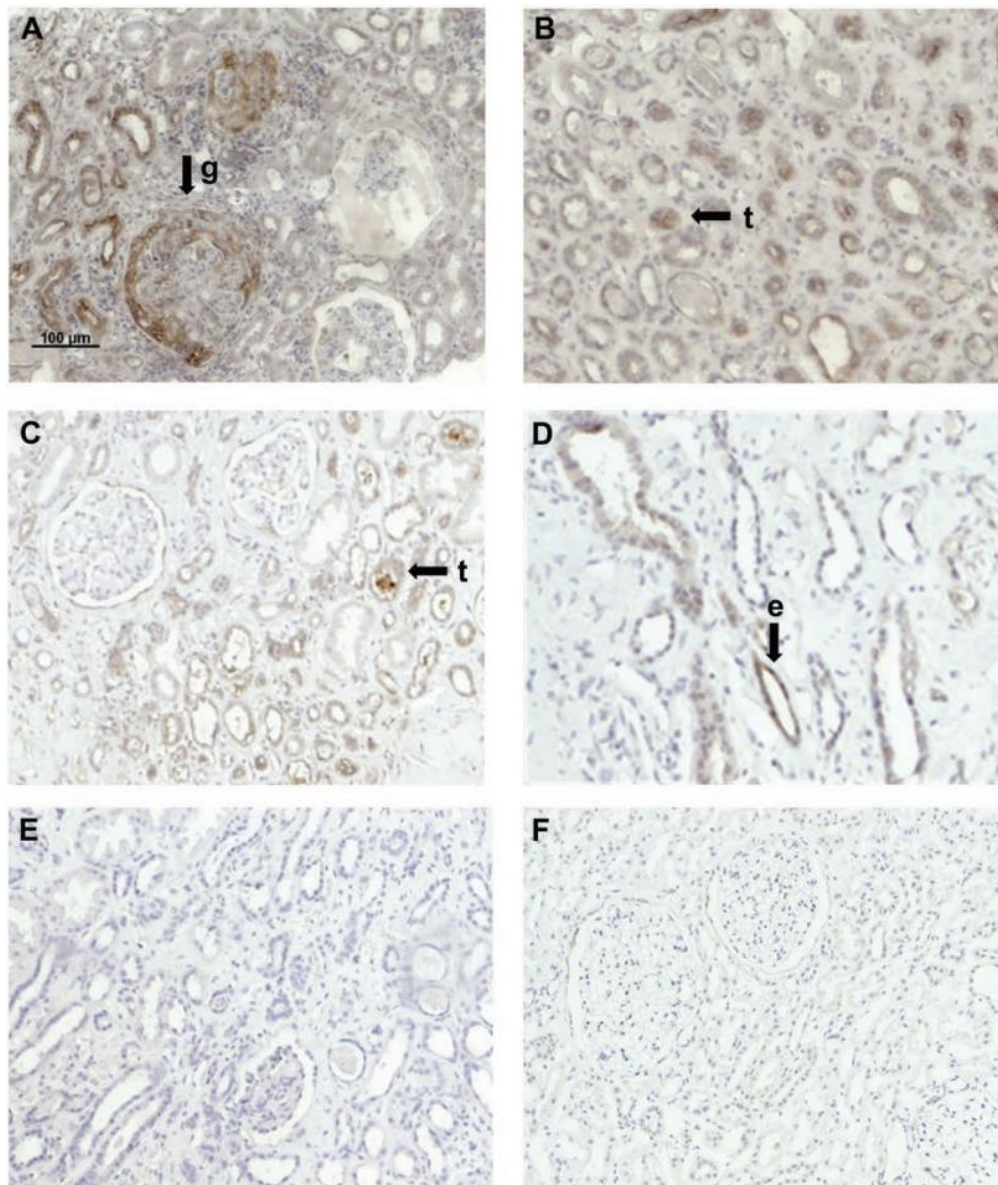
Altered tissue expression of GPATCH 2L and CTNND2 in scleroderma renal crisis

To explore further the significance of the SNPs identified in the original Royal Free cohort, I performed immunostaining for expression of the associated gene products in historical renal biopsy samples from both SRC cases and control kidney tissue. Given the significant genetic replication, I first stained for GPATCH2L (G patch domain containing 2-like) protein and compared expression of this protein in SRC cortical kidney tissue to that in normal renal cortex. Significantly increased expression of GPATCH2L protein was identified in the tubular and vascular regions of SRC tissue compared with controls. As described in the methods, a total biopsy staining score (median, range) was calculated for control (1, 0-4) and SRC (11, 9-21) and this confirmed increased expression in SRC ( $p=0.0009$ ). Tubular epithelial staining was the most striking difference between SRC and control biopsies. Assessing this categorically, there was positive staining (ie a total score  $\geq 1$ ) in all 8 SRC biopsies but only in 3/8 controls ( $p=0.026$  by Fisher's exact test). Figure 3 illustrates GPATCH2L staining of sections of SRC tissue (3A-3D), IgG control staining in this tissue (3E) and GPATCH 2L staining in normal control tissue (3F). Full scores for each biopsy specimen in both SRC and control (normal healthy kidney, NHK) groups are given in Table 3A (see end of chapter).

These immunohistochemistry findings support the genetic analysis in identifying GPATCH2L as potentially relevant to susceptibility, but also raise the intriguing possibility that this gene of unknown function might be implicated in the pathogenesis of SRC. The fact that polymorphisms in this region have been associated with hypertension in two previous studies adds further plausibility (176,177).

Figure 3: Immunohistochemistry images of normal human kidney (control) and renal crisis biopsy samples stained for GPATCH2L

Arrows indicate representative examples of positive staining in key renal structures: glomerulus (g), tubule (t) and vascular endothelium (e). Panels A-D show different staining intensity and distribution in 4 representative SRC samples. Panel E is an IgG control staining of SRC specimen and panel F shows GPATCH2L staining in normal control kidney.



Variants in the CTNND2 gene have previously been shown to associate with the appearance of pulmonary arterial hypertension in scleroderma (178). PAH has pathophysiological features in common with SRC, as both involve dysfunction of the medium-sized blood vessels. Additionally ARA antibodies are associated with increased long term risk of PAH, further supporting a shared pathogenic mechanism(179). Although the CTNND2 SNP identified in the discovery cohort did not meet my threshold for nominal statistical significance in the validation cohort, given the possibility that CTNND2 might play a pathogenetic role in more than one major form of scleroderma vasculopathy, I went on to perform further histological validation for this gene's product.

Similar to the methods described above, I performed immunostaining for CTNND2 in kidney biopsy tissue from SRC patients and controls. Using the same blinded scoring method I identified no difference in distribution or intensity of staining with anti-CTNND2 antibody in any of the tubular, interstitial or vascular compartments.

However, unlike GPATCH2L, glomerular staining was significant. It was entirely absent in 8/8 normal kidney control samples and present to a significant degree in 5/8 SRC samples ( $p=0.026$  by Fisher's exact test). The total biopsy staining score (median, range) was higher in SRC patients (12, 1-17) than in controls (3, 0-8,  $p=0.0135$ ) (Figure 4A). The full scoring for these samples is documented in Table 3B.

In order to localise the CTNND2 expression within the glomerulus, Immunofluorescence was performed and showed anti-CTNND2 antibody staining localising to the capillary loops and apparently distinct from the nucleated glomerular cells (Figure 4B).

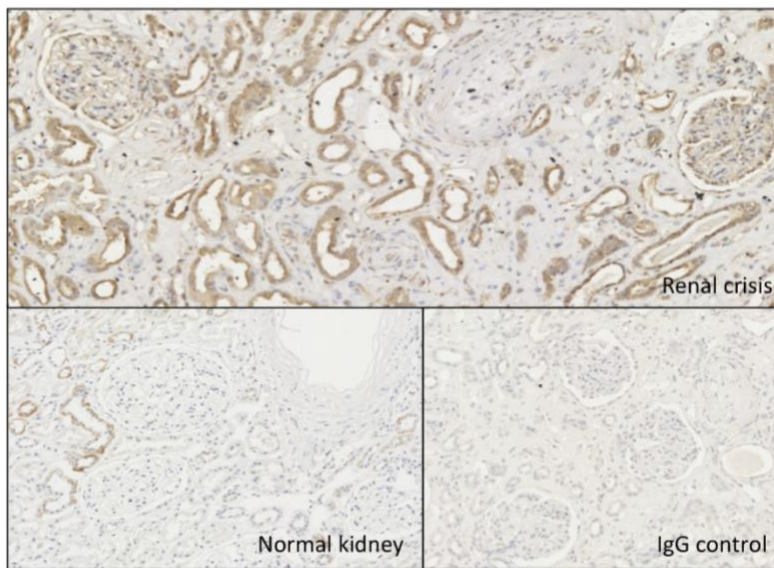


Figure 4: Immunohistochemistry images of normal human kidney (control) and renal crisis core biopsy samples stained for CTNND2 (Delta-2 catenin).

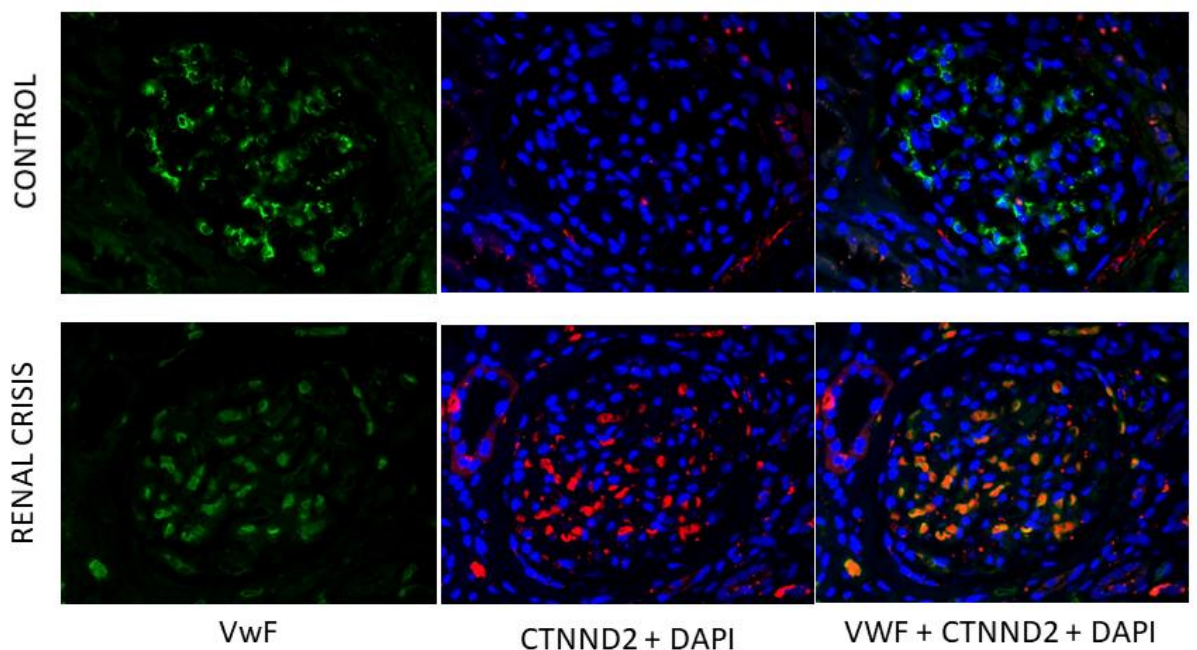
A. Immunoperoxidase demonstrates staining of anti-CTNND2 antibodies in renal crisis versus normal tissue. Bottom right panel shows IgG control staining of renal crisis kidney.

B. Immunofluorescence images demonstrate glomerular endothelial cells (VWF—green) as a background capillary tuft. Cell nuclei within the glomerulus are demonstrated in conjunction with CTNND2 (DAPI—blue + CTNND2—red). CTNND2 appears to collocate with the capillary endothelium but is distinct from the cell nuclei (VWF + CTNND2 + DAPI).

**A**



**B**



## Discussion

In this study I used the high prevalence of SRC in ARA positive scleroderma patients and the rarity of SRC developing late in the course of the disease, to enrich a study population which is necessarily small in view of the rarity of this disease and its specific organ complications. A much smaller number of cases was used than would traditionally be expected for a conventional GWAS analysis. The use of this approach allowed the identification of factors potentially reflecting susceptibility to SRC. The identified genes may also reveal further insight into the pathogenesis of SRC, which is poorly understood.

ARA-positive patients make up about half of those who have SRC, looked at conversely, about a third of patients with this circulating antibody will go on to develop SRC(115). The finding that the onset of SRC in ARA-positive cases in the discovery cohort was distinctly earlier and more frequent than in other antibody groups made this a logical subgroup for genetic analysis. The underlying presumption used is that after five years of follow-up patients can be divided into those who are susceptible and those who are “protected”. The prior hypothesis is also that at least some of the difference between these two groups is genetically determined. This approach has some similarity to what has been described as an “extreme phenotype” in rare diseases. In this approach, careful phenotyping attempts to overcome limitations of a small sample size. The difference in the approach I present here is that I have not made any attempt to phenotype difference in the presenting condition (eg mild versus severe SRC). Also unlike the extreme phenotype method, I am focussing via GWAS analysis on common risk

variants rather than rare variants, meaning that I am more likely to discover susceptibility alleles than functional gene mutations.

Previous work looking at the genetic associations of ARA identified MHC variants associated with this antibody(180). As described above, MHC variants have also been associated with the occurrence of scleroderma renal crisis (117). A distinct feature of this GWAS analysis compared with other analogous studies in autoimmune disease is that there is no Manhattan “peak” at chromosome 6, representing the loci associated with the Major Histocompatibility Complex. The deliberate homogeneity in autoantibody profile of our study cohort presumably underlies this meaningful absence. MHC associations are close to a universal finding in GWAS studies of complex autoimmune conditions (181). The lack of this association in the current study demonstrates the purpose of the method I have used to enrich the study group. The study design has “stripped-out” the reasons why this subgroup is likely to share a specific autoimmune antibody profile and set those aside to allow for better interrogation of why some individuals within this group develop SRC, while others appear to have some form of protection despite their equivalent high risk when viewed from an immunological perspective.

GWAS analysis identified only one SNP that was statistically associated with SRC in both the “discovery” and “validation” cohorts. Nevertheless, this finding is potentially of substantial relevance given the association between SNPs in the same region and hypertension in previous general population genetic studies. It is

possible therefore that a common genetic susceptibility to hypertension is one contributor to SRC susceptibility within the high-risk subgroup.

Although only the association for GPATCH2L was reproduced in the validation cohort, an intriguing potential pathogenetic relevance was presented by another of the SNPs associated with SRC risk in the original Royal Free patient cohort. SNP rs1859082 ( $p=0.000029$ ) is within the CTNND2 or delta 2 catenin (D2C) gene on chromosome 5. This same gene was significantly associated with PAH in scleroderma in a genetic study which used whole exome sequencing (178). I will explore the potential pathogenetic role of CTNND2 and its relationship with Wnt signalling further in the discussion chapter of this thesis.(183–186)(187)(188)(189)(190)(179)(185)(186)(191,192)(193,194)(195,196)(197)

Limitations of this study

The sample size and therefore the strengths of association seen in this analysis are well below the threshold traditionally used to impute significance in genome wide association studies (182). I have attempted to overcome this limitation, which is an intrinsic challenge of rare disease research, with the novel subgroup phenotyping method described above and this justifies the further investigation of associations observed at a lower  $p$  value in both our cohorts.

A second limitation is that there may be differences in clinical features and ethnicity between the two genetic cohorts studied which have not been captured by the

available demographic information and this might explain the absence of replication of the majority of the candidate genes developed in the first cohort.

The GWAS approach only detects common variant SNPs and in a low frequency disease such as scleroderma, rare causal variants might be more important to detect. An adaptive approach with the “extreme phenotype” method described above could identify such variants in a future study.

A further limitation at the time cases were selected for this study was a lack of agreed consensus definition for SRC. Consensus criteria are now proposed (183) and retrospective analysis suggests a large majority if not all cases included in this study would have fulfilled the proposed diagnostic criteria.

Finally, as a corollary of the phenotype design used, this study provides no data on genetic susceptibility for SRC among patients who are ARA negative, and these make up about half of SRC cases. Nevertheless, this is the first study to combine antibody status and disease duration phenotyping together with a GWAS approach to explore genetic risk of scleroderma renal crisis.

#### Future work

This study would be complemented by higher resolution genotyping approaches such as whole exome or whole genome sequencing, using the same novel cohort design. The potential and feasibility of such an approach is supported by the findings presented here. Direct sequencing of candidate loci identified in this study and

others could detect rare variants that are functionally important but difficult to identify using GWAS.

Table 3: Immunohistochemical staining scores for control (NHK) or scleroderma renal crisis (SRC) renal biopsies

Comparison of scores between NHK and SRC scores was by Mann-Whitney U test. If only 1 sample showed positive staining no formal comparison was made (NA).

**3A GPATCH2L staining score**

	NHK1	NHK2	NHK3	NHK4	NHK5	NHK6	NHK7	NHK8	SRC1	SRC2	SRC3	SRC4	SRC5	SRC6	SRC7	SRC8	p-value
<b>Tubular</b>	0	1	1	1	2	4	4	1	6	9	6	6	4	4	9	9	0.0098
<b>Vascular</b>	0	0	0	0	0	0	0	0	3	2	2	2	1	3	2	3	0.0009
<b>Glomerular</b>	0	0	0	0	2	0	0	0	0	2	2	2	6	4	2	9	0.0085
<b>Interstitial</b>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	NA
<b>Total</b>	0	1	1	1	4	4	4	1	9	13	10	10	11	11	13	21	0.0009

3B CTNND2 staining score

	NHK1	NHK2	NHK3	NHK4	NHK5	NHK6	NHK7	NHK8	SRC1	SRC2	SRC3	SRC4	SRC5	SRC6	SRC7	SRC8	p-value
<b>Tubular</b>	4	1	8	1	0	2	3	6	4	1	4	6	8	8	6	8	0.0929
<b>Vascular</b>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	NA
<b>Glomerular</b>	1	0	0	0	0	0	1	1	8	0	8	8	1	4	0	8	0.0455
<b>Interstitial</b>	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	NA
<b>Total</b>	5	1	8	1	0	2	4	7	12	1	12	14	9	14	6	17	0.0135



# **Chapter 3: Urine and serum markers of chronic kidney disease in scleroderma**

## Introduction

As described in previous chapters, at least half of patients recover well from the acute kidney injury (AKI) of scleroderma renal crisis. Despite this, there is an important minority who do not recover adequately and SRC is an important cause of chronic kidney disease (CKD) in Scleroderma. In addition, a large proportion of scleroderma patients without a history of renal crisis have CKD as a result of the systemic vasculopathy and fibrosis associated with the disease as well as other mechanisms which include overlap connective tissue diseases (lupus nephritis and ANCA-associated vasculitis). Other pathologies commonly contributing to CKD in scleroderma include interstitial nephritis and drug toxicity. Previous studies of sequential unselected patients found CKD in up to 50% of scleroderma cases (104). Although it often presents as only mild renal impairment, a recent study based on a large European registry of patients with scleroderma (EUSTAR) demonstrated that CKD was an independent risk factor for death from scleroderma (184). 13% of patients had eGFR < 60 ml/min in this registry dataset, compared with a prevalence of 1-6%, depending on region, in the European population as a whole (185). The association between CKD and death in the EUSTAR patients was “dose dependent” ie the higher the CKD stage, the higher the risk of death.

Creatinine-derived GFR measures (which include the commonly used MDRD and CKD-EPI eGFR equations) only deteriorate once significant parenchymal abnormality is established in the kidney so there has been a longstanding desire to develop more sensitive markers of current disease activity in chronic kidney disease(186). This includes assessing the CKD that occurs in the context of scleroderma. Such new markers could help to discriminate scleroderma-related processes from other causes of renal deterioration so that management can be tailored according to the cause. New markers of kidney disease could also assist in separating progressive or clinically important CKD from stable biochemical abnormalities with a good prognosis that require no further action. Readily available, non-invasive biomarkers could be used as outcome measures in clinical trials as well as in clinical practice, thereby providing early indication of therapeutic response that would anticipate a more significant future clinical benefit. Such sensitive disease measures could be used to gain meaningful outcome results from trials where the population size is necessarily small as in scleroderma.

Many previous studies have looked at potential biomarkers in scleroderma. Typically, these have focused on examination of protein expression in skin biopsies or peripheral blood, cell based-approaches including gene expression and the examination of microparticles (187,188). Composite serum markers have been designed including the enhanced liver fibrosis (ELF) test and assessment has correlated this with disease activity in the skin and with interstitial lung disease (189). Unbiased biomarker discovery techniques have looked for new serum markers of disease activity with proteomics and aptamer-based protein analysis (187,190).

With regards to kidney disease activity in scleroderma, urine offers a theoretically relevant and easily available substrate in addition to blood, for the exploration of candidate

biomarkers in scleroderma CKD (SSc-CKD). Urine could have meaningful advantages over plasma or serum as a fluid to sample for renal biomarker studies. It is produced in direct contact with (and dependent on active transport across) the epithelial surface of the renal tract, so any relevant proteins which are expressed in kidney injury could be shed directly into the urine(190) Based on this presumption, urine has previously been described as a “fluid biopsy” of the kidney (191). Additionally, urine can be obtained without any invasive procedure and is typically available in larger volumes than either serum or plasma. In this chapter I describe the use of multiplex technology to measure candidate proteins, selected as plausible markers of SSc-CKD, in both the blood and urine of patients with SSc-CKD. I have also included control samples with CKD of other cause, samples from cases of scleroderma without CKD and samples from healthy volunteers. This is the first study looking at biomarkers in the urine of patients with scleroderma.

## Methods

### Selection of candidate serum and urine markers

Drawing on data from the existing literature, I defined plausible candidate biomarkers of renal involvement in scleroderma which could be measured in urine and serum of patients. My aim was to measure a selection of proteins that would identify activity in the three “compartments” of scleroderma pathophysiology described in my introduction i.e. biomarkers of inflammatory, fibrotic and vasculopathic processes in the kidney that could in principle augment the conventional clinical assessment of nephropathy measured via serum creatinine and its derivatives (i.e. eGFR) and albuminuria. The following candidate proteins were selected for measurement:

**Interleukin 6 (IL-6)**, as described in my introduction, is a likely pathogenic mediator of inflammation and connective tissue dysfunction in SSc. Its expression in urine has been correlated with renal disease in several contexts including the autoimmune connective tissue disease Systemic Lupus Erythematosus (SLE)(192).

**Interleukin 18 (IL-18)**—a member of the IL-1 super-family) has been shown to be a mediator of ischaemic damage to the renal tubule in mice (193) and urine concentrations have been validated as a marker of acute kidney injury in humans(194).

**Tumour necrosis factor alpha (TNF $\alpha$ )** is a putative mediator of endothelial damage in systemic sclerosis. Serum and urinary concentrations have been demonstrated to be raised in other forms of kidney disease(195,196).

**Vascular endothelial growth factor (VEGF)** is overexpressed in tissue biopsies and sera from patients with SSc(197). It is expressed in urine in disease states and concentrations are independent of serum concentration(198).

**Monocyte chemoattractant proteins 1** (MCP-1 or CCL2) and **3** (MCP-3 or CCL7) have been described as pathogenic fibroblast activators in scleroderma(90) and high serum levels have been associated with organ-specific disease activity (199). Urine concentrations of MCP-1 have shown promise as a marker of renal involvement in SLE (200,201).

**Soluble ICAM-1** has been associated with disease severity in scleroderma when measured in serum and is considered a marker of activated endothelium, epithelial cells and fibroblasts (202). It is expressed and shed in greater quantities in scleroderma fibroblasts than in control cells (31).

**Soluble VCAM-1** has been associated with fibroblast activation, epithelial-to-mesenchymal transition and is a marker of immune cell and endothelial cell activation. It is elevated in SRC in other series and has been shown to be markedly increased in the serum in some cases of SRC (63,202). Urinary levels have not previously been examined in scleroderma.

#### Study design and participants

This study was approved locally by the Royal Free Research and Development team and externally by the Newcastle and North Tyneside Research Ethics Committee (REC). All individuals provided informed consent for their participation, according to the guidance set out by the REC.

The two largest groups of study participants were recruited from adult patients attending the national scleroderma referral clinic at the Royal Free Hospital and all had confirmed scleroderma according to the 2013 American College of Rheumatology (ACR)/European League Against Rheumatism (EULAR) classification (203). Kidney function was measured according to the Modification of Diet in Renal Disease Estimated Glomerular Filtration Rate equation (MDRD eGFR)(204). Patients were enrolled in the main study cohort (SSc-CKD) if

they had eGFR < 60mls/min/1.73m<sup>2</sup> or <90mls/min with persistent urinary blood or protein—consistent with CKD stages 2-5 in the 2002 guidelines from Kidney Disease Outcomes Quality Initiative (KDOQI) (205). As well as the 40 patients enrolled in this group a further 40 scleroderma patients with no evidence of CKD were recruited to the scleroderma control group (SSc). To act as CKD controls, a further 10 patients with CKD of other causes were recruited from the general nephrology clinic at the Royal Free Hospital. Patients without nephrotic range proteinuria were selected to avoid gross protein overspill from serum that would not be seen in the scleroderma groups who had at most mild proteinuria. Finally, 12 healthy volunteers, with no diagnosis of scleroderma or other chronic conditions and normal renal function, were also included for analysis.

#### Clinical data

As well as baseline demographic data on age, gender and ethnicity I recorded patients' significant medical history. In the two scleroderma groups this included data on organ complications, skin subgroup (lcSSc versus dcSSc) and the disease-defining circulating autoantibody. These data are summarised in Table 1.

Table 1 Demographics and clinic features of biomarker study groups.

Data expressed as n (% total population) or mean (SD)

	<b>SSc- no CKD (n=40)</b>	<b>SSc-CKD (n=39)</b>	<b>History of SRC (n=14)</b>	<b>CKD (n=11)</b>	<b>CONTROL (n=12)</b>
<b>Age</b>	57 (2.1)	63 (1.7)		58 (5.1)	34 (2.4)
<b>eGFR (ml/min/1.73m<sup>2</sup>)</b>	80 (1.5)	45 (2.3)		49 (7.7)	87 (1.3)
<b>Albumin:creatinine ratio</b>	<3	9 (22)	28 (33)	12 (22)	<3
<b>dcSSc</b>	10 (25%)	20 (51%)			
<b>lcSSc</b>	30 (75%)	19 (49%)			
<b>ACA (centromere)</b>	13 (33%)	12 (30%)	0		
<b>ATA (Scl-70)</b>	8 (20%)	2 (5%)	1		
<b>ARA (RNAPol)</b>	6 (15%)	12 (30%)	7		
<b>AFA (U3RNP)</b>	0	3 (8%)	2		
<b>Other ANA</b>	13 (33%)	11 (27%)	4		
<b>ADPKD diagnosis</b>				4	
<b>Obstructive uropathy diagnosis</b>				5	
<b>Other CKD diagnosis</b>				2	

## Sample collection and management

From each patient or control enrolled in his study, urine and blood samples were collected on the same day. Clotted blood and fresh mid-stream urine were both centrifuged at 3000 RPM at 4°C, for 10 minutes, within 60 minutes of collection. After centrifuging, serum was separated from clotted blood and then both serum and urine were divided into aliquots and frozen at -80°C.

Additional blood and urine samples taken on the same day were sent to the Royal Free Hospital clinical laboratories for measurement of serum creatinine, eGFR and urinary albumin:creatinine ratio.

## Multiplex analysis of serum and urine

Multiplex analysis was performed using a bead-based immunoassay allowing simultaneous measurement for all eight analytes. This analysis was performed according to the manufacturer's protocol (Luminex Corporation, Austin, USA). Analytic standards provided by the manufacturer, urine and serum samples were each analysed in duplicate wells for validation. As in standard clinical assessment of urinary protein concentration in spot samples, urinary biomarker concentrations (pg/ml) were expressed as a ratio to the urine creatinine concentration ( $\mu\text{mol/l}$ ) to compensate for diurnal variation in the water volume of urine samples.

## Statistical analysis

For each of the eight candidate markers I compared four subject groups ("SSc-CKD", "SSc-no CKD", "CKD" and "Control"). Overall difference between these four groups was assessed for each biomarker using Kruskal-Wallis test. SSc-CKD group was then also compared individually with each of the other three groups using Mann Whitney U test in a pair-wise fashion. To assess the degree of independence of urine biomarker concentrations from



glomerular filtration of blood, correlation between eGFR and biomarker concentrations was assessed with Pearson's coefficient. Each of the above statistical tests was performed using GraphPad Prism version 8.2.1 for Windows, GraphPad Software, La Jolla California USA.

## Results

Findings for each of the candidate analytes in serum and urine are described below with a view to assessing their suitability as markers of CKD in scleroderma. Given that these candidate biomarkers were selected based on previously published data, a key objective was to select those candidates, which would be most suitable for future validation as potential markers of SSc-CKD and particularly those that might act as markers of drug efficacy in future interventional studies for scleroderma. The summary data for the 8 proteins analysed in this study are detailed in Table 2 for serum and Table 3 for urine. Plots comparing the four subject groups for each protein are shown in Figure 1 (serum levels) and Figure 2 (urine levels expressed in ratio to the urinary creatinine concentration).

Table 2 Summary of serum analysis with Kruskal-Wallis group comparison (KW) and pairwise comparison versus SSc-CKD group (all units are pg/ml)

Analyte		Control	CKD	SSc-CKD	SSc	KW (p=)	SSc v. SSc-CKD (p=)	SSc-CKD v. CKD (p=)	SSc-CKD v. control (p=)
IL6	median	0	0.57	1.7	1.2	0.3748	0.5614	0.3041	0.133
	min	0	0	0	0				
	max	5.1	3.8	11	8.2				
	mean	1.3	1.1	2.7	2.1				
	SD	2	1.2	3.1	2.5				
MCP1	median	35	30	53	106	0.0001	0.0015	0.0236	0.2854
	min	3.4	2.5	3.4	5.1				
	max	166	66	207	263				
	mean	53	31	66	118				
	SD	53	21	49	76				
TNFa	median	0.36	0.65	1.1	1.5	0.0196	0.1393	0.2136	0.2231
	min	0	0	0	0				
	max	1.7	1.5	3.1	5				
	mean	0.54	0.56	1.1	1.5				
	SD	0.6	0.47	0.98	1.2				
VEGF	median	1.6	9.9	15	32	<0.0001	0.001	0.0963	0.1453
	min	0	0	0	1				
	max	60	19	74	131				
	mean	13	9.1	20	43				
	SD	19	8.3	18	34				
MCP3	median	0	0	0	0	NA			
	min	0	0	0	0				
	max	0	0	0.45	0				
	mean	0	0	0.019	0				
	SD	0	0	0.092	0				
IL18	median	6.6	3.2	17	20	0.0002	0.3991	0.0016	0.0299
	min	0	1.5	0	0.72				
	max	31	8	60	60				
	mean	9.2	3.7	21	23				
	SD	9.8	2.2	18	14				
ICAM-1	median	16083	14762	23082	23412	0.0004	0.413	0.0067	0.0079
	min	2030	10564	4453	9580				
	max	21329	17534	40541	56872				
	mean	14287	14321	22118	25449				
	SD	6055	2554	9090	11316				
VCAM-1	median	17561	10749	27047	22538	0.0113	0.0832	0.0028	0.1945
	min	7436	4373	4167	1196				
	max	48673	25272	58652	51776				
	mean	22427	13609	28734	23291				
	SD	14297	7021	14814	11645				

Table 3 Summary of urine analysis with Kruskal-Wallis group comparison (KW) and pairwise comparison versus SSc-CKD group (all units are pg/ml:mmol urine creatinine)

Analyte		Control	CKD	SSc-CKD	SSc	KW (p=)	SSc v. SSc-CKD (p=)	SSc-CKD v. CKD (p=)	SSc-CKD v. control (p=)
IL6	median	0.22	0.65	0.52	0.42	0.008	0.2243	0.7273	0.0018
	min	0.11	0.24	0.048	0.082				
	max	0.46	1.9	1.5	1.3				
	mean	0.23	0.69	0.57	0.47				
	SD	0.1	0.51	0.36	0.3				
MCP1	median	9.8	23	23	19	0.0032	0.0868	0.5803	<0.0001
	min	2.2	5.9	0.81	2.1				
	max	12	54	70	54				
	mean	8.7	26	30	22				
	SD	3.4	21	20	15				
TNFa	median	0.15	0.4	0.38	0.38	0.0164	>0.9999	0.7162	0.004
	min	0.016	0.18	0.028	0.018				
	max	0.49	0.84	1.5	1.3				
	mean	0.17	0.44	0.44	0.43				
	SD	0.16	0.21	0.34	0.3				
VEGF	median	31	29	34	36	0.9828	0.9081	0.7654	0.739
	min	17	19	11	1.8				
	max	52	61	90	129				
	mean	33	34	38	39				
	SD	10	14	19	27				
MCP3	median	0.84	1.6	3	2	0.0101	0.4744	0.2185	0.0008
	min	0	0.55	0	0				
	max	2.1	3.6	10	9				
	mean	0.94	1.9	3.5	3				
	SD	0.64	0.95	3	2.6				
IL18	median	3.9	5.7	8.9	7.9	0.0053	0.6337	0.0493	0.0026
	min	1.6	2.4	1.1	1.2				
	max	7.4	12	31	29				
	mean	2.2	2.9	7.1	6.8				
	SD	0.65	0.97	1.2	1.1				
ICAM-1	median	60	855	968	570	<0.0001	0.0134	0.7049	<0.0001
	min	8.8	73	9	13				
	max	275	3991	4678	2391				
	mean	94	1307	1499	807				
	SD	82	1211	1302	704				
VCAM-1	median	382	336	491	342	0.4377	0.2239	0.198	0.2928
	min	44	40	5.2	8.1				
	max	735	857	2371	1435				
	mean	400	387	704	509				
	SD	207	294	632	450				

Figure 1: Serum analytes for candidate biomarkers in SSc-CKD and controls

Panels show distribution for each analyte in serum for the normal controls (red), CKD without scleroderma (blue), scleroderma + CKD (green) and scleroderma without CKD (yellow).

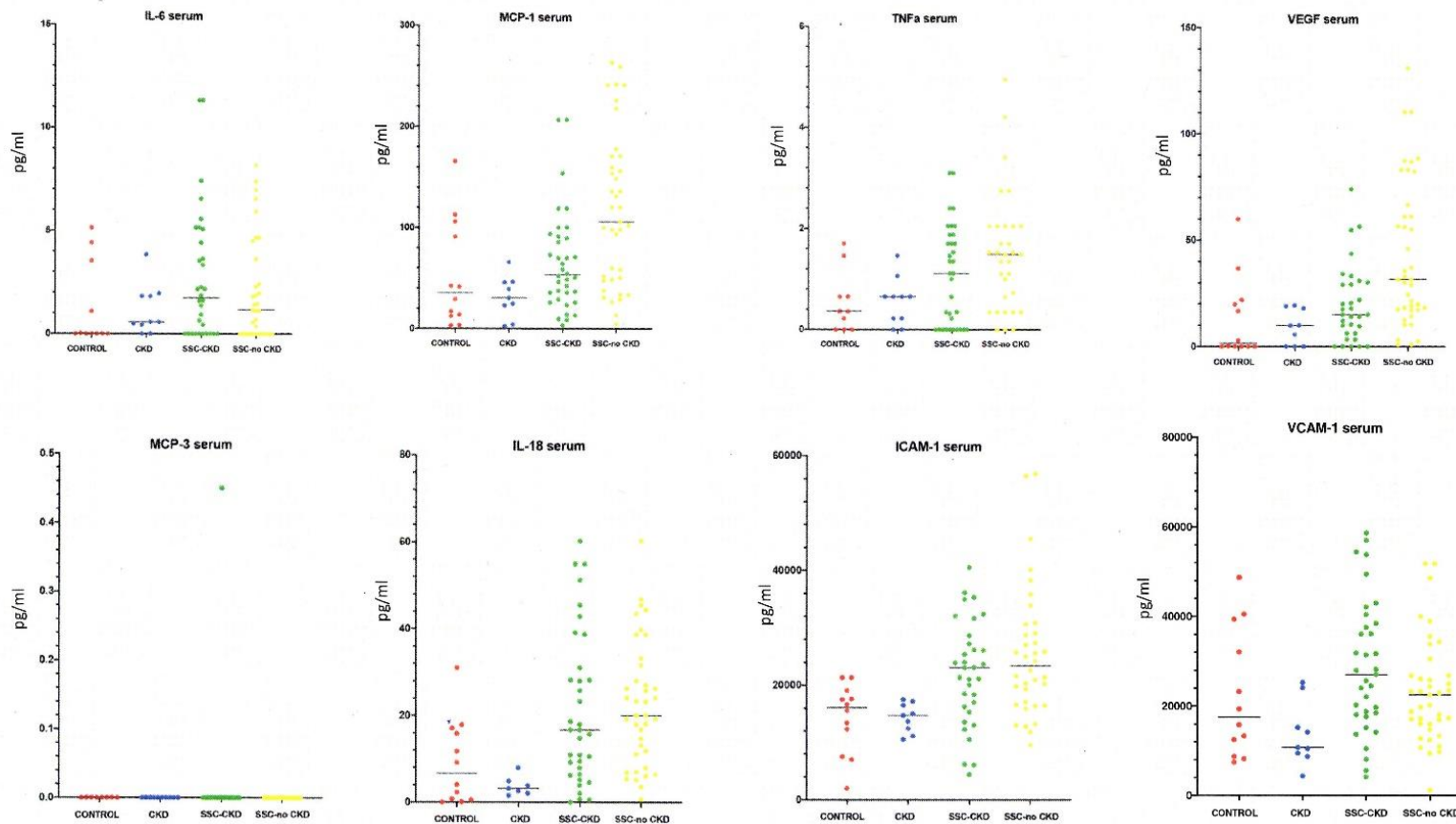
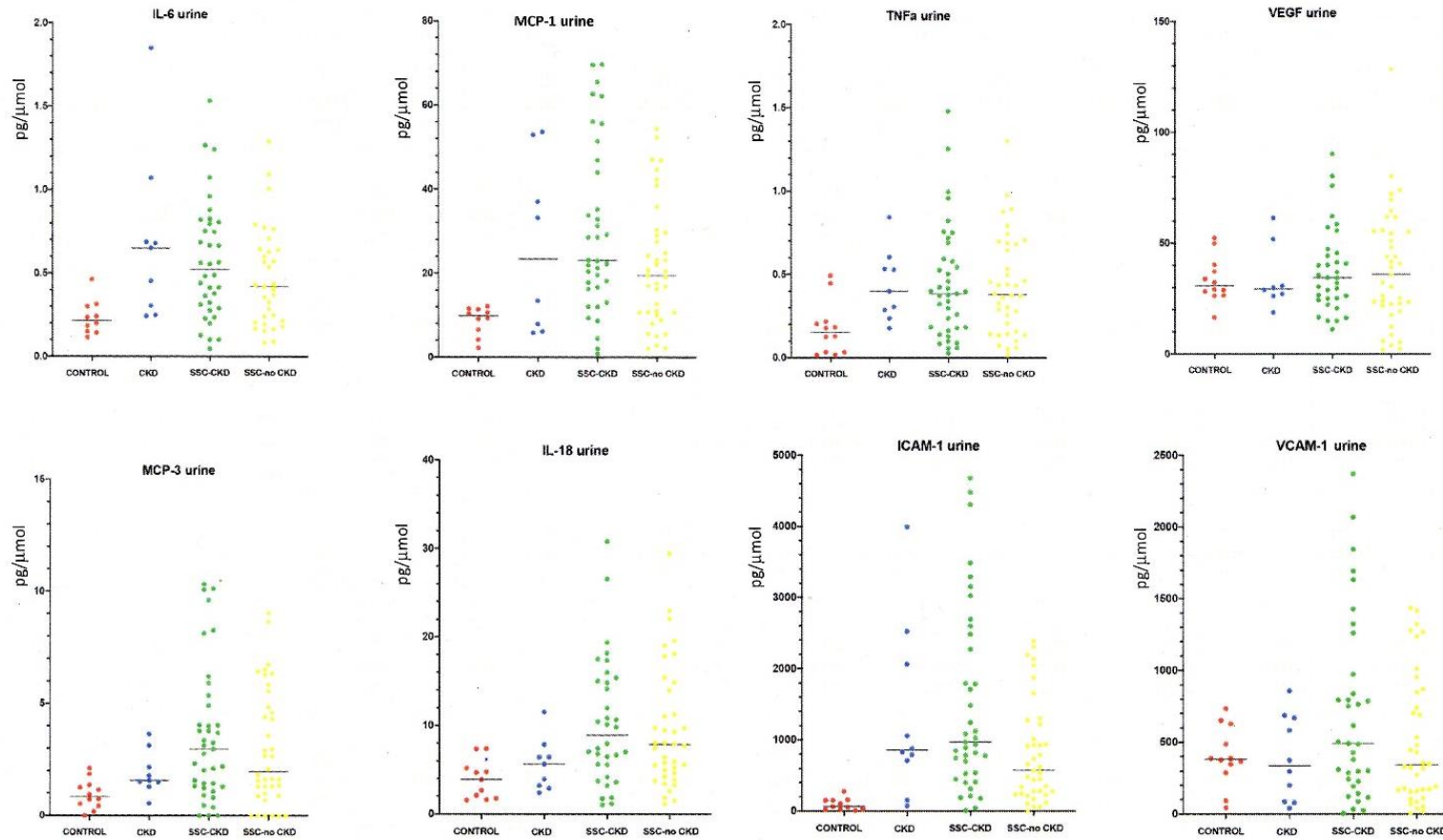


Figure 2: Urinary analyte:creatinine ratio for candidate biomarkers in SSC-CKD and controls.

Panels show distribution for each analyte in urine for the normal controls (red), CKD without scleroderma (blue), scleroderma + CKD (green) and scleroderma without CKD (yellow).



## Summary of biomarker results

### IL6

**Serum** IL6 is undetectable in most (6/10, 60%) healthy controls. Average concentration in CKD controls is lower than in SSc. The very wide range of spread in healthy controls where this was measurable suggests that it would be a poor discriminator for SSc despite the highly significant difference in average values between the four groups.

**Urine** concentrations of IL6 were elevated in all three disease groups and overall, this was significant ( $p=0.008$ ). The highest concentrations were found in the CKD control group. Combined with the very widespread within SSc, this suggests urine IL-6 will not be a robust marker of CKD from scleroderma although it might reflect more general pathological renal processes.

### MCP-1 (CCL2)

**Serum** concentrations of MCP-1 were markedly different between the four study groups ( $p=0.0001$ ), and the most statistically significant individual pairwise comparison was between the SSc group (without CKD) and the SSc-CKD group ( $p=0.0015$ ). Serum MCP-1 was similarly low in both the CKD and normal control groups compared to much higher levels in both scleroderma groups. As with serum IL-6, the range of spread in healthy individuals indicates this is unlikely to be a good discriminator for SSc-CKD at an individual patient level, despite the highly significant difference in average values.

**Urine** MCP-1: creatinine ratio showed particular promise as a marker of SSc-CKD compared to the other analytes in this study as there was both a significant overall comparison between groups ( $p=0.0032$ ), and it demonstrated the highest significance for any of the candidate analytes as a marker of SSc-CKD compared with healthy control samples ( $p<0.0001$ ). The elevated levels observed in some CKD controls suggested that this was a viable discriminator from healthy individuals that reflected renal function. In addition, there was a strong trend for difference between SSc with CKD and without ( $p=0.0868$ ) suggesting that although serum levels of MCP-1 might reflect overall disease process, MCP-1 level in urine was more likely to reflect renal involvement. Within the SSc-CKD group, patients with a history of SRC had higher average MCP-1:creatinine ratio—100 (22-244) versus 44 (26-66) for those with no history of SRC ( $p=0.019$ ).

TNF $\alpha$

**Serum** levels of TNF $\alpha$  showed only modest significant difference overall ( $p=0.0196$ ) and none of the pairwise group comparisons are statistically significant although the trend is for higher levels in SSc compared with either control group and the average level is greatest in SSc without CKD. This suggests that serum TNF $\alpha$  is not a reliable marker of kidney involvement in SSc.

In **urine**, whilst there was elevation of TNF $\alpha$ : creatinine ratio in the three disease groups of CKD and SSc with or without CKD, this was only modestly significant at a groups level ( $p=0.0164$ ). Whilst a potential reflection of renal disease, it did not appear to have value in discriminating between the groups of interest.



## VEGF

Overall differences for **serum** concentration between groups are highly significant ( $p < 0.0001$ ), with the clearest pairwise difference between SSc with or without CKD. The highest values occur in the non-CKD SSc group and this is in line with previous association of elevated VEGF levels with complications such as pulmonary hypertension in SSc. As with previous markers discussed, the range of spread in healthy controls suggests that it would be a poor discriminator between individuals in practice.

There was no evidence of difference between **urine** VEGF results for any of the 4 groups analysed or between the groups. This suggested that urinary VEGF does not have value as a molecular marker of either SSc or CKD.

## MCP3 (CCL7)

This was detected in the **serum** of only one patient and so appears to have limited value as a serum marker of SSc or CKD.

In **urine**, MCP3: creatinine levels were significantly elevated in SSc-CKD compared to healthy controls ( $p = 0.0008$ ) but did not differ as markedly from the other subgroups ( $p = 0.01$ ). Presence in the urine without any detectable presence in blood does suggest that levels of this chemokine may be relevant to pathogenesis of SSc-CKD and that local production of this chemokine might be relevant to renal complications but compared to other urinary analytes this appears to have lower potential as a molecular marker, especially for the elevated levels seen in SSc overall where those with or without CKD very much overlap. Along with the findings in urine MCP-1 this suggests that urinary

measurement of CC chemokines is more informative than serum levels, perhaps reflecting local production of these mediators in the renal tract.

#### IL18

There was a significant difference in average **serum** levels between the different subgroups ( $p=0.0002$ ), with the lowest levels in the non-SSc CKD control group, but no difference between SSc with or without CKD. Again, there is a wide range of spread in healthy controls, suggesting it would have little utility at the individual level despite the highly significant difference in average values.

There was a overall significant difference between groups for **urine** IL18: creatinine ratio ( $p=0.0053$ ) and this was most significant for the SSc-CKD versus control ( $p=0.0026$ ). In addition, borderline significance between SSc-CKD and the CKD controls was observed ( $p=0.049$ ).

#### ICAM-1

There is a clear difference in average levels in **serum** for SSc, compared to both control groups ( $p=0.0004$ ). This supports the utility of serum ICAM-1 as a marker that may be relevant to scleroderma. However, there is no difference between SSc with or without CKD suggesting that this is a serum marker of the disease, but not of renal involvement.

In contrast, the average values for **urine** ICAM-1: creatinine are significantly greater in cases of SSc-CKD than those SSc cases without CKD. This suggests that local production or shedding of ICAM-1 may reflect specific aspects of renal disease in SSc. The peak levels in SSc-CKD are well above the peak for SSc without CKD. Other forms of CKD all have elevated levels compared with healthy controls supporting the value as a marker of renal pathology although there was no difference

between SSc-CKD and other forms of CKD. The overall significance at a group levels ( $p < 0.0001$ ) together with this being driven by SSc-CKD, and a significant difference between SSc-CKD and SSc without CKD ( $p = 0.0134$ ), suggest that urinary ICAM-1: creatinine ratio may be a useful candidate marker for SSc-CKD.

#### VCAM-1

Although the comparison of **serum** level between groups shows a modest statistical significance ( $p = 0.01$ ), this is mostly due to the difference between SSc-CKD and CKD. This is notable as there have been other studies suggesting association of elevated serum VCAM-1 and scleroderma renal crisis. As in previous markers discussed, the spread of values in healthy controls is too wide for this to be widely used in practice but at the group level, VCAM-1 was the best serum discriminator of SSc versus non-SSc CKD.

There was no evidence of difference between **urine** VCAM-1 results for any of the 4 groups analysed or between the groups. This suggested that urinary VCAM-1 does not have value as a molecular marker of either SSc or CKD.

Overall, although serum levels of several of the candidate markers were elevated in scleroderma patients, all had significant weakness as candidate markers of SSc-CKD. In contrast, urinary levels of several of the candidates are elevated in SSc urine and for some of these the difference is most marked between SSc-CKD and healthy controls. Based on the results described above I selected two urinary analytes for further validation: MCP-1 and ICAM-1. These showed the most significant

difference between groups, compared with the other analytes but also demonstrated a marked pairwise difference between SSc-CKD and normal controls driving this statistical significance. They also both showed some potential to discriminate SSc-CKD from other causes of CKD, and more unusually at least a trend toward significance in differentiating between scleroderma patients with and without renal involvement.

Relationship of candidate urinary markers MCP-1 and ICAM-1 with renal function

Detectable concentration of low molecular weight proteins in the urine will always reflect to a varying degree the blood concentration of the proteins and the volume of blood filtered by the kidneys to produce urine (i.e. the renal plasma flow and glomerular filtration rate). To investigate the relationship between kidney function and concentrations of MCP-1 and ICAM1 in urine, I plotted urine concentrations of these analytes against MDRD eGFR. These data, along with those comparing uncorrected serum creatinine to the urinary analyte concentration, are illustrated in Figure 3.

ICAM-1: creatinine ratio showed a significant negative correlation with eGFR ( $R=-0.42$ ,  $p=0.0001$ ).

The correlation for urinary MCP-1: creatinine with eGFR followed the same trend but was weaker ( $R=-0.32$ ,  $p=0.0046$ ). Examination of the dot plot distributions for each marker suggests that in keeping with the lower significance value, the relationship between eGFR and urinary MCP:1 was less robust than that between eGFR and ICAM-1. This suggests that urinary MCP-1 concentrations may be more likely to reflect pathogenic processes in the kidney itself than ICAM-1. This is consistent

with previous work from within our lab showing expression of MCP-1 in scleroderma renal biopsy specimens (206).

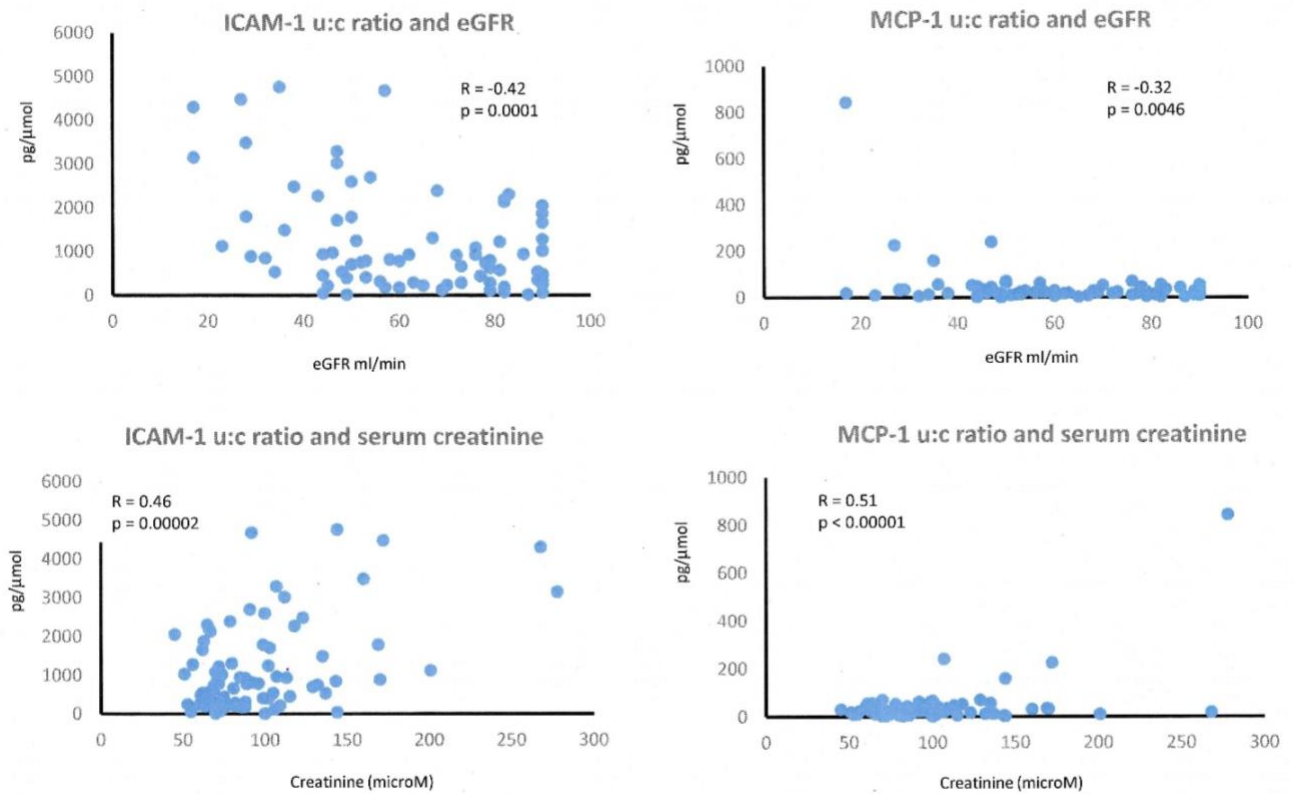


Figure 3: The relationship between urine biomarkers and renal function (eGFR and creatinine).

Panels illustrate the degree of correlation between renal function (both serum creatinine and MDRD eGFR are illustrated) compared with urinary MCP-1: creatinine ratio and urinary ICAM-1: creatinine ratio.

Correlation coefficient  $R$  and associated p-value are annotated for each panel.

## Discussion

In this study I showed that urinary levels of key proteins implicated in scleroderma pathogenesis may have the potential to be biomarkers of SSc-CKD that can detect or monitor renal involvement in this multi-system disease. Based upon previous studies of serum markers in SSc and emerging data on urinary analytes in other renal diseases I selected 8 candidates to assess in our well characterised cohort of scleroderma patients and relevant controls. Although many of the proteins were elevated in scleroderma serum compared with controls, this probably reflects disease occurring in multiple organs as there was no clear difference between serum levels in SSc-CKD and SSc without CKD. In fact, for half of the proteins the average level was higher in SSc without CKD suggesting that disease outside the kidneys had the most influence on serum levels. Whilst not significant, it is notable that VCAM1 levels on average were higher in SSc with CKD since the levels have been shown previously to be increased in SRC (31,63).

These findings confirm previous studies of serum cytokines and adhesion molecules in the serum of SSc patients. These have demonstrated correlation of MCP-1 level with skin sclerosis and with lung function change in clinical trials (207). However, most of these studies have focused on serum levels and this is a challenge for a multicompartiment disease like SSc where elevated levels may reflect disease in skin, lung, kidney, or other vascular structures. This may explain why it has been difficult to identify strong correlations with lung fibrosis or pulmonary arterial hypertension in general cohorts (199). In more selected cases, such as those recruited into the scleroderma lung study, there was a correlation of change in MCP-1 and treatment response (207). Likewise, cross sectional

studies have shown that in idiopathic pulmonary fibrosis, where other organ systems than the lungs are less likely to be abnormal, there is a strong predictive value of MCP-1 for future disease progression (208).

Urinary analytes appear to better reflect renal pathology evidenced by average levels being greater for SSC-CKD than SSc without renal dysfunction in six of the eight analytes examined. Although several of the proteins that were increased in SSC-CKD are of interest in SSc pathogenesis, the overall goal of the present study was to identify the most promising markers in the urine that could reflect CKD in SSc and be used as future biomarkers in observational cohort studies or interventional trials. As outlined in the Results section above I have selected MCP-1:cr and ICAM-1:cr as the most promising candidate markers to take forward because they show the most significant difference across all groups, highest discrimination from healthy controls, and the most potential to differentiate SSC-CKD from other CKD.

There have also been reports of correlation of ICAM-1 with skin or lung involvement although, as expected from the serum results in the present study, the relationship to renal involvement compared with other adhesion molecules is less clear (63). However, ICAM-1 has been shown to change over time in previous interventional studies supporting its possible value as a molecular surrogate of the disease process (209). Whilst I am describing the first study of urinary ICAM-1 in scleroderma, there have been several studies of urinary ICAM-1 in SLE that have overall shown elevated levels compared with controls (210). There is an association with renal involvement in SLE,



but a recent meta-analysis concluded that the current evidence does not support urinary ICAM-1 as an effective marker of the activity of lupus nephritis (211).

This is also the first study to investigate concentrations of MCP-1 in the urine of patients with scleroderma. The SSc-CKD group had lower serum and higher urine concentrations of MCP-1. This group with renal impairment has reduced clearance overall and therefore lower total filtered MCP-1, so it is likely that these findings represent upregulated local expression of MCP-1 in the kidney in those with renal involvement, rather than increased renal clearance from the blood.

Immunohistochemical staining has shown marked expression of this chemokine in kidney biopsy specimens from patients with SRC. I will explore the pathogenic role of MCP-1 and its potential role in research or clinical practice in the discussion chapter of this thesis.

Strengths of this study are that non-CKD scleroderma cases are included and that the cases were carefully stratified, and levels of analyte can be linked to renal function. Another positive aspect of the study is inclusion of cases of non-SSc CKD controls. These were selected to have non-inflammatory underlying diseases so that this would not confound results and the group therefore serves as a control for poor renal function.

Limitations include the small number of samples (as previously discussed, a limitation in all research in this rare disease) and the cross-sectional design. Nevertheless, the number of cases included was sufficient to include some representatives of most of the major patterns and subsets of SSc as evidenced by the serological and clinical features of our study cohort. Another limitation is the grouping together of CKD patients with a wide range of degree of renal impairment and likely with a

wide variety of disease processes in the kidney, for categorical comparison against those with normal renal function. This in part explains the diversity of results for some analytes including MCP-1. It is possible that a threshold level might be important and future studies could compare high and low level urinary MCP-1 cases to explore clinical or other associations.

In a cohort where renal biopsy data or other more discriminatory diagnostics were available, separation by renal abnormalities could segregate cases further and account for other differences that might affect urinary protein concentration.

Future work can explore use of urinary MCP-1 and ICAM-1 as potential longitudinal markers in observational cohort and explore how baseline levels might reflect long term outcome or progression. A similar approach has been fruitful in scleroderma for serum IL6 and lung function decline and in idiopathic pulmonary fibrosis, with serum MCP-1 predicting longterm decline in lung function.

A further approach would be to observe changes in these candidate markers in response to experimental treatment in a clinical trial setting and assess their suitability as an outcome measure. I will describe an experiment using this method in the next chapter of this thesis.

# **Chapter 4: Evaluation of the highly selective endothelin A receptor antagonist zibotentan in scleroderma-associated renal disease**

In previous chapters I have illustrated the importance of kidney disease in scleroderma (135) and have discussed the causes and treatments of both its acute form (SRC) and CKD in scleroderma (133)(118). In chapter 2 of this thesis, I identified potential genetic contributors to SRC susceptibility in the subgroup of patients with ARA antibodies. In chapter 3 I explored urine and serum biomarkers that may reflect the chronic disease process in the kidneys in scleroderma, in patients both with and without a history of SRC. In patients with scleroderma and CKD, information from renal biopsy studies is limited but post-mortem case series suggest that significant vasculopathy is common in the kidneys of patients even without a history of SRC(212,213). As discussed in chapter 2, CKD has been shown to predict poor outcome in SSc (184).

The role of the endothelin system in scleroderma

Endothelin-1 (ET-1) , acting via the ETRA and ETRB receptors, is a significant contributor to the vasculopathic phenotype of scleroderma in the kidneys and elsewhere(230) (see chapter 1). Previous

clinical studies using antagonists that target both receptors relatively unselectively have demonstrated positive results in the management of patients with scleroderma vasculopathies including macitentan for pulmonary hypertension (214) and bosentan for digital vasculopathy(231)(232). As well as the above, I have previously described, an open label study of bosentan in SRC that showed a possible clinical benefit (45) as well as a further study, again open label with no control group, which failed to show improved outcome in patients with SRC (149).

#### *Endothelin receptor selectivity*

In practice, all commercially available endothelin receptor antagonists (ERAs) show some degree of relative affinity for the ETRA receptor sub-type but this degree of selectivity varies dramatically from bosentan (10-fold selectivity for ETRA approximately)(215) and macitentan (50-fold)(216), which are both conventionally described as “non-selective antagonists”, to the highly selective antagonist atrasentan (1000-fold relative affinity for ETRA over ETRB)(215). The degree of selectivity may have a significant effect on both the efficacy and the tolerability of this drug class in clinical practice (217).

In this chapter I will describe the design, conduct and results of a clinical research project examining zibotentan (ZD4054) as a treatment for patients with scleroderma kidney disease. Zibotentan is an ERA with much higher selectivity for ETRA than any of the medications discussed above (estimated at  $1 \times 10^7$ -fold by the manufacturer) (215).

The largest population in this multi-part clinical trial is of patients with scleroderma and CKD, but the study also included a smaller trial subgroup of acute SRC patients and, in a separate cohort without

scleroderma, pharmacokinetic evaluation of zibotentan dosing in patients on intermittent haemodialysis (HD).

ZEBRA was a three-part trial with the following aims in summary:

1. ZEBRA 1: to measure tolerability, safety, and efficacy of 6 months of treatment with zibotentan in patients with scleroderma and CKD stage 3A.
2. ZEBRA 2A: to measure tolerability, safety, and efficacy of 6 months of treatment with zibotentan in patients with SRC who do not require renal replacement therapy (RRT).
3. ZEBRA 2B: to measure tolerability, safety, and pharmacokinetic profile of a single dose of zibotentan in patients with endstage kidney disease on intermittent haemodialysis.

In the first two sub-studies, efficacy was measured with both conventional clinical outcomes (eGFR) and novel renal biomarkers (serum VCAM-1, urine MCP-1 and urine ICAM-1) so in addition to the other aims, these studies provide a proof of concept for the biomarker development objectives described in chapter 3.

## Methods

### Study Design

The full trial protocol is available with this thesis as Appendix A. I summarise the study design below.

ZEBRA 1 is a conventional double-blind placebo control trial with 1:1 randomisation of subjects to either:

- a. Oral zibotentan 10 mg once daily (with the option of dose reductions to a minimum dose of 5 mg once daily where side effects limited tolerability)
- b. A matched placebo

To be eligible for ZEBRA 1 patients had to have a diagnosis of scleroderma according to the 2013 ACR/EULAR criteria(203) AND an eGFR 45-60 ml/min/1.73 m<sup>2</sup> ie CKD3A in the current KDIGO clinical practice guidelines [17]. They could not have had SRC within the previous 12 months.

ZEBRA 2A is a single blind trial with 2:1 (treatment:placebo) randomisation of subjects to either:

- a. Oral zibotentan at an escalating dose over 4 weeks, starting at 2.5mg once daily and escalating by 2.5mg per week to the maximum tolerated dose or 10mg once daily, whichever is lower.
- b. A matched placebo with sham dose escalation

To be eligible for ZEBRA 2A patients were selected who had a diagnosis of scleroderma (see above) AND had been diagnosed with SRC more than one month and less than 12 months prior to recruitment. SRC was defined conservatively as new onset hypertension (blood pressure >150/85

mmHg obtained at least twice over a consecutive 24-hour period) AND an increase in serum creatinine of at least 10% from baseline (baseline value was required to be within 12 months). These diagnostic criteria are in keeping with the proposed international SRC classification criteria discussed in chapter 1 (109). Although there was no pre-specified exclusion for degree of renal dysfunction, patients were ineligible for recruitment to ZEBRA 2A if clinically adjudged to require RRT.

ZEBRA 2B was a single dose pharmacokinetic study of zibotentan 2.5 mg to 5mg orally in patients treated with maintenance intermittent haemodialysis for end stage kidney disease (ESKD). Individual patients received up to two single doses of zibotentan (at different dose levels). A diagnosis of scleroderma was not required for enrolment in ZEBRA 2B.

Participants in all 3 studies were > 18 years old and all were recruited from either the Royal Free scleroderma cohort (a national referral centre) or from the Royal Free maintenance haemodialysis programme (ZEBRA 2B). In view of the unknown teratogenic risk associated with zibotentan, pre-menopausal females and all males had to use one highly effective and one other method of contraception from enrolment into the study up to 6 weeks after cessation of study drug. For all 3 studies, patients were excluded if they had been exposed to any ERA in the 3 months prior to recruitment. There were no other excluded concomitant medications.

The ZEBRA trial protocol was approved by the local R&D and Research Ethics Committees. All participants gave informed consent in writing before any assessment or intervention was made and all study processes were performed as per the International Council for Harmonisation of Technical

Requirements for Pharmaceuticals for Human Use (ICH) and Good Clinical Practice (GCP) requirements(110).

The study visit schedule following consent and screening is described below.

ZEBRA 1:

Baseline (randomisation and start of investigational medicinal product), week 1, every 4 weeks up to and including week 24, week 26 (discontinuation of IMP), week 27, week 30, week 52.

ZEBRA 2A:

Baseline (randomisation and start of investigational medicinal product), weekly until week 4 (dose escalation visits) and then as per ZEBRA 1.

ZEBRA 2B:

Baseline (IMP administration and 3-hour post dose blood sampling), day 1 (pre and post dialysis sampling), day 7.

Endpoints

As this was a phase II study, the most important pre-defined primary endpoint for ZEBRA 1 was safety and tolerability of the IMP and this was measured by the number and character of adverse events (AEs) and serious AEs (SAEs). The primary pre-defined efficacy measure was change in serum VCAM-1 from baseline to end of treatment period (week 26). The primary endpoints for ZEBRA 2A were safety and tolerability measured as above and change in eGFR from baseline to end of treatment. Previous studies have confirmed the reliability of both MDRD and CKD-EPI calculations of eGFR in scleroderma [18]. For ZEBRA 2B the primary endpoints were safety and tolerability as



defined above and zibotentan plasma concentrations (ng/ml) recorded at the following times (in hours) since dosage: 0 (baseline), 3 (peak), 24 (pre-dialysis), 30 (post-dialysis).

Based on the work done in parallel on novel biomarkers and described in chapter 3, a further panel of outcome measures was designated, although these were not pre-defined as primary outcomes in the protocol: serum and urine concentrations of ET-1, VCAM-1, MCP-1 and ICAM-1. All urine analytes were expressed as urinary analyte: creatinine ratio to correct for differences in urinary volume for each patient sample as described in chapter 3.

Standard haematology and biochemistry samples were processed by the routine clinical laboratories of the Royal Free Hospital. eGFR was calculated using the MDRD equation as described earlier.

Standard bedside dipstick urinalysis was used to measure urine protein, blood, and glucose.

Biomarker analysis for the experimental trial endpoints (ET-1, VCAM-1, MCP-1 and ICAM-1) was conducted using commercial ELISA assay kits (R&D Systems, Abingdon, Oxford, UK) according to the manufacturer's instructions. PK plasma samples were anticoagulated with lithium, centrifuged and frozen in aliquots locally before transfer to a commercial laboratory for analysis using liquid chromatography-mass spectrometry.

Statistical analysis and reporting methods

The target sample sizes for ZEBRA were calculated according to the previous data on the frequency of CKD in the Royal Free scleroderma cohort, predicted variability in primary and secondary outcome measures as well as the likely extent of any treatment effect on eGFR (45). Recruitment target was

initially 48 patients for ZEBRA 1 and 12 patients for ZEBRA 2A. For ZEBRA 2B the target was to achieve at least 8 dose administrations in up to 12 patients.

I stratify the number of adverse events by group (zibotentan or placebo) where appropriate and for serious adverse events I describe both the nature of the adverse event and whether it was adjudged to be related to the IMP.

Outcome measures are stratified according to treatment group where appropriate, and include summary statistics for ZEBRA 1 (mean, median, standard deviation, maximum and minimum).

Outcome measures for ZEBRA 2A are reported directly by individual due to the small number of patients recruited. No summary statistics are presented and comparison between groups was not possible.

For each patient in ZEBRA 2B the zibotentan plasma concentration is shown individually at each time point. Where individual patients received a second dose, the concentrations are plotted in a separate figure.

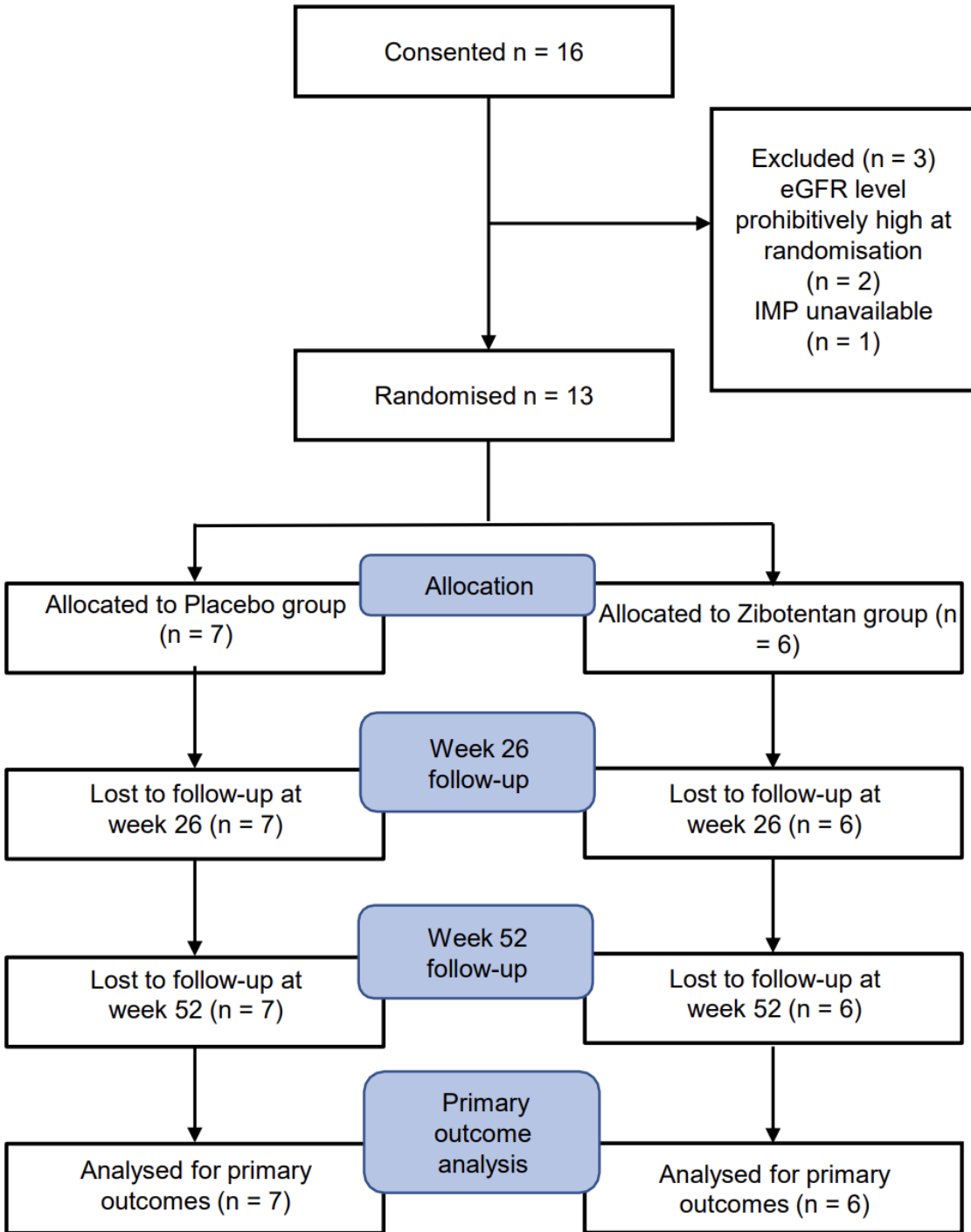
## Results

### Baseline patient characteristics

Target recruitment numbers were not achieved in either ZEBRA 1 or ZEBRA 2A and any conclusions drawn from the data have to be interpreted in that context. The failure to achieve recruitment targets was related to several factors including a lower than predicted prevalence of CKD in the target patient cohort and challenges external to the Royal Free study team which included a substantial pause in manufacture and supply of study medication and a change of external database supplier. Notwithstanding these challenges I successfully recruited patients to all three sub-studies and despite its limitations, this remains the first randomised placebo-controlled trial examining any treatment of kidney disease in scleroderma. In the ZEBRA 2B sub-study for which no diagnosis of scleroderma was required, the planned number of doses was achieved.

Figure 1 describes the screening and randomisation of patients in ZEBRA 1. 16 patients consented to undergo full screening, of whom 2 failed screening and 1 was excluded due to lack of availability of the IMP. 13 patients went forward to randomisation, of whom 6 were randomised to zibotentan and 7 were randomised to matched placebo.

Figure 1 Recruitment and randomisation of patients for ZEBRA 1



Only four patients were successfully screened for ZEBRA 2A and all four went on to randomisation. Of these, despite the 2:1 randomisation schedule, two were randomised to receive zibotentan and two were randomised to placebo. One patient allocated to the zibotentan group (Patient “D”) was diagnosed with severe community acquired pneumonia requiring hospitalisation in week 2 of follow-up and was withdrawn from treatment, although the acute illness was not adjudicated to be secondary to IMP. No further follow-up data were available for this patient.

Eight patients consented to screening for ZEBRA 2B. Two patients failed screening and the remaining six went forward to treatment in the study. Four of these patients received one dose only of zibotentan (2.5mg) and the remaining two completed both first and second dosing schedules.

The baseline demographics and key clinical features of all 3 study cohorts are summarised in Table 1.

There were no clinically significant imbalances between patient groups at baseline.

Table 1: Demographic and clinical characteristics for 3 ZEBRA sub-study populations.

		<b>Placebo</b>	<b>Zibotentan</b>
<b>ZEBRA 1</b>			
Number of subjects		7	6
Sex	Male	1	2
	Female	6	4
Ethnicity	Caucasian	7	6
Skin subgroup	dcSSc	2	1
	lcSSc	5	5
Autoantibody	ACA	5	3
	ARA	1	2
	ATA	0	0
	Other	1	1
<b>ZEBRA 2A</b>			
Number of subjects		2	2
Sex	Male	0	1
	Female	2	1
Ethnicity	Caucasian	2	1
	South Asian	0	1
<b>ZEBRA 2B</b>			
Number of subjects		NA	6
Sex	Male	NA	5
	Female	NA	1
Ethnicity	Caucasian	NA	4
	Afro-Caribbean	NA	1
	Mixed-race	NA	1

## Safety analysis

There were a total of 47 non-serious adverse events (AEs) among patients in the ZEBRA 1 sub-study. 27 of these occurred in patients on placebo and 20 in patients on zibotentan. 6/7 patients on placebo and 5/6 on zibotentan experienced at least one AE. AEs related to fluid retention and weight gain were more frequent on active treatment and in some cases this required dose adjustment or discontinuation. One serious adverse event (SAE) occurred in the ZEBRA 1 sub-study. This was a case of community acquired pneumonia which occurred in the placebo group.

There were a total of eight AEs among patients in ZEBRA 2A (four each in placebo and zibotentan groups). All patients in both groups experienced at least one AE. There were two SAEs among patients in ZEBRA 2A. The first was a pericardial effusion that occurred after screening but before start of IMP in a patient in the placebo group. This was automatically classified as an SAE as it required hospital admission. The second SAE was community acquired pneumonia in a patient in the zibotentan group and this adverse event, which resulted in withdrawal from the trial, is described above.

There were a total of three AEs in ZEBRA 2B occurring in 3 separate patients, none of which were considered related to study drug. There was a single SAE in ZEBRA 2B, pseudoaneurysm of arteriovenous fistula (AVF), which occurred 7 weeks after the zibotentan dosing but within a 12-week monitoring period for AEs. It was adjudicated not to be related to IMP.

## Efficacy data

Table 2 records primary and secondary outcome measures for ZEBRA 1. The pre-specified outcome measure was serum sVCAM-1 based on previous data as discussed above (202). Given that this data was from a historical SRC cohort it is perhaps not surprising that no significant effect was found on this marker in the patients without a history of SRC in ZEBRA 1 and there was no observable difference in VCAM-1 levels at end of treatment or end of follow-up.

As seen in Figure 3A there was a wide distribution of baseline levels that limits interpretation of any change over time. This is in keeping with the data collected in parallel to this trial and described in my biomarker study (chapter 3 of this thesis) which suggest that serum VCAM-1 is not a useful marker of SSc-CKD.

Formal statistical analysis of the other outcome measures is limited by the small sample size but nevertheless there are striking findings among some of the secondary efficacy measures that potentially merit further consideration. Most notable is the change in eGFR over time compared between placebo and zibotentan groups. eGFR (ml/min/1.73 m<sup>2</sup>) was evenly matched at the time of randomisation with mean (SD) of 52.0 (4.7) in the placebo group and 52.8 (4.5) in the zibotentan group. At end of treatment there was a slight reduction in eGFR in the placebo group 50 (7.1) compared with a trend towards improvement in the zibotentan group (54.3 (3.2)). This difference was more clearly apparent at the end of follow-up (52 weeks) where the mean eGFR continued to move in opposite directions: 47 (6.8) for placebo group compared with 60.8 (8.4) for zibotentan group ( $p=0.012$  by Mann-Whitney U for the difference at 52 weeks). This finding is of particular



importance as zibotentan is a vasodilator and so any changes in estimated renal function while still on treatment might be due to haemodynamic effects rather than disease modification in the kidney. An effect that persists and intensifies after the end of treatment in those exposed to the study drug implies true effect.

Four candidate biomarkers were measured in serum and urine and the results for all of these are shown in Table 2. Results are reported by Optical Density Units (ODU) and illustrative standard curves for these analytes are shown in Figure 2. As discussed in chapter 3, the markers of particular interest based on my most recent data were urine MCP-1 and ICAM-1. The results for these two biomarkers are illustrated in Figure 3B. Urine ICAM-1 did not show any observable difference between the treatment groups at any time point. Urine MCP-1: creatinine ratio fell in those in the zibotentan group and increased in those in the placebo group. At baseline mean urine MCP1/creatinine (ODU/mmol/L) was 9.4 (SD 5.8) in placebo compared with 9.5 (9.8) in zibotentan group. This rose to 25.2 (34.3) in placebo at end of treatment, whereas it fell to 5.9 (3.2) in the zibotentan group. The difference persisted at end of follow-up (52 weeks): 22.2 (33.3) in placebo versus 4.8 (0.9) in zibotentan group.

The opportunity for efficacy analysis was considerably more limited in ZEBRA 2A due to the recruitment difficulties and very small sample size. Follow-up data at end of treatment and end of follow-up was only available for one patient in the active treatment arm. Data are presented in full in Table 3. In summary all three patients with follow-up data available, including two in the placebo group, had improvement in their eGFR over the study period. This is in keeping with the very good

prognosis for patients with SRC who do not require RRT in the acute phase, as discussed in chapter 1.

Endothelin-1 concentrations in patients receiving zibotentan

Tables 2 and 3, include the serum ET-1 concentrations for subjects in ZEBRA 1 and 2A. There was no observable change from baseline in either the placebo or zibotentan group in either study at either of the later timepoints. This is a notable finding given the dramatic rise in serum ET-1 concentration seen in patients treated with the relatively non-selective ERA bosentan in previous studies [12].

Table 2 Outcome measures in ZEBRA 1 subjects

Outcomes are recorded at randomisation (T1), end of treatment (T2) and end of follow-up (T3). Experimental biomarkers are measured in Optical Density Units (ODU). Urine biomarkers are expressed as a ratio to urine creatinine concentration (ODU/mmol/l).

Outcome measure	Group	n	Mean	Std. Dev.	Median	Min.	Max.
Serum VCAM-1 T1	Placebo	7	0.28	0.19	0.17	0.15	0.56
	Zibotentan	6	0.19	0.09	0.17	0.11	0.34
Serum VCAM-1 T2	Placebo	7	0.28	0.22	0.17	0.08	0.7
	Zibotentan	6	0.2	0.05	0.19	0.15	0.26
Serum VCAM-1 T3	Placebo	7	0.29	0.15	0.23	0.14	0.53
	Zibotentan	5	0.26	0.13	0.2	0.14	0.41
eGFR T1 (ml/min/1.73m <sup>2</sup> )	Placebo	7	52	4.69	51	44	58
	Zibotentan	6	52.83	4.45	50.5	49	59
eGFR T2 (ml/min/1.73m <sup>2</sup> )	Placebo	7	50	7.09	53	37	58
	Zibotentan	6	54.33	3.20	54	50	58
eGFR T3 (ml/min/1.73m <sup>2</sup> )	Placebo	7	47	6.83	50	36	55
	Zibotentan	6	60.83	8.35	60.5	50	74
Serum ET-1 T1	Placebo	7	0.2	0.08	0.17	0.15	0.38
	Zibotentan	6	0.19	0.06	0.18	0.11	0.3
Serum ET-1 T2	Placebo	7	0.19	0.09	0.16	0.1	0.4
	Zibotentan	6	0.18	0.04	0.18	0.13	0.24
Serum ET-1 T3	Placebo	7	0.17	0.07	0.15	0.11	0.33
	Zibotentan	5	0.24	0.04	0.22	0.21	0.3
Serum MCP-1 T1	Placebo	7	0.24	0.12	0.2	0.11	0.48
	Zibotentan	6	0.22	0.07	0.21	0.13	0.32
Serum MCP-1 T2	Placebo	7	0.27	0.17	0.23	0.11	0.59
	Zibotentan	6	0.17	0.04	0.15	0.14	0.25
Serum MCP-1 T3	Placebo	7	0.23	0.09	0.22	0.12	0.4
	Zibotentan	5	0.29	0.19	0.16	0.13	0.57
Serum ICAM-1 T1	Placebo	7	0.67	0.22	0.7	0.31	1.03
	Zibotentan	6	0.74	0.16	0.7	0.58	0.98
Serum ICAM-1 T2	Placebo	7	0.68	0.33	0.66	0.14	1.21

	Zibotentan	6	0.77	0.14	0.79	0.56	0.91
Serum ICAM-1 T3	Placebo	7	0.74	0.3	0.74	0.37	1.22
	Zibotentan	5	0.83	0.17	0.92	0.56	0.98
Urine MCP-1 T1	Placebo	7	9.4	5.82	7.1	5.23	21.9
	Zibotentan	6	9.49	9.82	5.41	3.09	28.94
Urine MCP-1 T2	Placebo	7	25.21	34.29	9.49	6.33	99.92
	Zibotentan	6	5.85	3.52	4.37	2.94	11.21
Urine MCP-1 T3	Placebo	7	22.21	33.32	8.08	6.39	97.24
	Zibotentan	4	4.77	0.91	4.51	4.06	5.98
Urine ICAM- 1 T1	Placebo	7	2.61	1.2	2.47	1.17	3.98
	Zibotentan	6	1.18	0.76	0.96	0.47	2.35
Urine ICAM- 1 T2	Placebo	7	3.11	2.24	2.13	0.91	6.96
	Zibotentan	6	10.83	22.43	2.01	0.88	56.6
Urine ICAM- 1 T3	Placebo	7	2.58	1.4	2.37	0.48	4.82
	Zibotentan	4	1.35	0.78	1.48	0.4	2.03
Urine VCAM- 1 T1	Placebo	7	85.73	207.91	3.45	0.36	556.61
	Zibotentan	6	2.63	4.31	0.88	0.71	11.43
Urine VCAM- 1 T2	Placebo	7	58.53	131.85	5.51	0.33	356.79
	Zibotentan	6	10.76	22.04	2.11	0.09	55.64
Urine VCAM- 1 T3	Placebo	7	98.87	237.13	2.65	0	635.95
	Zibotentan	4	1.58	0.44	1.41	1.29	2.23

Table 3 Outcome measures in ZEBRA 2A subjects

Outcomes are recorded at randomisation (T1), end of treatment (T2) and end of follow-up (T3). Experimental biomarkers are measured in Optical Density Units (ODU). Urine biomarkers are expressed as a ratio to urine creatinine concentration (ODU/mmol/l). Anonymised individual patients are designated as A to D. Data for patient D were absent at T2 and T3 (NA).

Outcome measure	Patient	Treatment group	Value
Serum VCAM-1 T1	A	Placebo	0.27
	B	Placebo	0.44
	C	Zibotentan	0.65
	D	Zibotentan	0.41
Serum VCAM-1 T2	A	Placebo	0.20
	B	Placebo	0.20
	C	Zibotentan	0.76
	D	Zibotentan	NA
Serum VCAM-1 T3	A	Placebo	0.16
	B	Placebo	0.22
	C	Zibotentan	0.81
	D	Zibotentan	NA
eGFR T1 (ml/min/1.73m <sup>2</sup> )	A	Placebo	57
	B	Placebo	34
	C	Zibotentan	27
	D	Zibotentan	21
eGFR T2 (ml/min/1.73m <sup>2</sup> )	A	Placebo	65
	B	Placebo	43
	C	Zibotentan	26
	D	Zibotentan	NA
eGFR T3 (ml/min/1.73m <sup>2</sup> )	A	Placebo	71
	B	Placebo	51
	C	Zibotentan	34
	D	Zibotentan	NA
Serum ET-1 T1	A	Placebo	0.13
	B	Placebo	0.15
	C	Zibotentan	0.24
	D	Zibotentan	0.29
Serum ET-1 T2	A	Placebo	0.14

	B	Placebo	0.12
	C	Zibotentan	0.38
	D	Zibotentan	NA
Serum ET-1 T3	A	Placebo	0.7
	B	Placebo	0.12
	C	Zibotentan	0.39
	D	Zibotentan	NA
Serum MCP-1 T1	A	Placebo	0.95
	B	Placebo	0.21
	C	Zibotentan	0.26
	D	Zibotentan	0.77
Serum MCP-1 T2	A	Placebo	0.64
	B	Placebo	0.42
	C	Zibotentan	0.36
	D	Zibotentan	NA
Serum MCP-1 T3	A	Placebo	0.18
	B	Placebo	0.43
	C	Zibotentan	0.31
	D	Zibotentan	NA
Serum ICAM-1 T1	A	Placebo	0.46
	B	Placebo	1.13
	C	Zibotentan	0.67
	D	Zibotentan	0.94
Serum ICAM-1 T2	A	Placebo	0.46
	B	Placebo	0.59
	C	Zibotentan	0.73
	D	Zibotentan	NA
Serum ICAM-1 T3	A	Placebo	0.25
	B	Placebo	0.65
	C	Zibotentan	0.69
	D	Zibotentan	NA
Urine MCP-1 T1	A	Placebo	9.50
	B	Placebo	9.49
	C	Zibotentan	6.24
	D	Zibotentan	8.63
Urine MCP-1 T2	A	Placebo	4.79
	B	Placebo	7.79

	C	Zibotentan	45.92
	D	Zibotentan	NA
Urine MCP-1 T3	A	Placebo	3.63
	B	Placebo	4.77
	C	Zibotentan	12.99
	D	Zibotentan	NA
Urine ICAM-1 T1	A	Placebo	4.55
	B	Placebo	1.67
	C	Zibotentan	0.84
	D	Zibotentan	0.41
Urine ICAM-1 T2	A	Placebo	2.74
	B	Placebo	1.20
	C	Zibotentan	7.10
	D	Zibotentan	NA
Urine ICAM-1 T3	A	Placebo	3.17
	B	Placebo	2.60
	C	Zibotentan	2.83
	D	Zibotentan	NA
Urine VCAM-1 T1	A	Placebo	129.75
	B	Placebo	282.44
	C	Zibotentan	272.89
	D	Zibotentan	13.30
Urine VCAM-1 T2	A	Placebo	23.97
	B	Placebo	36.57
	C	Zibotentan	877.49
	D	Zibotentan	NA
Urine VCAM-1 T3	A	Placebo	334.29
	B	Placebo	66.33
	C	Zibotentan	295.76
	D	Zibotentan	NA

Table 4 ZEBRA 2B results

Plasma zibotentan concentrations (ng/ml) for each single dose administration are given at four time points: Baseline (pre-dose), Peak (3h post-dose), trough (pre-dialysis, 24h post-dose), post-dialysis (30h post-dose). The individuals who received two doses are shaded for ease of reference.

<b>Patient</b>	<b>Dose</b>	<b>Timepoint</b>	<b>Zibotentan concentration (ng/ml)</b>
A	2.5mg	Baseline	0.0
		Peak	87.0
		Trough	16.3
		Post-dialysis	5.6
B	2.5mg	Baseline	0.0
		Peak	54.7
		Trough	33.2
		Post-dialysis	17.0
C	2.5mg	Baseline	0.0
		Peak	106.0
		Trough	37.8
		Post-dialysis	8.7
D	2.5mg	Baseline	0.0
		Peak	107.0
		Trough	22.0
		Post-dialysis	4.5
E	2.5mg	Baseline	0.0
		Peak	107.0
		Trough	22.0
		Post-dialysis	4.6
F	2.5mg	Baseline	0.0
		Peak	138.0
		Trough	43.2
		Post-dialysis	9.1
B	5mg	Baseline	0.0
		Peak	131.0
		Trough	67.8
		Post-dialysis	25.5
C	5mg	Baseline	0.0
		Peak	177.0
		Trough	52.4
		Post-dialysis	19.3



Figure 2 Illustrative standard Curves for ELISA analysis of ZEBRA biomarkers.

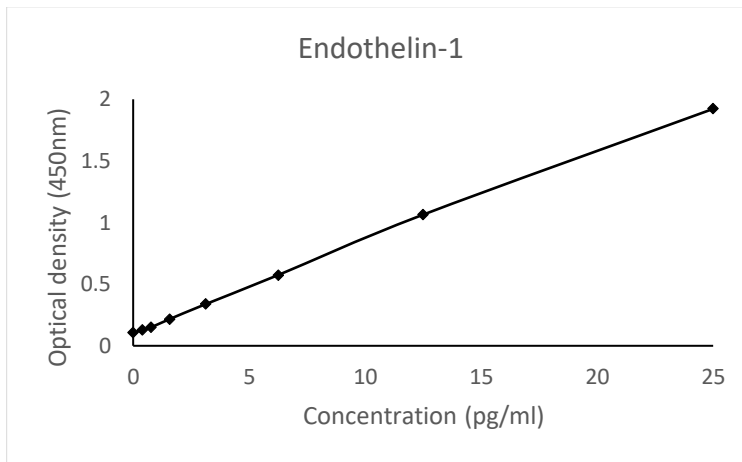
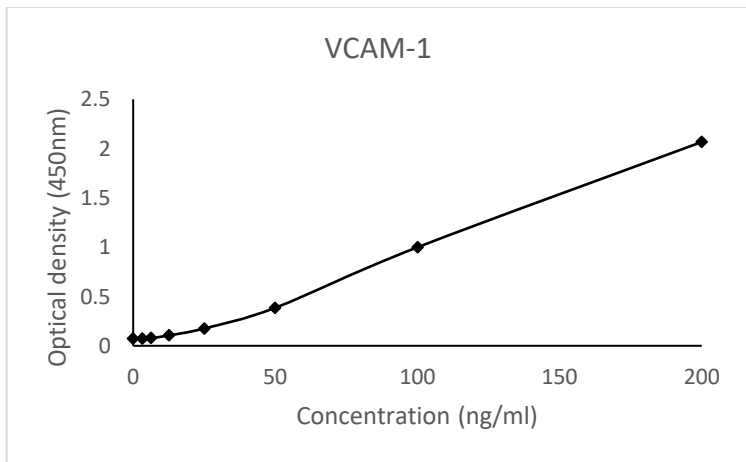
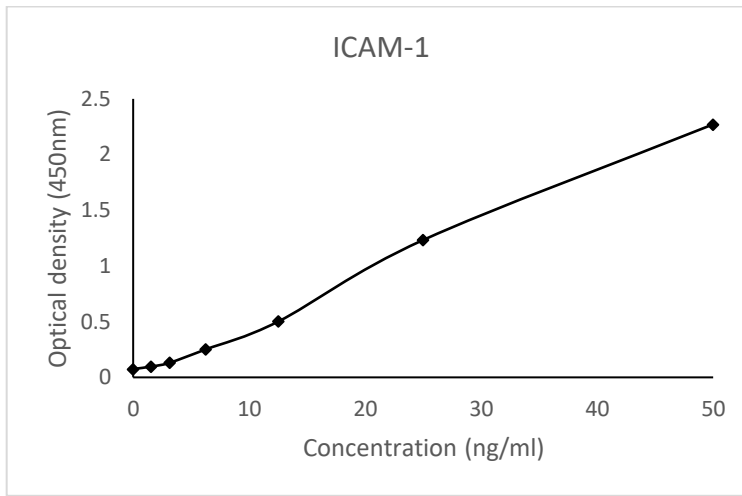
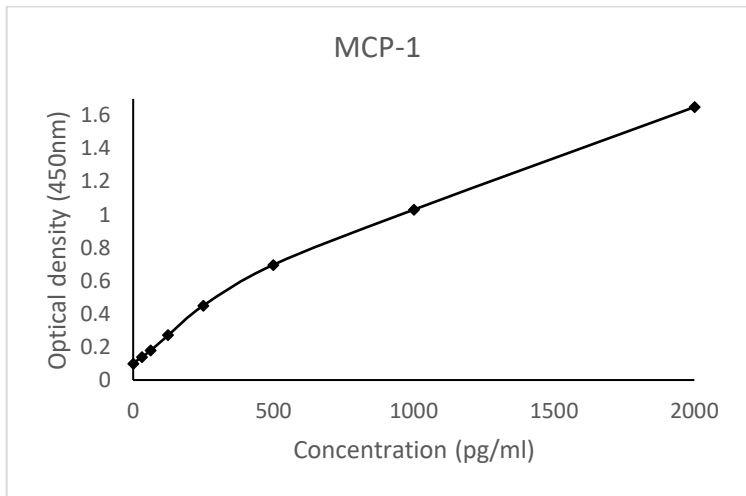
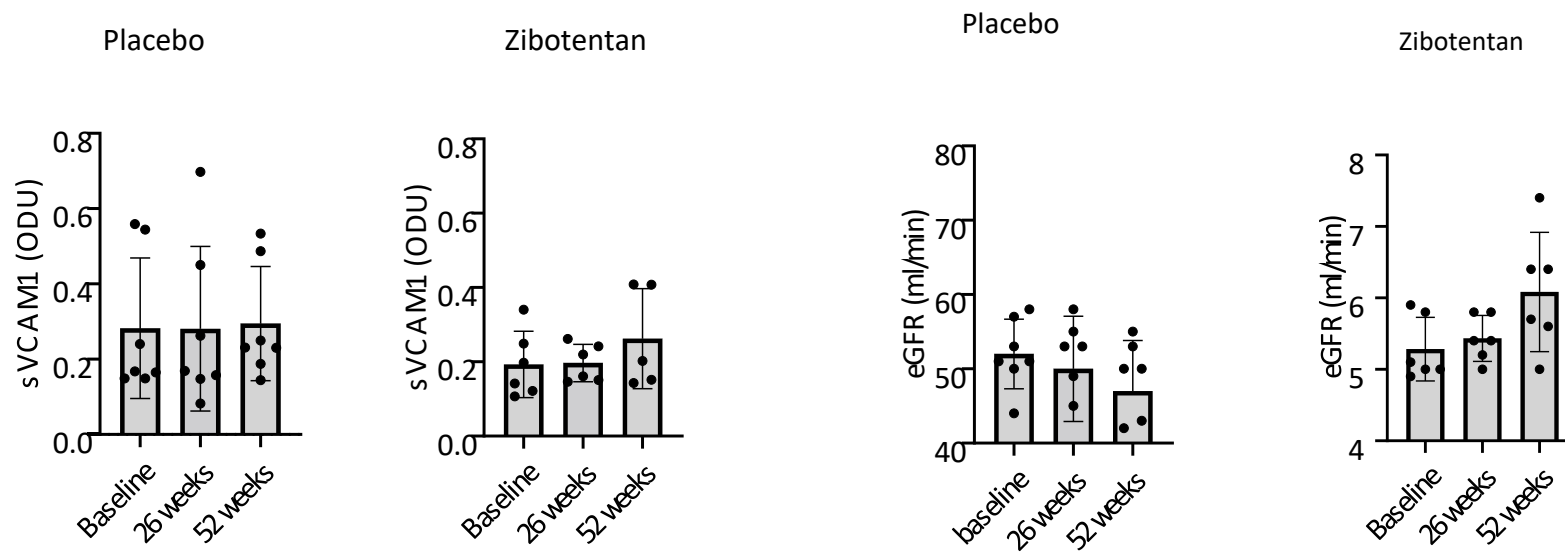
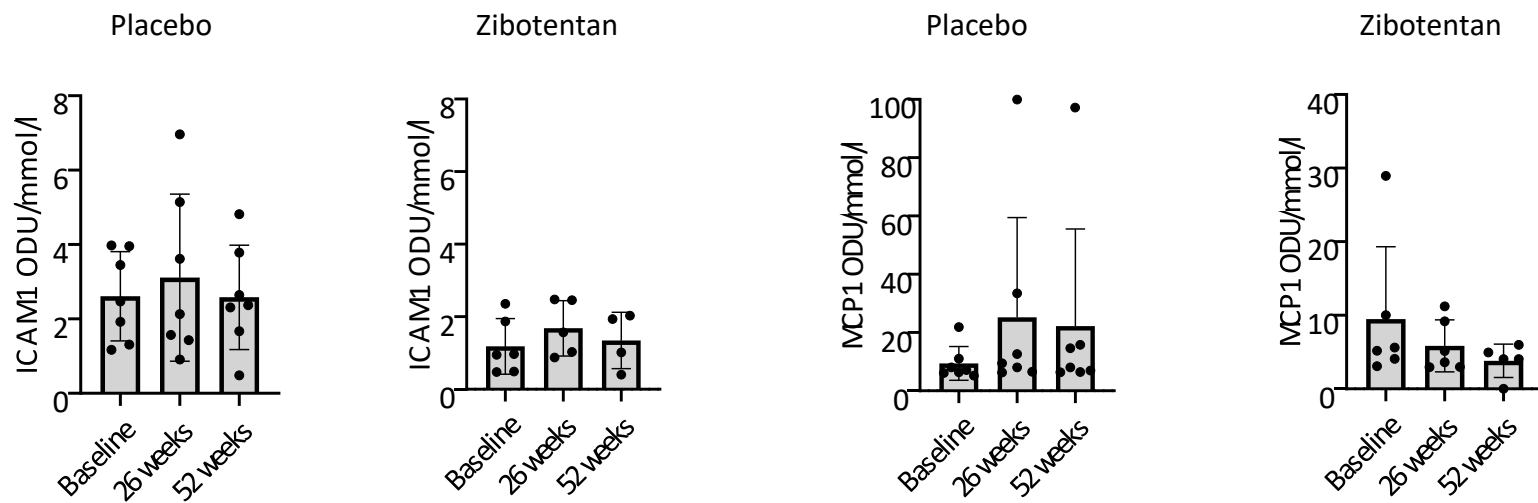


Figure 3 Outcome data for ZEBRA 1

A: Serum VCAM-1 and eGFR at randomisation, end of treatment and end of follow-up



B: Novel urine biomarker outcomes for ZEBRA 1



C: *eGFR in placebo and zibotentan groups compared at each study time-point.*

Zibotentan concentration in ZEBRA 2B

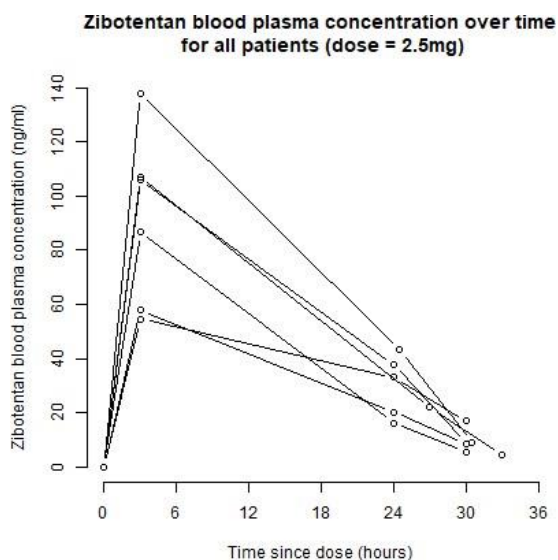
ZEBRA 2B patients all underwent pharmacokinetic evaluation after receiving a single dose of zibotentan. The pre-specified time points for sampling are described above and plasma concentrations are illustrated in Figure 3: Figure 3A shows the concentrations at each time point super-imposed for the first dose in all six individuals; Figure 3B shows the concentrations individually for the two patients later receiving a 5 mg dose.

Although the drug has been observed to be primarily renally excreted(218), it is notable that despite minimal native renal clearance in these patients, there was a marked fall from the post-dose peak in all patients after 24 hours. These pre-dialysis zibotentan concentrations (range 16-43 ng/ml) were in the same approximate range as the trough levels at 24h observed in healthy individuals given zibotentan 2.5mg (described in the manufacturer's Investigator's Brochure). As there was additional clearance across 4 hours of dialysis observed, this suggests that daily dosing in haemodialysis will be feasible in future. The concentrations achieved at both doses are well within the safe range described for zibotentan in previous pharmacokinetic studies in CKD patients not on dialysis (218)

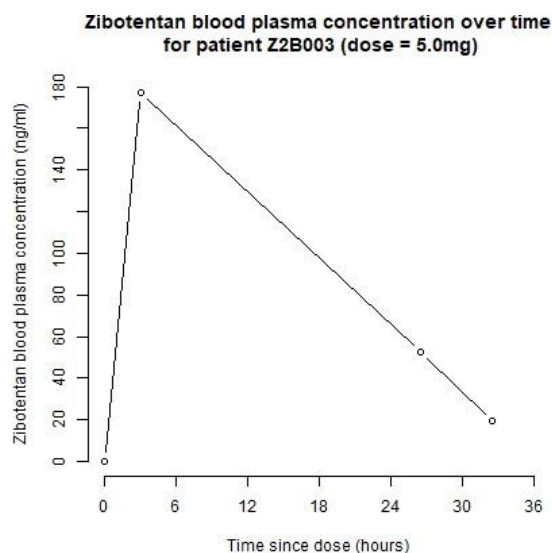
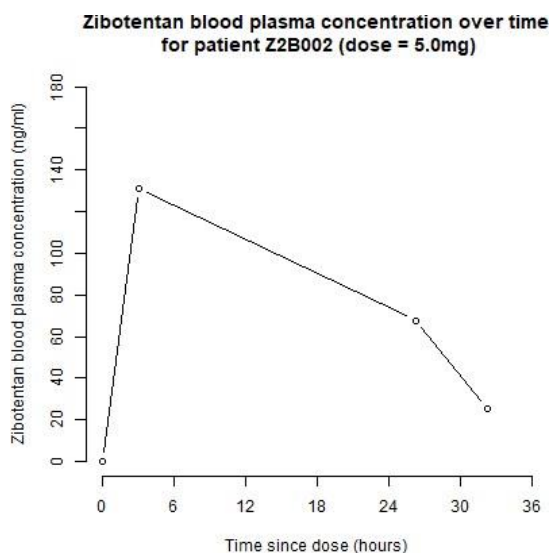
Figure 4 Zibotentan concentrations in ZEBRA 2B patients.

Concentration is in pg/ml and is shown at four timepoints for all patients (pre-dose, peak, trough and post-dialysis).

A: *Zibotentan plasma concentration following a single 2.5 mg dose*



B: *Zibotentan plasma concentration following a single 5 mg dose*



## Discussion

In this chapter I have examined the suitability of the highly selective ETRA antagonist zibotentan as a treatment for kidney disease in scleroderma. This is on a background of previous clinical trials showing a benefit for less selective ERAs in other scleroderma vasculopathies as described above, and also a proof-of-concept study carried out at the Royal Free, looking at Bosentan in scleroderma renal crisis(45).

In all three ZEBRA sub-studies zibotentan was shown to be safe in patients with renal impairment ranging from mild (CKD 3A) to severe (CKD stage 5 on haemodialysis). There were no serious adverse events in any study group adjudicated to be caused by zibotentan. In general, patients tolerated Zibotentan well and there was no overall excess of adverse events in the active treatment group. However, a variety of adverse events did occur with a large majority experiencing at least one, in ZEBRA 1/2A. In patients on active treatment with zibotentan, adverse events included fluid retention and weight gain. While these were not classified as serious according to the pre-specified AE criteria, they required medical management and on occasion led to treatment discontinuation. These findings are in keeping with other studies in zibotentan (219) and in other highly selective ERAs(220).

The selection of zibotentan for treatment of patients with renal impairment was based on the hypothesis that reduced activity at the ETRB receptor would improve tolerability for patients with CKD, who have a high incidence of hypervolaemia and hypertension. This hypothesis drew on three important observations from previous research:

- ET-1 binding to ETRB promotes the release of vasodilators including nitric oxide and prostacyclin and therefore ETRB acts as a tonic vasodilator in opposition to the vasoconstrictive effects of ETRA (221).

- ETRB acts as a scavenger receptor for ET-1 and therefore reduces circulating concentration of the hormone(222).
- ET-1 acting on ETRB in the collecting duct promotes natriuresis and absence of this activity results in volume expansion(223).

Based on this experimental evidence, previously observed adverse events in clinical trials of endothelin antagonists recruiting CKD patients (salt and water overload and hypertension) were hypothesised to be secondary to antagonism of ETRB. As the most highly selective ETRA antagonist available, zibotentan had the theoretical potential to reduce fluid overload side effects and reduce the incidence of rebound hypertension secondary to high circulating ET-1 levels on drug discontinuation. The first of these hypotheses was disproved and other studies of selective ERAs have supported our finding that fluid overload from the drug class does not seem to be mediated purely via ETRB(220). Regarding the second hypothesis, there was no evidence of worsening hypertension or rebound hypertension in any of the study groups at any dose and there was no increase in circulating ET-1 levels on zibotentan. This finding is especially significant in considering ERAs for the management of scleroderma renal crisis, an acute hypertensive emergency.

The prior study design for the ZEBRA trials drew on historical data on potential kidney biomarkers in scleroderma. Since that time, the experiments presented in chapter 3 of this thesis (which were collected in parallel to the early phases of ZEBRA) have provided more detailed and up-to-date information about possible biomarkers for CKD in scleroderma, especially those measured in urine. VCAM-1, the pre-determined primary endpoint for ZEBRA 1 does not appear to reflect disease activity in chronic kidney disease patients. As would be predicted from the findings in those experiments, this pre-specified primary end



point for ZEBRA 1 was negative. I also measured the two most promising novel urine biomarkers developed in this earlier experiment at the key timepoints in ZEBRA 1 and ZEBRA 2A. Of these two experimental urine biomarkers measured as secondary outcomes, MCP-1 showed the most discriminating results and appeared to act as a local disease activity marker with levels highest in those pre-treatment or treated with placebo and lowest in patients post-treatment with zibotentan whose renal impairment had improved on average. These data in a small population do not provide definitive evidence but offer a starting point for further investigation.

While urine MCP-1 may give insight into the disease process in the kidneys, a further secondary outcome measured (eGFR) makes a particularly compelling case that there was a real treatment effect from zibotentan, notwithstanding the limitations of this small trial. Inversely to urine MCP1, eGFR had fallen on average at the end of treatment in patients treated with placebo and it continued to fall over the following six months. On the other hand, in those treated with zibotentan, there was a small numerical increase in eGFR at the end of treatment and this trend continued over the following six months so that 12 months after starting treatment the mean eGFR had gone from 53 to 61, compared to a mean eGFR of 47 at the end of 12 months in the placebo group ( $p=0.0072$ , see Figure 3C). This suggestion of a treatment effect that persists after temporary exposure to study drug has been seen in previous scleroderma clinical trials (e.g. a trial of oral cyclophosphamide in scleroderma-associated interstitial lung disease (224) but it is an unexpected finding in a vasoactive drug rather than a disease modifying immunosuppressant. The intriguing possibility that an ERA could have a disease modifying or remodelling effect is potentially supported by another recent clinical trial and I will discuss this further in chapter 5 (225).

Zibotentan is mainly excreted renally (218) and its safety in patients with advanced renal impairment has not been determined previous to this study. Administration for patients on renal replacement therapy is a particular concern in the absence of data. As was seen in the ZEBRA 2A results, the prognosis is generally good for patients with SRC and only moderate acute kidney injury whereas mortality or failure to recover remain high in those who require dialysis (see chapter 1). Therefore, this is the group of patients most in need of new therapeutics to improve their outcome (151,164). For that reason, it was an important objective of this study to improve our understanding of the potential for dosing zibotentan in patients on renal replacement therapy (the most common form of which after SRC is haemodialysis).

The data presented here on zibotentan PK on HD provide valuable first insights in this area. 2.5mg and 5mg doses both provided plasma concentrations at peak that would be considered safe and therapeutic in keeping with the data provided in the Investigator's Brochure. Peak plasma concentrations at these doses did not exceed those seen in previous studies in patients with either normal or impaired renal function (218). Safe trough levels 24 hours post-dose (with more clearance achieved after 4 hours of HD) suggests that daily dosing could be assessed in an extended study without any significant safety concerns. It is also notable that the peak concentrations showed fairly linear increase in the escalation from 2.5 to 5mg in two patients. The ZEBRA 2B pharmacokinetic findings are especially relevant for future study designs because they were not restricted to patients with scleroderma. Nevertheless, they do provide support for investigation of the SRC population with severe AKI, which would ameliorate some of the recruitment difficulties seen in ZEBRA 2A.

There are very notable limitations to the studies presented in this chapter: in particular, neither ZEBRA 1 or 2A came close to its recruitment target and any findings are limited therefore by the small population sizes. Nevertheless ZEBRA 1 and 2A were the first randomised controlled trials of any treatment for renal involvement in scleroderma, and although their conduct highlighted some of the difficulties of clinical research in this disease group, the findings of ZEBRA 1, in particular, offer promise for therapeutics in this population and potentially others with CKD. The studies also provided an important opportunity to test the utility of novel urine biomarkers which I described the development of in an earlier chapter.

## Conclusions

The data presented in this chapter are an important contribution to the management of kidney disease in scleroderma and provide a platform for further research in the use of highly selective ERAs in this condition and others. They also provide a starting point for the investigation of zibotentan in patients on renal replacement therapy.

# **Chapter 5: Final Discussion**

## Summary

This project has investigated the processes that contribute to kidney disease in scleroderma with the aim of furthering our understanding of its pathobiology and improving the clinical management of the condition.

In the first chapter I summarised current knowledge on the aetiopathogenesis of scleroderma as a whole and described the specific processes observed in the kidney, both at a molecular level and in the clinical presentation of patients with renal involvement in scleroderma. I also described the current consensus as to the appropriate clinical management of these patients.

In the second chapter I investigated potential genetic risks for scleroderma renal crisis by focussing on an immunological subgroup of patients at particularly high risk of this complication. I then assessed whether there are common genetic variants to account for some of the excess risks in those individuals within this enriched subgroup who went on to develop SRC. Plausible candidate genes were investigated further with immunohistochemistry looking at the relevant gene products in kidney tissue.

In the third chapter I investigated potential novel biomarkers for kidney disease in scleroderma using a multiplex immunoassay on urine and serum samples from patients and control groups. Candidate biomarkers were assessed both for their potential pathogenic role in the progression of kidney disease and in terms of their utility as future clinical or research outcome measures.

In the fourth chapter I described a multi-part clinical trial, evaluating the safety and effectiveness of zibotentan, a highly selective endothelin receptor antagonist (ERA), in

improving clinical outcomes in scleroderma kidney disease, with a particular focus on chronic kidney disease (CKD). I also evaluated the safety and pharmacokinetic profile of zibotentan administration in patients on intermittent haemodialysis (HD).

In this final chapter I highlight findings from each of the investigations above which are of particular interest in view of the current research literature, and suggest how further research in these areas could build on the work in this thesis.

GWAS results: CTNND2, Wnt signalling and scleroderma

The genome wide association project described in chapter 2 identified a strong statistical association in our “discovery cohort” between a common single nucleotide polymorphism within the CTNND2 gene area and the development of renal crisis in our high-risk subgroup. This association was supported by findings in renal crisis biopsy tissue. SNPs within this gene had previously been demonstrated to associate with pulmonary arterial hypertension in scleroderma. PAH shares immunological distribution (ie high in ARA positive patients) and pathophysiology (chronic medium vessel vasculopathy) with SRC so this link is suggestive of a pathobiological role of a disordered CTNND2 gene in the disease process of scleroderma vasculopathies.

CTNND2 (also known as delta-catenin) is a protein in the armadillo-repeat family which interacts with adhesive junction proteins including E-cadherin and is thereby implicated in the regulation of cell-to-cell adhesion (226–229). Its role is best understood in the central nervous system, with mutations previously associated with several neurodevelopmental and neurodegenerative disorders (229). A close regulatory association between the CTNND2 gene and the Wnt signalling pathway across the cell surface has been identified via a study of cell migration and adhesion in lung adenocarcinoma(230). A study in hepatocellular

carcinoma showed that hypoxia could upregulate expression of CTNND2 via HIF1 $\alpha$  and that this in turn influences activity in the Wnt pathway(231). A role for hypoxic drivers via HIF1 $\alpha$  has already been recognised in the scleroderma disease process (232).

Wnt signalling has become a field of increasing research interest in recent years with the development of novel therapeutics targeting this pathway (233). In scleroderma, the Wnt pathway is understood to drive extracellular matrix expansion by upregulating TGF $\beta$  (234). In a clinical trial setting, a  $\beta$ -catenin inhibitor that downregulates Wnt -promoted genes was applied to the skin of scleroderma patients and found to prevent the differentiation of fibroblasts into the disease-forming phenotype (235). This is in keeping with experiments in transgenic mice which confirmed that Wnt signalling plays a role in the release of endothelial progenitor cells and their eventual fate in differentiation (236,237). Therefore the CTNND2 gene identified in our study may be linked to the the dysregulation of endothelial to mesenchymal transformation that plays a key role in the pathobiology of scleroderma vasculopathies, including SRC and PAH (238,239).

In chapter 2 I describe immunohistochemistry experiments on scleroderma renal crisis tissue which showed that CTNND2 was present in discrete fragments within the glomerular capillary loops but distinct from the resident nucleated endothelial cells. As I described in chapter 1, the typical pathological findings within the kidney in SRC are those of thrombotic microangiopathy (TMA), where a fibrin mesh typically causes occlusion of small and medium-sized vessels and red cell or platelet fragments are often sequestered within the diseased vasculature (240,241). A recent study inducing TMA on human blood in vitro suggested that rather than simple mechanical sequestration in the fibrin mesh, as previously had been assumed, red cells may be fixed to the endothelium by active cell-to-cell-adhesion pathways (242). The possibility that these CTNND2-positive fragments are such sequestered

red cells or platelets merits further investigation into the role of this gene in scleroderma renal crisis and other forms of TMA in the kidney. This potential avenue of research is particularly important in the context of the development of pharmacological interventions for Wnt-mediated disease processes.

Biomarker results: MCP-1 as a urine biomarker

In chapter 3 I demonstrated that monocyte chemoattractant protein-1 (MCP-1 or CCL2) could be measured in the urine using an immunoassay and that its concentration could help to distinguish between patients with scleroderma with CKD and healthy controls as well as other disease subgroups. In chapter 4, I showed that urine MCP-1 concentrations were dynamically responsive to an experimental treatment that appeared to stabilise or improve kidney function in scleroderma patients with CKD.

MCP-1 is a cytokine in the C-C group. It has been shown to be expressed by resident cells in the vascular endothelium, the epithelium and in fibroblasts within connective tissue (243). Nevertheless, immune cells (monocytes/macrophages) are the predominant source of its production (244). MCP-1 acts via the chemokine receptor CCR2. In skin biopsy samples and cell culture from scleroderma patients the interaction with this cytokine and its receptor was demonstrated to initiate differentiation of fibroblasts into myofibroblasts (94). As inferred by its name it also acts as chemoattractant, promoting migration of T lymphocytes and Natural Killer cells as well as monocytes (244). Thickened skin in scleroderma patients shows increased MCP-1 expression compared to unaffected areas (245), it is found in high concentration in bronchioalveolar lavage fluid from scleroderma patients with active lung disease (246) but its concentrations in sera of scleroderma patients have been shown to be variable (200). So although MCP-1 has long been considered a potential pathogenic

mediator in scleroderma, its role as a biomarker has been limited by the availability of a suitable non-invasive tissue substrate in which to measure it dynamically. Concentrations of the protein in circulating blood appear to be independent of the extent of MCP-1 expression in tissues where there is active scleroderma disease process (94). Therefore, the findings of this study, that urine may offer a local guide to disease activity in the kidney via the measurement of MCP-1, is potentially of great significance for future management of patients with scleroderma kidney disease.

Research in the related connective tissue disease systemic lupus (SLE) supports my findings. Multiple studies in this disease show that urine concentrations of MCP-1 in patients with active nephritis are higher than in patients without active kidney disease or in healthy controls(200,201,247). A more recent study has proposed a composite biomarker designer with urine MCP-1 in lupus measured together with urinary TWEAK to indicate the onset of lupus nephritis earlier than would otherwise be possible (248). Studies of urine MCP-1 to predict drug toxicity also suggest its potential as a marker that acts as an early sentinel for what later becomes clinically apparent pathology in the kidney (249). Taken together with the findings I have presented above, these studies support the further development of urinary MCP-1 as both a diagnostic and a dynamic longitudinal tool in measuring the extent of kidney involvement in scleroderma.

Clinical trial results: the role of highly selective ETRA antagonists in the treatment of kidney disease

In chapter 4 of this thesis I described a series of parallel clinical trials of the highly selective endothelin A receptor (ETRA) antagonist zibotentan. As I discussed in chapter 1, Endothelin



(ET-1) is a potent vasoconstrictor and exerts this effect primarily through its action at ETRA whereas the endothelin B receptor (ETRB) provides some tonic vasodilatation (250). ETRB is an important scavenger receptor for plasma endothelin in the lung, liver and kidney [21], and it has previously been hypothesised that its blockade is responsible for the increase in circulating ET-1 that has been observed during treatment with non-selective ERAs such as bosentan. It is theorised that these high circulating hormone levels are then responsible for rebound hypertension that has been observed on discontinuation of these medications in clinical studies (217). The follow-up visits I conducted with patients one week after drug discontinuation did not reveal any instances of rebound hypertension in any of the three studies with patients with varying degrees of CKD. This suggests that a selective antagonist with minimal activity at ETRB may be especially suitable for the treatment of CKD patients, in whom hypertension is particular clinical challenge.

Further support for the use of selective ETRA antagonists has been provided since completion of my study by the results of a large multicentre trial of another highly selective ERA in patients with diabetes, moderate CKD and albuminuria (251) This double blind, randomised control trial (SONAR) examined 2648 patients on atrasentan or placebo for an average of two years and found a 35% reduction in the pre-specified negative renal endpoints among those on active treatment compared to controls [25]. The SONAR study was performed in a two-phase manner, with all eligible patients first given atrasentan for six weeks in order to identify a subgroup of patients for whom the study drug was tolerable without excessive fluid overload and who had a response in the intermediate biomarker (albuminuria) (220). The aim of this run-in period was to enrich the study group with patients likely to have a positive response over longer term observation on atrasentan therapy. Those classified as “responders” were randomised to treatment or placebo in the

full-length trial. Interestingly the outcomes for responders who had received atrasentan in phase one but went on to be randomised to placebo for the full trial showed a fall in urinary albumin during the first phase that was persistent throughout the trial, so that two or more years later, at the end of follow-up, their urine albumin:creatinine ratio was still significantly lower than it had been before their six weeks of exposure to atrasentan, which happened prior to randomisation. This unexpected finding supports my observation in patients with CKD and scleroderma, where those treated with zibotentan had a trend towards improved kidney function which continued, and was even amplified, six months after the discontinuation of the study drug.

A recent retrospective study of vasodilator treatments used for the treatment of PAH associated with connective tissue diseases looked at renal outcome depending on the timing of initiation of dual vasodilator therapy. The largest subgroup of patients had scleroderma, although SLE and mixed-connective tissue disease patients were also included. Those who were initiated on combination therapy at the same time, early after diagnosis of PAH, typically a phosphodiesterase-5 inhibitor (PD5i) plus an ERA, had lower incidence of CKD and less progression of CKD than the patients initially started on one vasodilator (typically a PD5i) and escalated to combination treatment as required depending on clinical need(252). The findings of this study provide further support for the possibility of a disease-modifying effect on CKD for ERAs and are of particular relevance for SSc-CKD.

As in the ZEBRA trials, fluid weight gain and its associated side effects was observed in SONAR, even among those who tolerated the drug adequately during the run-in period. This has lead to increased recent research interest in investigating the combination of highly selective endothelin antagonists with other drug classes shown to be beneficial in chronic

kidney disease and which have diuretic properties so might thereby improve the tolerability.

One such study of zibotentan combined with a sodium-glucose cotransporter inhibitor (dapagliflozin) is currently in progress and builds on the findings of the ZEBRA studies(253).

## Conclusion

The work described in this thesis has made a significant contribution to the understanding of kidney disease in scleroderma and its management. Areas of particular interest that I have highlighted for future study include:

- The role of CTNND2 and Wnt signalling in renal scleroderma and the potential role of Wnt inhibitors in modulating this.
- The use of urine MCP-1 measurement as a clinical or research tool in diagnosis and longitudinal measurement of renal involvement in scleroderma.
- Selective endothelin A receptor antagonists as treatments for chronic kidney disease both from scleroderma and other causes.

# References

1. Agarwal SK, Tan FK, Arnett FC. Genetics and genomic studies in scleroderma (systemic sclerosis). *Rheum Dis Clin North Am*. 2008 Feb;34(1):17–40; v.
2. Feghali-Bostwick C, Medsger TA, Wright TM. Analysis of systemic sclerosis in twins reveals low concordance for disease and high concordance for the presence of antinuclear antibodies. *Arthritis Rheum*. 2003 Jul;48(7):1956–63.
3. Tan FK, Arnett FC. Genetic factors in the etiology of systemic sclerosis and Raynaud phenomenon. *Curr Opin Rheumatol*. 2000 Nov;12(6):511–9.
4. Tan FK. Systemic sclerosis: The susceptible host (genetics and environment). Vol. 29, *Rheumatic Disease Clinics of North America*. 2003. p. 211–37.
5. Morel PA, Chang HJ, Wilson JW, Conte C, Falkner D, Tweardy DJ, et al. HLA and ethnic associations among systemic sclerosis patients with anticentromere antibodies. *Hum Immunol*. 1995;42(1):35–42.
6. Reveille JD, Durban E, MacLeod-St Clair MJ, Goldstein R, Moreda R, Altman RD, et al. Association of amino acid sequences in the HLA-DQB1 first domain with antitopoisomerase I autoantibody response in scleroderma (progressive systemic sclerosis). *J Clin Invest*. 1992 Sep;90(3):973–80.
7. Gilchrist FC, Bunn C, Foley PJ, Lympny PA, Black CM, Welsh KI, et al. Class II HLA associations with autoantibodies in scleroderma: a highly significant role for HLA-DP. *Genes Immun*. 2001 Apr;2(2):76–81.
8. Fatini C, Gensini F, Sticchi E, Battaglini B, Angotti C, Conforti ML, et al. High prevalence of polymorphisms of angiotensin-converting enzyme (I/D) and endothelial nitric oxide

- synthase (Glu298Asp) in patients with systemic sclerosis. *Am J Med.* 2002 May;112(7):540–4.
9. Fonseca C, Renzoni E, Sestini P, Pantelidis P, Lagan A, Bunn C, et al. Endothelin axis polymorphisms in patients with scleroderma. *Arthritis Rheum.* 2006 Sep;54(9):3034–42.
  10. Fonseca C, Lindahl GE, Ponticos M, Sestini P, Renzoni EA, Holmes AM, et al. A polymorphism in the CTGF promoter region associated with systemic sclerosis. *N Engl J Med [Internet].* 2007 Sep 20 [cited 2014 Oct 2];357(12):1210–20. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/17881752>
  11. Tan FK, Stivers DN, Foster MW, Chakraborty R, Howard RF, Milewicz DM, et al. Association of microsatellite markers near the fibrillin 1 gene on human chromosome 15q with scleroderma in a Native American population. *Arthritis Rheum.* 1998 Oct;41(10):1729–37.
  12. Zhou X, Tan FK, Reveille JD, Wallis D, Milewicz DM, Ahn C, et al. Association of novel polymorphisms with the expression of SPARC in normal fibroblasts and with susceptibility to scleroderma. *Arthritis Rheum.* 2002 Nov;46(11):2990–9.
  13. Rueda B, Broen J, Simeon C, Hesselstrand R, Diaz B, Suárez H, et al. The STAT4 gene influences the genetic predisposition to systemic sclerosis phenotype. *Hum Mol Genet.* 2009 Jun 1;18(11):2071–7.
  14. Rueda B, Gourh P, Broen J, Agarwal SK, Simeon C, Ortego-Centeno N, et al. BANK1 functional variants are associated with susceptibility to diffuse systemic sclerosis in Caucasians. *Ann Rheum Dis.* 2010 Apr;69(4):700–5.

15. Dieudé P, Guedj M, Wipff J, Avouac J, Fajardy I, Diot E, et al. Association between the IRF5 rs2004640 functional polymorphism and systemic sclerosis: a new perspective for pulmonary fibrosis. *Arthritis Rheum.* 2009 Jan;60(1):225–33.
16. Dieudé P, Guedj M, Wipff J, Ruiz B, Hachulla E, Diot E, et al. STAT4 is a genetic risk factor for systemic sclerosis having additive effects with IRF5 on disease susceptibility and related pulmonary fibrosis. *Arthritis Rheum.* 2009 Aug;60(8):2472–9.
17. Radstake TRDJ, Gorlova O, Rueda B, Martin JE, Alizadeh BZ, Palomino-Morales R, et al. Genome-wide association study of systemic sclerosis identifies CD247 as a new susceptibility locus. *Nat Genet.* 2010 May;42(5):426–9.
18. Allanore Y, Saad M, Dieudé P, Avouac J, Distler JHW, Amouyel P, et al. Genome-wide scan identifies TNIP1, PSORS1C1, and RHOB as novel risk loci for systemic sclerosis. *PLoS Genet.* 2011 Jul;7(7):e1002091.
19. Gorman CL, Russell AI, Zhang Z, Cunninghame Graham D, Cope AP, Vyse TJ. Polymorphisms in the CD3Z gene influence TCRzeta expression in systemic lupus erythematosus patients and healthy controls. *J Immunol.* 2008 Jan 15;180(2):1060–70.
20. Gorlova O, Martin JE, Rueda B, Koeleman BPC, Ying J, Teruel M, et al. Identification of novel genetic markers associated with clinical phenotypes of systemic sclerosis through a genome-wide association strategy. *PLoS Genet.* 2011 Jul 14;7(7):e1002178.
21. Binns CH. Vinyl chloride: a review. *J Soc Occup Med.* 1979 Oct;29(4):134–41.
22. Black CM, Welsh KI, Walker AE, Bernstein RM, Catoggio LJ, McGregor AR, et al. Genetic susceptibility to scleroderma-like syndrome induced by vinyl chloride. *Lancet.* 1983 Jan 1;1(8314–5):53–5.

23. Black C, Pereira S, McWhirter A, Welsh K, Laurent R. Genetic susceptibility to scleroderma-like syndrome in symptomatic and asymptomatic workers exposed to vinyl chloride. *J Rheumatol.* 1986 Dec;13(6):1059–62.
24. Itoh M, Yanaba K, Kobayashi T, Nakagawa H. Taxane-induced scleroderma. *Br J Dermatol.* 2007 Feb;156(2):363–7.
25. Tabuenca JM. Toxic-allergic syndrome caused by ingestion of rapeseed oil denatured with aniline. *Lancet.* 1981 Sep 12;2(8246):567–8.
26. Silver RM, Heyes MP, Maize JC, Quearry B, Vionnet-Fuasset M, Sternberg EM. Scleroderma, fasciitis, and eosinophilia associated with the ingestion of tryptophan. *N Engl J Med.* 1990 Mar 29;322(13):874–81.
27. Nietert PJ, Sutherland SE, Silver RM, Pandey JP, Knapp RG, Hoel DG, et al. Is occupational organic solvent exposure a risk factor for scleroderma? *Arthritis Rheum.* 1998 Jun;41(6):1111–8.
28. Marie I, Gehanno JF, Bubenheim M, Duval-Modeste AB, Joly P, Dominique S, et al. Prospective study to evaluate the association between systemic sclerosis and occupational exposure and review of the literature. *Autoimmun Rev.* 2014 Feb;13(2):151–6.
29. Kettaneh A, Al Moufti O, Tiev KP, Chayet C, Tolédano C, Fabre B, et al. Occupational exposure to solvents and gender-related risk of systemic sclerosis: a metaanalysis of case-control studies. *J Rheumatol.* 2007 Jan;34(1):97–103.
30. Marie I, Gehanno JF, Bubenheim M, Duval-Modeste AB, Joly P, Dominique S, et al. Prospective study to evaluate the association between systemic sclerosis and occupational exposure and review of the literature. *Autoimmun Rev.* 2014 Feb;13(2):151–6.

31. Shi-Wen X, Panesar M, Vancheeswaran R, Mason J, Haskard D, Black C, et al. Expression and shedding of intercellular adhesion molecule 1 and lymphocyte function-associated antigen 3 by normal and scleroderma fibroblasts. Effects of interferon-gamma, tumor necrosis factor alpha, and estrogen. *Arthritis Rheum* [Internet]. 1994 Nov [cited 2015 Jan 26];37(11):1689–97. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/7526872>
32. Maul GG, Jimenez SA, Riggs E, Ziemnicka-Kotula D. Determination of an epitope of the diffuse systemic sclerosis marker antigen DNA topoisomerase I: sequence similarity with retroviral p30gag protein suggests a possible cause for autoimmunity in systemic sclerosis. *Proc Natl Acad Sci U S A*. 1989 Nov;86(21):8492–6.
33. Lunardi C, Bason C, Navone R, Millo E, Damonte G, Corrocher R, et al. Systemic sclerosis immunoglobulin G autoantibodies bind the human cytomegalovirus late protein UL94 and induce apoptosis in human endothelial cells. *Nat Med*. 2000 Oct;6(10):1183–6.
34. Shah AA, Rosen A, Hummers L, Wigley F, Casciola-Rosen L. Close temporal relationship between onset of cancer and scleroderma in patients with RNA polymerase I/III antibodies. *Arthritis Rheum*. 2010 Sep;62(9):2787–95.
35. Moinzadeh P, Fonseca C, Hellmich M, Shah A a, Chighizola C, Denton CP, et al. Association of anti-RNA polymerase III autoantibodies and cancer in scleroderma. *Arthritis Res Ther*. 2014 Feb 14;16(1):R53.
36. Joseph CG, Darrah E, Shah AA, Skora AD, Casciola-Rosen LA, Wigley FM, et al. Association of the autoimmune disease scleroderma with an immunologic response to cancer. *Science*. 2014 Jan 10;343(6167):152–7.



37. Systemic sclerosis: current pathogenetic concepts and future prospects for targeted therapy. *Lancet*. 1996 May 25;347(9013):1453–8.
38. Pearson JD. The endothelium: its role in scleroderma. *Ann Rheum Dis*. 1991 Nov;50 Suppl 4:866–71.
39. Flavahan NA, Flavahan S, Liu Q, Wu S, Tidmore W, Wiener CM, et al. Increased alpha2-adrenergic constriction of isolated arterioles in diffuse scleroderma. *Arthritis Rheum*. 2000 Aug;43(8):1886–90.
40. Levin ER. Endothelins. *N Engl J Med*. 1995 Aug 10;333(6):356–63.
41. Kahaleh MB. Endothelin, an endothelial-dependent vasoconstrictor in scleroderma. Enhanced production and profibrotic action. *Arthritis Rheum*. 1991 Aug;34(8):978–83.
42. Yamane K, Miyauchi T, Suzuki N, Yuhara T, Akama T, Suzuki H, et al. Significance of plasma endothelin-1 levels in patients with systemic sclerosis. *J Rheumatol*. 1992 Oct;19(10):1566–71.
43. Vancheeswaran R, Magoulas T, Efrat G, Wheeler-Jones C, Olsen I, Penny R, et al. Circulating endothelin-1 levels in systemic sclerosis subsets--a marker of fibrosis or vascular dysfunction? *J Rheumatol*. 1994 Oct;21(10):1838–44.
44. Kobayashi H, Nishimaki T, Kaise S, Suzuki T, Watanabe K, Kasukawa R. Immunohistological study endothelin-1 and endothelin-A and B receptors in two patients with scleroderma renal crisis. *Clin Rheumatol [Internet]*. 1999 Jan [cited 2014 Oct 7];18(5):425–7. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/10524561>
45. Penn H, Quillinan N, Khan K, Chakravarty K, Ong V, Burns A, et al. Targeting the endothelin axis in scleroderma renal crisis: rationale and feasibility. *QJM*. 2013 May 21;

46. Vancheeswaran R, Azam A, Black C, Dashwood MR. Localization of endothelin-1 and its binding sites in scleroderma skin. *J Rheumatol.* 1994 Jul;21(7):1268–76.
47. Rolla G, Colagrande P, Scappaticci E, Chiavassa G, Dutto L, Cannizzo S, et al. Exhaled nitric oxide in systemic sclerosis: relationships with lung involvement and pulmonary hypertension. *J Rheumatol.* 2000 Jul;27(7):1693–8.
48. Andersen GN, Caidahl K, Kazzam E, Petersson AS, Waldenström A, Mincheva-Nilsson L, et al. Correlation between increased nitric oxide production and markers of endothelial activation in systemic sclerosis: findings with the soluble adhesion molecules E-selectin, intercellular adhesion molecule 1, and vascular cell adhesion molecule 1. *Arthritis Rheum.* 2000 May;43(5):1085–93.
49. Ibba-Manneschi L, Niissalo S, Milia AF, Allanore Y, Del Rosso A, Pacini A, et al. Variations of neuronal nitric oxide synthase in systemic sclerosis skin. *Arthritis Rheum.* 2006 Jan;54(1):202–13.
50. Carvalho D, Savage CO, Black CM, Pearson JD. IgG antiendothelial cell autoantibodies from scleroderma patients induce leukocyte adhesion to human vascular endothelial cells in vitro. Induction of adhesion molecule expression and involvement of endothelium-derived cytokines. *J Clin Invest.* 1996 Jan 1;97(1):111–9.
51. Sgonc R, Gruschwitz MS, Boeck G, Sepp N, Gruber J, Wick G. Endothelial cell apoptosis in systemic sclerosis is induced by antibody-dependent cell-mediated cytotoxicity via CD95. *Arthritis Rheum.* 2000 Nov;43(11):2550–62.
52. Ahmed SS, Tan FK, Arnett FC, Jin L, Geng YJ. Induction of apoptosis and fibrillin 1 expression in human dermal endothelial cells by scleroderma sera containing anti-endothelial cell antibodies. *Arthritis Rheum.* 2006 Jul;54(7):2250–62.

53. Sprott H, Müller-Ladner U, Distler O, Gay RE, Barnum SR, Landthaler M, et al. Detection of activated complement complex C5b-9 and complement receptor C5a in skin biopsies of patients with systemic sclerosis (scleroderma). *J Rheumatol*. 2000 Feb;27(2):402–4.
54. Grote K, Flach I, Luchtefeld M, Akin E, Holland SM, Drexler H, et al. Mechanical stretch enhances mRNA expression and proenzyme release of matrix metalloproteinase-2 (MMP-2) via NAD(P)H oxidase-derived reactive oxygen species. *Circ Res*. 2003 Jun 13;92(11):e80-6.
55. Riccieri V, Spadaro A, Fuksa L, Firuzi O, Saso L, Valesini G. Specific oxidative stress parameters differently correlate with nailfold capillaroscopy changes and organ involvement in systemic sclerosis. *Clin Rheumatol*. 2008 Feb;27(2):225–30.
56. Kuwana M, Okazaki Y, Yasuoka H, Kawakami Y, Ikeda Y. Defective vasculogenesis in systemic sclerosis. *Lancet*. Jan;364(9434):603–10.
57. Mulligan-Kehoe MJ, Drinane MC, Mollmark J, Casciola-Rosen L, Hummers LK, Hall A, et al. Antiangiogenic plasma activity in patients with systemic sclerosis. *Arthritis Rheum*. 2007 Oct;56(10):3448–58.
58. Springer TA. Adhesion receptors of the immune system. *Nature*. 1990 Aug 2;346(6283):425–34.
59. Sato S. Abnormalities of adhesion molecules and chemokines in scleroderma. *Curr Opin Rheumatol*. 1999 Nov;11(6):503–7.
60. Gruschwitz M, von den Driesch P, Kellner I, Hornstein OP, Sterry W. Expression of adhesion proteins involved in cell-cell and cell-matrix interactions in the skin of patients with progressive systemic sclerosis. *J Am Acad Dermatol*. 1992 Aug;27(2 Pt 1):169–77.

61. Carson CW, Beall LD, Hunder GG, Johnson CM, Newman W. Serum ELAM-1 is increased in vasculitis, scleroderma, and systemic lupus erythematosus. *J Rheumatol*. 1993 May;20(5):809–14.
62. Sfrikakis PP, Tesar J, Baraf H, Lipnick R, Klipple G, Tsokos GC. Circulating intercellular adhesion molecule-1 in patients with systemic sclerosis. *Clin Immunol Immunopathol*. 1993 Jul;68(1):88–92.
63. Stratton RJ, Coghlan JG, Pearson JD, Burns A, Sweny P, Abraham DJ, et al. Different patterns of endothelial cell activation in renal and pulmonary vascular disease in scleroderma. *QJM [Internet]*. 1998 Aug [cited 2014 Oct 7];91(8):561–6. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/9893759>
64. Casciola-Rosen L, Wigley F, Rosen A. Scleroderma autoantigens are uniquely fragmented by metal-catalyzed oxidation reactions: implications for pathogenesis. *J Exp Med*. 1997 Jan 6;185(1):71–9.
65. Chizzolini C, Raschi E, Rezzonico R, Testoni C, Mallone R, Gabrielli A, et al. Autoantibodies to fibroblasts induce a proadhesive and proinflammatory fibroblast phenotype in patients with systemic sclerosis. *Arthritis Rheum*. 2002 Jun;46(6):1602–13.
66. Riemekasten G, Philippe A, Näther M, Slowinski T, Müller DN, Heidecke H, et al. Involvement of functional autoantibodies against vascular receptors in systemic sclerosis. *Ann Rheum Dis*. 2011 Mar;70(3):530–6.
67. Baroni SS, Santillo M, Bevilacqua F, Luchetti M, Spadoni T, Mancini M, et al. Stimulatory autoantibodies to the PDGF receptor in systemic sclerosis. *N Engl J Med*. 2006 Jun 22;354(25):2667–76.

68. Stummvoll GH, Aringer M, Grisar J, Steiner CW, Smolen JS, Knobler R, et al. Increased transendothelial migration of scleroderma lymphocytes. *Ann Rheum Dis*. 2004 May;63(5):569–74.
69. Roumm AD, Whiteside TL, Medsger TA, Rodnan GP. Lymphocytes in the skin of patients with progressive systemic sclerosis. Quantification, subtyping, and clinical correlations. *Arthritis Rheum*. 1984 Jun;27(6):645–53.
70. Brent J, McMartin K, Phillips S, Burkhardt KK, Donovan JW, Wells M, et al. Fomepizole for the treatment of ethylene glycol poisoning. Methylpyrazole for Toxic Alcohols Study Group. *N Engl J Med*. 1999 Mar 18;340(11):832–8.
71. Murata M, Fujimoto M, Matsushita T, Hamaguchi Y, Hasegawa M, Takehara K, et al. Clinical association of serum interleukin-17 levels in systemic sclerosis: is systemic sclerosis a Th17 disease? *J Dermatol Sci*. 2008 Jun;50(3):240–2.
72. Radstake TRDJ, van Bon L, Broen J, Hussiani A, Hesselstrand R, Wuttge DM, et al. The pronounced Th17 profile in systemic sclerosis (SSc) together with intracellular expression of TGFbeta and IFNgamma distinguishes SSc phenotypes. *PLoS One*. 2009 Jan 17;4(6):e5903.
73. Antiga E, Quaglino P, Bellandi S, Volpi W, Del Bianco E, Comessatti A, et al. Regulatory T cells in the skin lesions and blood of patients with systemic sclerosis and morphea. *Br J Dermatol*. 2010 May;162(5):1056–63.
74. York MR. Novel insights on the role of the innate immune system in systemic sclerosis. *Expert Rev Clin Immunol*. 2011 Jul;7(4):481–9.
75. Bhattacharyya S, Kelley K, Melichian DS, Tamaki Z, Fang F, Su Y, et al. Toll-like receptor 4 signaling augments transforming growth factor- $\beta$  responses: a novel

- mechanism for maintaining and amplifying fibrosis in scleroderma. *Am J Pathol.* 2013 Jan;182(1):192–205.
76. Abraham D, Lupoli S, McWhirter A, Plater-Zyberk C, Piela TH, Korn JH, et al. Expression and function of surface antigens on scleroderma fibroblasts. *Arthritis Rheum.* 1991 Sep;34(9):1164–72.
77. Higley H, Persichitte K, Chu S, Waegell W, Vancheeswaran R, Black C. Immunocytochemical localization and serologic detection of transforming growth factor beta 1. Association with type I procollagen and inflammatory cell markers in diffuse and limited systemic sclerosis, morphea, and Raynaud's phenomenon. *Arthritis Rheum.* 1994 Feb;37(2):278–88.
78. Corrin B, Butcher D, McAnulty BJ, Dubois RM, Black CM, Laurent GJ, et al. Immunohistochemical localization of transforming growth factor-beta 1 in the lungs of patients with systemic sclerosis, cryptogenic fibrosing alveolitis and other lung disorders. *Histopathology.* 1994 Feb;24(2):145–50.
79. Dziadzio M, Smith RE, Abraham DJ, Black CM, Denton CP. Circulating levels of active transforming growth factor beta1 are reduced in diffuse cutaneous systemic sclerosis and correlate inversely with the modified Rodnan skin score. *Rheumatology (Oxford).* 2005 Dec;44(12):1518–24.
80. Kubo M, Ihn H, Yamane K, Tamaki K. Up-regulated expression of transforming growth factor beta receptors in dermal fibroblasts in skin sections from patients with localized scleroderma. *Arthritis Rheum.* 2001 Mar;44(3):731–4.
81. McWhirter A, Colosetti P, Rubin K, Miyazono K, Black C. Collagen type I is not under autocrine control by transforming growth factor-beta 1 in normal and scleroderma fibroblasts. *Lab Invest.* 1994 Dec;71(6):885–94.

82. Yamakage A, Kikuchi K, Smith EA, LeRoy EC, Trojanowska M. Selective upregulation of platelet-derived growth factor alpha receptors by transforming growth factor beta in scleroderma fibroblasts. *J Exp Med*. 1992 May 1;175(5):1227–34.
83. Leask A, Holmes A, Abraham DJ. Connective tissue growth factor: a new and important player in the pathogenesis of fibrosis. *Curr Rheumatol Rep*. 2002 Apr;4(2):136–42.
84. Dziadzio M, Usinger W, Leask A, Abraham D, Black CM, Denton C, et al. N-terminal connective tissue growth factor is a marker of the fibrotic phenotype in scleroderma. *QJM*. 2005 Jul;98(7):485–92.
85. Shi-wen X, Stanton LA, Kennedy L, Pala D, Chen Y, Howat SL, et al. CCN2 is necessary for adhesive responses to transforming growth factor-beta1 in embryonic fibroblasts. *J Biol Chem*. 2006 Apr 21;281(16):10715–26.
86. Andersen MB, Pingel J, Kjær M, Langberg H. Interleukin-6: a growth factor stimulating collagen synthesis in human tendon. *J Appl Physiol (1985)*. 2011 Jun;110(6):1549–54.
87. Sato S, Hasegawa M, Takehara K. Serum levels of interleukin-6 and interleukin-10 correlate with total skin thickness score in patients with systemic sclerosis. *J Dermatol Sci*. 2001 Oct;27(2):140–6.
88. Salmon-Ehr V, Serpier H, Nawrocki B, Gillery P, Clavel C, Kalis B, et al. Expression of interleukin-4 in scleroderma skin specimens and scleroderma fibroblast cultures. Potential role in fibrosis. *Arch Dermatol*. 1996 Jul;132(7):802–6.
89. Needleman BW, Wigley FM, Stair RW. Interleukin-1, Interleukin-2, Interleukin-4, Interleukin-6, Tumor Necrosis Factor  $\alpha$ , and Interferon- $\gamma$  Levels in Sera from Patients With Scleroderma. *Arthritis Rheum*. 1992 Jan;35(1):67–72.

90. Distler JHW, Jünger A, Caretto D, Schulze-Horsel U, Kowal-Bielecka O, Gay RE, et al. Monocyte chemoattractant protein 1 released from glycosaminoglycans mediates its profibrotic effects in systemic sclerosis via the release of interleukin-4 from T cells. *Arthritis Rheum* [Internet]. 2006 Jan [cited 2015 Jan 26];54(1):214–25. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/16385517>
91. Aden N, Nuttall A, Shiwen X, de Winter P, Leask A, Black CM, et al. Epithelial cells promote fibroblast activation via IL-1alpha in systemic sclerosis. *J Invest Dermatol*. 2010 Sep;130(9):2191–200.
92. Romagnani P, Maggi L, Mazzinghi B, Cosmi L, Lasagni L, Liotta F, et al. CXCR3-mediated opposite effects of CXCL10 and CXCL4 on TH1 or TH2 cytokine production. *J Allergy Clin Immunol*. 2005 Dec;116(6):1372–9.
93. van Bon L, Affandi AJ, Broen J, Christmann RB, Marijnissen RJ, Stawski L, et al. Proteome-wide analysis and CXCL4 as a biomarker in systemic sclerosis. *N Engl J Med*. 2014 Jan 30;370(5):433–43.
94. Carulli MT, Ong VH, Ponticos M, Shiwen X, Abraham DJ, Black CM, et al. Chemokine receptor CCR2 expression by systemic sclerosis fibroblasts: evidence for autocrine regulation of myofibroblast differentiation. *Arthritis Rheum* [Internet]. 2005 Dec [cited 2015 Jan 26];52(12):3772–82. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/16320328>
95. Shi-Wen X, Denton CP, McWhirter A, Bou-Gharios G, Abraham DJ, du Bois RM, et al. Scleroderma lung fibroblasts exhibit elevated and dysregulated type I collagen biosynthesis. *Arthritis Rheum*. 1997 Jul;40(7):1237–44.



96. Kähäri VM, Sandberg M, Kalimo H, Vuorio T, Vuorio E. Identification of fibroblasts responsible for increased collagen production in localized scleroderma by in situ hybridization. *J Invest Dermatol*. 1988 May;90(5):664–70.
97. Scharffetter K, Lankat-Buttgereit B, Krieg T. Localization of collagen mRNA in normal and scleroderma skin by in-situ hybridization. *Eur J Clin Invest*. 1988 Feb;18(1):9–17.
98. Denton CP, Khan K, Hoyles RK, Shiwen X, Leoni P, Chen Y, et al. Inducible lineage-specific deletion of TbetaRII in fibroblasts defines a pivotal regulatory role during adult skin wound healing. *J Invest Dermatol*. 2009 Jan;129(1):194–204.
99. Hoyles RK, Derrett-Smith EC, Khan K, Shiwen X, Howat SL, Wells AU, et al. An essential role for resident fibroblasts in experimental lung fibrosis is defined by lineage-specific deletion of high-affinity type II transforming growth factor  $\beta$  receptor. *Am J Respir Crit Care Med*. 2011 Jan 15;183(2):249–61.
100. LeRoy EC, Mercurio S, Sherer GK. Replication and phenotypic expression of control and scleroderma human fibroblasts: responses to growth factors. *Proc Natl Acad Sci U S A*. 1982 Feb;79(4):1286–90.
101. Wang Y, Fan PS, Kahaleh B. Association between enhanced type I collagen expression and epigenetic repression of the FLI1 gene in scleroderma fibroblasts. *Arthritis Rheum*. 2006 Jul;54(7):2271–9.
102. Wallis DD, Tan FK, Kielty CM, Kimball MD, Arnett FC, Milewicz DM. Abnormalities in fibrillin 1-containing microfibrils in dermal fibroblast cultures from patients with systemic sclerosis (scleroderma). *Arthritis Rheum*. 2001 Aug;44(8):1855–64.
103. Lemaire R, Farina G, Kissin E, Shipley JM, Bona C, Korn JH, et al. Mutant fibrillin 1 from tight skin mice increases extracellular matrix incorporation of microfibril-associated glycoprotein 2 and type I collagen. *Arthritis Rheum*. 2004 Mar;50(3):915–26.

104. KINGDON EJ, BURNS A; HOSSEINI M, REYNOLDS TP, TAYLOR F, MENDONCA K, THOMAS M, POWIS SH, CHOUDHARY MR, BUNN CC, BLACK CM DC. Tubular and glomerular proteinuria as markers of occult renal involvement in systemic sclerosis. *Arthritis Rheum.* 2003;48:10(Supp.
105. Arnaud L, Huart A, Plaisier E, Francois H, Mougenot B, Tiev K, et al. ANCA-related crescentic glomerulonephritis in systemic sclerosis: revisiting the “normotensive scleroderma renal crisis”. *Clin Nephrol* [Internet]. 2007 Sep [cited 2014 Oct 7];68(3):165–70. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/17915619>
106. Derrett-Smith EC, Nihtyanova SI, Harvey J, Salama AD, Denton CP. Revisiting ANCA-associated vasculitis in systemic sclerosis: clinical, serological and immunogenetic factors. *Rheumatology (Oxford)* [Internet]. 2013 Oct 5 [cited 2015 Mar 10];52(10):1824–31. Available from: <http://rheumatology.oxfordjournals.org/content/early/2013/07/05/rheumatology.kest213.full>
107. Batal I, Domsic RT, Shafer A, Medsger TA, Kiss LP, Randhawa P, et al. Renal biopsy findings predicting outcome in scleroderma renal crisis. *Hum Pathol.* 2009;40(3):332–40.
108. Steen VD, Mayes MD, Merkel PA. Assessment of kidney involvement. *Clin Exp Rheumatol* [Internet]. 2003 Jan [cited 2014 Oct 7];21(3 Suppl 29):S29-31. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/12889219>
109. Butler EA, Baron M, Fogo AB, Frech T, Ghossein C, Hachulla E, et al. Generation of a Core Set of Items to Develop Classification Criteria for Scleroderma Renal Crisis Using Consensus Methodology. *Arthritis & Rheumatology* [Internet]. 2019 [cited 2019 Apr

- 12];0(0):1–8. Available from:  
<https://onlinelibrary.wiley.com/doi/pdf/10.1002/art.40809>
110. Hoa S, Stern EP, Denton CP, Hudson M, Scleroderma Clinical Trials Consortium Scleroderma Renal Crisis Working Group M, Investigators of the Scleroderma Clinical Trials Consortium Scleroderma Renal Crisis Working Group T, et al. Towards developing criteria for scleroderma renal crisis: A scoping review. *Autoimmun Rev* [Internet]. 2017;16(4):407–15. Available from:  
<http://linkinghub.elsevier.com/retrieve/pii/S1568997217300526>  
<http://www.ncbi.nlm.nih.gov/pubmed/28212921>
111. Mouthon L, Mehrenberger M, Teixeira L, Fakhouri F, Bérezné A, Guillevin L, et al. Endothelin-1 expression in scleroderma renal crisis. *Hum Pathol* [Internet]. 2011 Jan [cited 2014 Oct 7];42(1):95–102. Available from:  
<http://www.ncbi.nlm.nih.gov/pubmed/20971496>
112. Stone RA, Tisher CC, Hawkins HK, Robinson RR. Juxtaglomerular hyperplasia and hyperreninemia in progressive systemic sclerosis complicated acute renal failure. *Am J Med* [Internet]. 1974 Jan [cited 2014 Oct 7];56(1):119–23. Available from:  
<http://www.ncbi.nlm.nih.gov/pubmed/4129267>
113. Cannon PJ, Hassar M, Case DB, Casarella WJ, Sommers SC, LeRoy EC. The relationship of hypertension and renal failure in scleroderma (progressive systemic sclerosis) to structural and functional abnormalities of the renal cortical circulation. *Medicine* [Internet]. 1974 Jan [cited 2014 Oct 7];53(1):1–46. Available from:  
<http://www.ncbi.nlm.nih.gov/pubmed/4808710>
114. Steen VD, Medsger TA, Osial TA, Ziegler GL, Shapiro AP, Rodnan GP. Factors predicting development of renal involvement in progressive systemic sclerosis. *Am J*

- Med [Internet]. 1984 May [cited 2014 Oct 7];76(5):779–86. Available from:  
<http://www.ncbi.nlm.nih.gov/pubmed/6372452>
115. Steen VD. Autoantibodies in systemic sclerosis. *Semin Arthritis Rheum* [Internet]. 2005 Aug [cited 2014 Oct 7];35(1):35–42. Available from:  
<http://www.ncbi.nlm.nih.gov/pubmed/16084222>
116. Turk M, Pope JE. The frequency of scleroderma renal crisis over time: A metaanalysis. *Journal of Rheumatology*. 2016 Jul 1;43(7):1350–5.
117. Penn H, Howie a J, Kingdon EJ, Bunn CC, Stratton RJ, Black CM, et al. Scleroderma renal crisis: patient characteristics and long-term outcomes. *QJM* [Internet]. 2007 Aug [cited 2014 Sep 25];100(8):485–94. Available from:  
<http://www.ncbi.nlm.nih.gov/pubmed/17601770>
118. Traub YM, Shapiro AP, Rodnan GP, Medsger TA, McDonald RH, Steen VD, et al. Hypertension and renal failure (scleroderma renal crisis) in progressive systemic sclerosis. Review of a 25-year experience with 68 cases. *Medicine* [Internet]. 1983 Nov [cited 2014 Oct 6];62(6):335–52. Available from:  
<http://www.ncbi.nlm.nih.gov/pubmed/6355755>
119. Morgan ND, Shah AA, Mayes MD, Domsic RT, Medsger TA, Steen VD, et al. Clinical and serological features of systemic sclerosis in a multicenter African American cohort: Analysis of the genome research in African American scleroderma patients clinical database. *Medicine (United States)*. 2017 Dec 1;96(51).
120. Steen VD, Costantino JP, Shapiro AP, Medsger TA. Outcome of renal crisis in systemic sclerosis: relation to availability of angiotensin converting enzyme (ACE) inhibitors. *Ann Intern Med* [Internet]. 1990 Sep 1 [cited 2014 Oct 6];113(5):352–7. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/2382917>

121. LeRoy EC, Black C, Fleischmajer R, Jablonska S, Krieg T, Medsger TA, et al. Scleroderma (systemic sclerosis): classification, subsets and pathogenesis. *J Rheumatol* [Internet]. 1988 Feb [cited 2014 Oct 7];15(2):202–5. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/3361530>
122. Steen VD, Medsger TA. The palpable tendon friction rub: an important physical examination finding in patients with systemic sclerosis. *Arthritis Rheum* [Internet]. 1997 Jun [cited 2014 Oct 7];40(6):1146–51. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/9182926>
123. Okano Y, Steen VD, Medsger TA. Autoantibody reactive with RNA polymerase III in systemic sclerosis. *Ann Intern Med* [Internet]. 1993 Nov 15 [cited 2014 Oct 7];119(10):1005–13. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/8214977>
124. Bunn CC, Denton CP, Shi-Wen X, Knight C, Black CM. Anti-RNA polymerases and other autoantibody specificities in systemic sclerosis. *Br J Rheumatol* [Internet]. 1998 Jan [cited 2014 Oct 7];37(1):15–20. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/9487245>
125. Steen VD, Medsger TA. Epidemiology and natural history of systemic sclerosis. *Rheum Dis Clin North Am* [Internet]. 1990 Feb [cited 2014 Oct 7];16(1):1–10. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/2406801>
126. Steen VD, Ziegler GL, Rodnan GP, Medsger TA. Clinical and laboratory associations of anticentromere antibody in patients with progressive systemic sclerosis. *Arthritis Rheum* [Internet]. 1984 Feb [cited 2014 Oct 7];27(2):125–31. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/6607734>
127. Lynch BM, Stern EP, Ong V, Harber M, Burns A, Denton CP. UK Scleroderma Study Group (UKSSG) guidelines on the diagnosis and management of scleroderma renal

- crisis. *Clin Exp Rheumatol* [Internet]. 2016 [cited 2016 Oct 25];(5):106–9. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/27749244>
128. LUNSETH JH, BAKER LA, SHIFRIN A. Chronic scleroderma with acute exacerbation during corticotropin therapy; report of a case with autopsy observations. *AMA Arch Intern Med* [Internet]. 1951 Dec [cited 2014 Oct 7];88(6):783–92. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/14867982>
129. SHARNOFF JG, CARIDEO HL, STEIN ID. Cortisone-treated scleroderma; report of a case with autopsy findings. *J Am Med Assoc* [Internet]. 1951 Apr 21 [cited 2014 Oct 7];145(16):1230–2. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/14813909>
130. Trang G, Steele R, Baron M, Hudson M. Corticosteroids and the risk of scleroderma renal crisis: a systematic review. *Rheumatol Int* [Internet]. 2012 Mar [cited 2014 Sep 25];32(3):645–53. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/21132302>
131. Steen VD, Medsger TA. Case-control study of corticosteroids and other drugs that either precipitate or protect from the development of scleroderma renal crisis. *Arthritis Rheum* [Internet]. 1998 Sep [cited 2014 Oct 7];41(9):1613–9. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/9751093>
132. Guillevin L, Bérezné A, Seror R, Teixeira L, Pourrat J, Mahr A, et al. Scleroderma renal crisis: a retrospective multicentre study on 91 patients and 427 controls. *Rheumatology (Oxford)* [Internet]. 2012 Mar [cited 2014 Sep 25];51(3):460–7. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/22087012>
133. Bütikofer L, Varisco PA, Distler O, Kowal-Bielecka O, Allanore Y, Riemekasten G, et al. ACE inhibitors in SSc patients display a risk factor for scleroderma renal crisis - A EUSTAR analysis. *Arthritis Res Ther*. 2020 Mar 24;22(1).

134. Hudson M, Baron M, Tatibouet S, Furst DE, Khanna D. Exposure to ACE inhibitors prior to the onset of scleroderma renal crisis—results from the International Scleroderma Renal Crisis Survey. *Semin Arthritis Rheum* [Internet]. 2014 Apr [cited 2014 Sep 25];43(5):666–72. Available from:  
<http://www.sciencedirect.com/science/article/pii/S0049017213002047>
135. Penn H, Denton CP. Diagnosis, management and prevention of scleroderma renal disease. *Curr Opin Rheumatol* [Internet]. 2008 Nov [cited 2014 Sep 25];20(6):692–6. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/18946330>
136. KDIGO. Clinical Practice Guideline for Acute Kidney Injury (AKI). *Kidney Int Suppl* (2011) [Internet]. 2012 Mar [cited 2014 Oct 7];2(1):4. Available from:  
<http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4089764&tool=pmcentrez&rendertype=abstract>
137. Yusin J, Lewin K, Clements P. Thrombotic thrombocytopenia purpura in a patient with systemic sclerosis. *J Clin Rheumatol* [Internet]. 2001 Apr [cited 2014 Oct 7];7(2):106–11. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/17039106>
138. Kapur A, Ballou SP, Renston JP, Luna E, Chung-Park M. Recurrent acute scleroderma renal crisis complicated by thrombotic thrombocytopenic purpura. *J Rheumatol* [Internet]. 1997 Dec [cited 2014 Oct 7];24(12):2469–72. Available from:  
<http://www.ncbi.nlm.nih.gov/pubmed/9415660>
139. Manadan AM, Harris C, Block JA. Thrombotic thrombocytopenic purpura in the setting of systemic sclerosis. *Semin Arthritis Rheum* [Internet]. 2005 Feb [cited 2014 Oct 7];34(4):683–8. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/15692962>
140. Abudiab M, Krause ML, Fidler ME, Nath KA, Norby SM. Differentiating scleroderma renal crisis from other causes of thrombotic microangiopathy in a postpartum

- patient. Clin Nephrol [Internet]. 2013 Oct [cited 2014 Oct 6];80(4):293–7. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/22579274>
141. Lian ECY. Pathogenesis of thrombotic thrombocytopenic purpura: ADAMTS13 deficiency and beyond. Semin Thromb Hemost [Internet]. 2005 Dec [cited 2014 Oct 7];31(6):625–32. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/16388413>
142. Stern EP, Steen VD, Denton CP. Management of Renal Involvement in Scleroderma. Curr Treatm Opt Rheumatol [Internet]. 2015 Jan 8 [cited 2015 Jan 11];1–13. Available from: <http://link.springer.com/10.1007/s40674-014-0004-1>
143. Kowal-Bielecka O, Landewé R, Avouac J, Chwiesko S, Miniati I, Czirjak L, et al. EULAR recommendations for the treatment of systemic sclerosis: a report from the EULAR Scleroderma Trials and Research group (EUSTAR). Ann Rheum Dis [Internet]. 2009 May [cited 2014 Oct 1];68(5):620–8. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/19147617>
144. Caskey FJ, Thacker EJ, Johnston PA, Barnes JN. Failure of losartan to control blood pressure in scleroderma renal crisis. Lancet [Internet]. 1997 Mar 1 [cited 2014 Oct 7];349(9052):620. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/9057740>
145. Cheung WY, Gibson IW, Rush D, Jeffery J, Karpinski M. Late recurrence of scleroderma renal crisis in a renal transplant recipient despite angiotensin II blockade. Am J Kidney Dis [Internet]. 2005 May [cited 2014 Oct 7];45(5):930–4. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/15861360>
146. McAlister FA, Zhang J, Tonelli M, Klarenbach S, Manns BJ, Hemmelgarn BR. The safety of combining angiotensin-converting-enzyme inhibitors with angiotensin-receptor blockers in elderly patients: a population-based longitudinal analysis. CMAJ :



- Canadian Medical Association Journal [Internet]. 2011 Apr 4 [cited 2022 Jun 7];183(6):655. Available from: [/pmc/articles/PMC3071385/](#)
147. Izzedine H, Rouvier P, Deray G. Endothelin Receptor Antagonism-Based Treatment for Scleroderma Renal Crisis. *Am J Kidney Dis*. 2013 Jun 12;
148. Wipff J, Gallier G, Dieude P, Avouac J, Tiev K, Hachulla E, et al. Angiotensin-converting enzyme gene does not contribute to genetic susceptibility to systemic sclerosis in European Caucasians. *J Rheumatol* [Internet]. 2009 Feb [cited 2014 Nov 24];36(2):337–40. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/19132786>
149. Berezne A, Abdoul H, Karras A, Bienvenu B, Imbert B, Marie I, et al. ScS REINBO : évaluation de l'efficacité du bosentan au cours de la crise rénale sclérodermique (CRS) en adjonction au traitement de référence comprenant un IEC ± autres anti-hypertenseurs. *Rev Med Interne* [Internet]. 2017 Jun [cited 2019 Sep 25];38:A76–7. Available from: <https://linkinghub.elsevier.com/retrieve/pii/S0248866317301583>
150. Scorza R, Rivolta R, Mascagni B, Berruti V, Bazzi S, Castagnone D, et al. Effect of iloprost infusion on the resistance index of renal vessels of patients with systemic sclerosis. *J Rheumatol* [Internet]. 1997 Oct [cited 2014 Oct 3];24(10):1944–8. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/9330936>
151. Steen VD. Long-Term Outcomes of Scleroderma Renal Crisis. *Ann Intern Med* [Internet]. 2000 Oct 17 [cited 2014 Oct 3];133(8):600. Available from: <http://annals.org/article.aspx?articleid=713931>
152. MOIST LM, PORT FK, ORZOL SM, YOUNG EW, OSTBYE T, WOLFE RA, et al. Predictors of Loss of Residual Renal Function among New Dialysis Patients. *J Am Soc Nephrol* [Internet]. 2000 Mar 1 [cited 2014 Oct 6];11(3):556–64. Available from:

[http://jasn.asnjournals.org/content/11/3/556.abstract?ijkey=eee49c593dd629fea752847fec2f94e2b3bf4357&keytype2=tf\\_ipsecsha](http://jasn.asnjournals.org/content/11/3/556.abstract?ijkey=eee49c593dd629fea752847fec2f94e2b3bf4357&keytype2=tf_ipsecsha)

153. Jansen MAM, Hart AAM, Korevaar JC, Dekker FW, Boeschoten EW, Krediet RT. Predictors of the rate of decline of residual renal function in incident dialysis patients. *Kidney Int* [Internet]. 2002 Sep [cited 2014 Oct 6];62(3):1046–53. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/12164889>
154. Liu X, Dai C. Advances in Understanding and Management of Residual Renal Function in Patients with Chronic Kidney Disease. *Kidney Diseases* [Internet]. 2016 [cited 2022 Jun 7];2(4):187–96. Available from: <https://www.karger.com/Article/FullText/449029>
155. Siva B, McDonald SP, Hawley CM, Rosman JB, Brown FG, Wiggins KJ, et al. End-stage kidney disease due to scleroderma--outcomes in 127 consecutive ANZDATA registry cases. *Nephrol Dial Transplant* [Internet]. 2011 Oct 1 [cited 2014 Oct 6];26(10):3165–71. Available from: <http://ndt.oxfordjournals.org/content/26/10/3165.long>
156. Denton CP, Sweny P, Abdulla A, Black CM. Acute renal failure occurring in scleroderma treated with cyclosporin A: a report of three cases. *Br J Rheumatol* [Internet]. 1994 Jan [cited 2014 Oct 7];33(1):90–2. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/8162467>
157. Nunokawa T, Akazawa M, Yokogawa N, Shimada K, Hiramatsu K, Nishio Y, et al. Late-onset scleroderma renal crisis induced by tacrolimus and prednisolone: a case report. *Am J Ther* [Internet]. [cited 2014 Oct 7];21(5):e130-3. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/22836123>
158. Pham PTT, Pham PCT, Danovitch GM, Gritsch HA, Singer J, Wallace WD, et al. Predictors and risk factors for recurrent scleroderma renal crisis in the kidney allograft: case report and review of the literature. *Am J Transplant* [Internet]. 2005

- Oct [cited 2014 Oct 3];5(10):2565–9. Available from:  
<http://www.ncbi.nlm.nih.gov/pubmed/16162209>
159. Bertrand D, Dehay J, Ott J, Sberro R, Brunelle C, Kamar N, et al. Kidney transplantation in patients with systemic sclerosis: a nationwide multicentre study. *Transplant International* [Internet]. 2017 Mar 1 [cited 2022 Jun 7];30(3):256–65. Available from:  
<https://onlinelibrary.wiley.com/doi/full/10.1111/tri.12923>
160. Gibney EM, Parikh CR, Jani A, Fischer MJ, Collier D, Wiseman AC. Kidney transplantation for systemic sclerosis improves survival and may modulate disease activity. *Am J Transplant* [Internet]. 2004 Dec [cited 2014 Oct 6];4(12):2027–31. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/15575905>
161. Hruskova Z, Pippias M, Stel VS, Abad-Díez JM, Benítez Sánchez M, Caskey FJ, et al. Characteristics and Outcomes of Patients With Systemic Sclerosis (Scleroderma) Requiring Renal Replacement Therapy in Europe: Results From the ERA-EDTA Registry. *American Journal of Kidney Diseases* [Internet]. 2019 Feb 1 [cited 2022 Jun 7];73(2):184–93. Available from:  
<http://www.ajkd.org/article/S0272638618307686/fulltext>
162. Teixeira L, Mouthon L, Mahr A, Berezné A, Agard C, Mehrenberger M, et al. Mortality and risk factors of scleroderma renal crisis: a French retrospective study of 50 patients. *Ann Rheum Dis* [Internet]. 2008 Jan [cited 2014 Oct 7];67(1):110–6. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/17557890>
163. Walker JG, Ahern MJ, Smith MD, Coleman M, Pile K, Rischmueller M, et al. Scleroderma renal crisis: poor outcome despite aggressive antihypertensive treatment. *Intern Med J* [Internet]. [cited 2014 Oct 7];33(5–6):216–20. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/12752889>

164. Kim H, Lefebvre F, Hoa S, Hudson M. Mortality and morbidity in scleroderma renal crisis: A systematic literature review. *J Scleroderma Relat Disord*. 2020;
165. Abbott KC, Trespalacios FC, Welch PG, Agodoa LYC. Scleroderma at end stage renal disease in the United States: patient characteristics and survival. *J Nephrol* [Internet]. [cited 2014 Oct 7];15(3):236–40. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/12113593>
166. Chang YJ, Spiera H. Renal transplantation in scleroderma. *Medicine* [Internet]. 1999 Nov [cited 2014 Oct 3];78(6):382–5. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/10575420>
167. Denton CP, Khanna D. Systemic sclerosis. [www.thelancet.com](http://www.thelancet.com). 2017;
168. Morelli S, Barbieri C, Sgreccia A, Ferrante L, Pittoni V, Conti F, et al. Relationship between cutaneous and pulmonary involvement in systemic sclerosis. *J Rheumatol*. 1997 Jan;24(1):81–5.
169. Walker UA, Tyndall A, Czirjak L, Denton C, Farge-Bancel D, Kowal-Bielecka O, et al. Clinical risk assessment of organ manifestations in systemic sclerosis: a report from the EULAR Scleroderma Trials And Research group database. *Ann Rheum Dis*. 2007 Jan 12;66(6):754–63.
170. Nguyen B, Assassi S, Arnett FC, Mayes MD. Association of RNA polymerase III antibodies with scleroderma renal crisis. *J Rheumatol*. 2010 May;37(5):1068; author reply 1069.
171. Nguyen B, Mayes MD, Arnett FC, del Junco D, Reveille JD, Gonzalez EB, et al. HLA-DRB1\*0407 and \*1304 are risk factors for scleroderma renal crisis. *Arthritis Rheum*. 2011 Feb;63(2):530–4.

172. van den Hoogen F, Khanna D, Fransen J, Johnson SR, Baron M, Tyndall A, et al. 2013 classification criteria for systemic sclerosis: an American College of Rheumatology/European League against Rheumatism collaborative initiative. *Arthritis Rheum*. 2013 Nov;65(11):2737–47.
173. Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira MAR, Bender D, et al. PLINK: A Tool Set for Whole-Genome Association and Population-Based Linkage Analyses. *The American Journal of Human Genetics*. 2007 Sep;81(3):559–75.
174. R Development Core Team. *R: A Language and Environment for Statistical Computing*. Vienna, Austria; 2017.
175. Shen GQ, Abdullah KG, Wang QK. The TaqMan method for SNP genotyping. *Methods Mol Biol [Internet]*. 2009 [cited 2022 Jun 7];578:293–306. Available from: [https://link.springer.com/protocol/10.1007/978-1-60327-411-1\\_19](https://link.springer.com/protocol/10.1007/978-1-60327-411-1_19)
176. Levy D, Larson MG, Benjamin EJ, Newton-Cheh C, Wang TJ, Hwang SJ, et al. Framingham Heart Study 100K Project: genome-wide associations for blood pressure and arterial stiffness. *BMC Med Genet [Internet]*. 2007 Sep 19 [cited 2017 Oct 10];8 Suppl 1(Suppl 1):S3. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/17903302>
177. Warren HR, Evangelou E, Cabrera CP, Gao H, Ren M, Mifsud B, et al. Genome-wide association analysis identifies novel blood pressure loci and offers biological insights into cardiovascular risk. *Nat Genet*. 2017 Mar;49(3):403–15.
178. Gao L, Emond MJ, Louie T, Cheadle C, Berger AE, Rafaels N, et al. Identification of Rare Variants in *ATP8B4* as a Risk Factor for Systemic Sclerosis by Whole-Exome Sequencing. *Arthritis & Rheumatology*. 2016 Jan;68(1):191–200.

179. Nihtyanova SI, Schreiber BE, Ong VH, Rosenberg D, Moinzadeh P, Coghlan JG, et al. Prediction of pulmonary complications and long term survival in systemic sclerosis. *Arthritis Rheumatol.* 2014 Mar 3;66(6):1625–35.
180. Kuwana M, Pandey JP, Silver RM, Kawakami Y, Kaburaki J. HLA class II alleles in systemic sclerosis patients with anti-RNA polymerase I/III antibody: associations with subunit reactivities. *J Rheumatol.* 2003 Nov 1;30(11):2392–7.
181. Matzaraki V, Kumar V, Wijmenga C, Zhernakova A. The MHC locus and genetic susceptibility to autoimmune and infectious diseases. *Genome Biol.* 2017;18(1):76.
182. Fadista J, Manning AK, Florez JC, Groop L. The (in)famous GWAS P-value threshold revisited and updated for low-frequency variants. *European Journal of Human Genetics.* 2016 Aug 6;24(8):1202–5.
183. Butler EA, Baron M, Fogo AB, Frech T, Ghossein C, Hachulla E, et al. Generation of a core set of items to develop classification criteria for Scleroderma renal crisis using consensus methodology. *Arthritis & Rheumatology.* 2019 Jan 7;
184. Gigante A, Hoffmann-Vold AM, Alunni Fegatelli D, Gabrielli A, Leodori G, Coleiro B, et al. Estimated glomerular filtration rate is a marker of mortality in the European Scleroderma Trials and Research Group (EUSTAR) database. *Rheumatology (Oxford).* 2021;61(1).
185. Brück K, Stel VS, Gambaro G, Hallan S, Völzke H, Ärnlöv J, et al. CKD prevalence varies across the European general population. *Journal of the American Society of Nephrology [Internet].* 2016 Jul 1 [cited 2022 Jun 7];27(7):2135–47. Available from: <https://jasn.asnjournals.org/content/27/7/2135>

186. Lopez-Giacoman S, Madero M. Biomarkers in chronic kidney disease, from kidney function to kidney damage. *World J Nephrol* [Internet]. 2015 Feb 2 [cited 2022 Jun 12];4(1):57. Available from: [/pmc/articles/PMC4317628/](#)
187. Rice LM, Ziemek J, Stratton EA, Mclaughlin SR, Padilla CM, Mathes AL, et al. A longitudinal biomarker for the extent of skin disease in patients with diffuse cutaneous systemic sclerosis. *Arthritis Rheumatol* Nov; 2015;67:11.
188. Skaug B, Assassi S. Biomarkers in systemic sclerosis. *Curr Opin Rheumatol* Nov; 2019;31(6):595–602.
189. Abignano G, Blagojevic J, Bissell LA, Dumitru RB, Eng S, Allanore Y, et al. European multicentre study validates elf test as biomarker of fibrosis in systemic sclerosis. In: Poster Presentations [Internet]. {BMJ} Publishing Group Ltd and European League Against Rheumatism; 2017. Available from: <https://doi.org/10.1136/annrheumdis-2017-eular.3236>
190. Galdo F del, Shaw MA, Jimenez SA. Proteomic Analysis Identification of a Pattern of Shared Alterations in the Secretome of Dermal Fibroblasts from Systemic Sclerosis and Nephrogenic Systemic Fibrosis. *Am J Pathol* [Internet]. 2010 Oct;177(4):1638–46. Available from: <https://doi.org/10.2353/ajpath.2010.091095>
191. McMahon GM, Waikar SS. Biomarkers in Nephrology: Core Curriculum 2013. *American Journal of Kidney Diseases* [Internet]. 2013 Jul;62(1):165–78. Available from: <https://doi.org/10.1053/ajkd.2012.12.022>
192. BRUGOS, B., VINCZE, Z., SIPKA, S., SZEGEDI, G. & ZEHER M. Serum and urinary cytokine levels of SLE patients. *Pharmazie*. 2012;67:411–3.
193. Melnikov VY, Ecder T, Fantuzzi G, Siegmund B, Lucia MS, Dinarello CA, et al. Impaired IL-18 processing protects caspase-1-deficient mice from ischemic acute

- renal failure. *Journal of Clinical Investigation* [Internet]. 2001 May;107(9):1145–52. Available from: <https://doi.org/10.1172%2Fjci12089>
194. Parikh CR, Mishra J, Thiessen-Philbrook H, Dursun B, Ma Q, Kelly C, et al. Urinary IL-18 is an early predictive biomarker of acute kidney injury after cardiac surgery. *Kidney Int* [Internet]. 2006 Jul 17 [cited 2015 Dec 18];70(1):199–203. Available from: <http://dx.doi.org/10.1038/sj.ki.5001527>
195. Vanmassenhove J, Vanholder R, Nagler E, Biesen W van. Urinary and serum biomarkers for the diagnosis of acute kidney injury: an in-depth review of the literature. *Nephrology Dialysis Transplantation* [Internet]. 2012 Oct;28(2):254–73. Available from: <https://doi.org/10.1093%2Fndt%2Fgfs380>
196. Kalantarinia K, Awad AS, Siragy HM. Urinary and renal interstitial concentrations of TNF-alpha increase prior to the rise in albuminuria in diabetic rats. *Kidney Int* [Internet]. 2003 Oct [cited 2016 Jan 12];64(4):1208–13. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/12969138>
197. Maurer B, Distler A, Suliman YA, Gay RE, Michel BA, Gay S, et al. Vascular endothelial growth factor aggravates fibrosis and vasculopathy in experimental models of systemic sclerosis. *Ann Rheum Dis* [Internet]. 2013 Aug 5 [cited 2016 Jan 12];73(10):1880–7. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/23918036>
198. Kitamoto Y, Matsuo K, Tomita K. Different response of urinary excretion of VEGF in patients with chronic and acute renal failure. *Kidney Int* [Internet]. 2001 Jan [cited 2016 Jan 12];59(1):385–6. Available from: <http://dx.doi.org/10.1046/j.1523-1755.2001.00504.x>
199. Carulli MT, Handler C, Coghlan JG, Black CM, Denton CP. Can CCL2 serum levels be used in risk stratification or to monitor treatment response in systemic sclerosis? *Ann*



- Rheum Dis [Internet]. 2008 Jan 1 [cited 2015 Oct 27];67(1):105–9. Available from:  
<http://ard.bmj.com/content/67/1/105.long>
200. Barbado J, Martin D, Vega L, Almansa R, Gonçalves L, Nocito M, et al. MCP-1 in urine as biomarker of disease activity in Systemic Lupus Erythematosus. *Cytokine* [Internet]. 2012 Nov [cited 2016 Jan 12];60(2):583–6. Available from:  
<http://www.sciencedirect.com/science/article/pii/S1043466612005716>
201. Singh RG, Usha, Rathore SS, Behura SK, Singh NK. Urinary MCP-1 as diagnostic and prognostic marker in patients with lupus nephritis flare. *Lupus* [Internet]. 2012 Oct [cited 2016 Jan 12];21(11):1214–8. Available from:  
<http://www.ncbi.nlm.nih.gov/pubmed/22759858>
202. Denton CP, Bickerstaff MC, Shiwen X, Carulli MT, Haskard DO, Dubois RM, et al. Serial circulating adhesion molecule levels reflect disease severity in systemic sclerosis. *Br J Rheumatol* Nov; 1995;34:11.
203. van den Hoogen F, Khanna D, Fransen J, Johnson SR, Baron M, Tyndall A, et al. 2013 Classification Criteria for Systemic Sclerosis: An American College of Rheumatology/European League Against Rheumatism Collaborative Initiative. *Arthritis Rheum* [Internet]. 2013 Nov 1 [cited 2020 Jul 29];65(11):2737–47. Available from: <http://doi.wiley.com/10.1002/art.38098>
204. Levey AS, Coresh J, Greene T, Stevens LA, Zhang YL, Hendriksen S, et al. Using standardized serum creatinine values in the modification of diet in renal disease study equation for estimating glomerular filtration rate. *Ann Intern Med* [Internet]. 2006 Aug 15 [cited 2020 Jul 29];145(4):247–54. Available from:  
<http://www.ncbi.nlm.nih.gov/pubmed/16908915>

205. K/DOQI clinical practice guidelines for chronic kidney disease: evaluation, classification, and stratification. *Am J Kidney Dis* [Internet]. 2002 Feb [cited 2014 Dec 10];39(2 Suppl 1):S1-266. Available from:  
<http://www.ncbi.nlm.nih.gov/pubmed/11904577>
206. STERN EP, HONG, C, ONG VH, BURNS A, UNWIN R DC. Monocyte Chemoattractant Protein-1 (MCP-1, CCL2) Is a Potential Local Marker of Renal Involvement in Scleroderma. *Arthritis Rheumatol*. 2015;67 (Suppl).
207. Wu M, Baron M, Pedroza C, Salazar GA, Ying J, Charles J, et al. {CCL}2 in the Circulation Predicts Long-Term Progression of Interstitial Lung Disease in Patients With Early Systemic Sclerosis: Data From Two Independent Cohorts. *Arthritis & Rheumatology* [Internet]. 2017 Aug;69(9):1871–8. Available from:  
<https://doi.org/10.1002%2Fart.40171>
208. Lauretis A de, Sestini P, Pantelidis P, Hoyles R, Hansell DM, Goh NSL, et al. Serum Interleukin 6 Is Predictive of Early Functional Decline and Mortality in Interstitial Lung Disease Associated with Systemic Sclerosis. *J Rheumatol* [Internet]. 2013 Feb;40(4):435–46. Available from: <https://doi.org/10.3899%2Fjrheum.120725>
209. Sfrikakis PP, Papamichael C, Stamatelopoulos KS, Tousoulis D, Fragiadaki KG, Katsichti P, et al. Improvement of vascular endothelial function using the oral endothelin receptor antagonist bosentan in patients with systemic sclerosis. *Arthritis Rheum* Jun; 2007;56(6):1985–93.
210. Howe HS, Kong KO, Thong BY, Law WG, Chia FL, Lian TY, et al. Urine sVCAM-1 and and sICAM-1 levels are elevated in lupus nephritis. *Int J Rheum Dis* Feb; 2012;15(1):13–6.
211. Liu RNG, Cheng QY, Zhou HY, Li BZ, Ye DQ. Elevated Blood and Urinary {ICAM}-1 is a Biomarker for Systemic Lupus Erythematosus: A Systematic Review and Meta-

- Analysis. *Immunol Invest* [Internet]. 2019 Jul;49(1–2):15–31. Available from:  
<https://doi.org/10.1080%2F08820139.2019.1624769>
212. Sandmeier B, Jäger VK, Nagy G, Carreira PE, Tzankov A, Widuchowska M, et al. Autopsy versus clinical findings in patients with systemic sclerosis in a case series from patients of the EUSTAR database. *Clin Exp Rheumatol*. 2015;33:75–9.
213. Trostle DC, Bedetti CD, Steen VD, Al-Sabbagh MR, Zee B, Medsger TA. Renal vascular histology and morphometry in systemic sclerosis. a case–control autopsy study. *Arthritis Rheum* [Internet]. 1988 Mar 1 [cited 2022 Jun 11];31(3):393–400. Available from: <https://onlinelibrary.wiley.com/doi/full/10.1002/art.1780310311>
214. Pulido T, Adzerikho I, Channick RN, Delcroix M, Galiè N, Ghofrani HA, et al. Macitentan and Morbidity and Mortality in Pulmonary Arterial Hypertension. *New England Journal of Medicine* [Internet]. 2013 Aug 29 [cited 2022 Jun 7];369(9):809–18. Available from: <https://www.nejm.org/doi/10.1056/NEJMoa1213917>
215. Davenport AP. Pharmacology of Renal Endothelin Receptors. 2011;172:1–17.
216. Iglarz M, Binkert C, Morrison K, Fischli W, Gatfield J, Treiber A, et al. Pharmacology of Macitentan, an Orally Active Tissue-Targeting Dual Endothelin Receptor Antagonist. *Journal of Pharmacology and Experimental Therapeutics* [Internet]. 2008 Dec 1 [cited 2022 Jun 11];327(3):736–45. Available from:  
<https://jpet.aspetjournals.org/content/327/3/736>
217. Maguire JJ, Davenport AP. Endothelin Receptors and Their Antagonists. *Semin Nephrol* [Internet]. 2015 Mar [cited 2015 Oct 12];35(2):125–36. Available from:  
<http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4437774&tool=pmcentr ez&rendertype=abstract>

218. Tomkinson H, Kemp J, Oliver S, Swaisland H, Taboada M, Morris T. Pharmacokinetics and tolerability of zibotentan (ZD4054) in subjects with hepatic or renal impairment: two open-label comparative studies. *BMC Clin Pharmacol* [Internet]. 2011 Mar 17 [cited 2022 Jun 7];11:3. Available from: [/pmc/articles/PMC3070638/](https://pubmed.ncbi.nlm.nih.gov/23070638/)
219. James ND, Caty A, Borre M, Zonnenberg BA, Beuzeboc P, Morris T, et al. Safety and Efficacy of the Specific Endothelin-A Receptor Antagonist ZD4054 in Patients with Hormone-Resistant Prostate Cancer and Bone Metastases Who Were Pain Free or Mildly Symptomatic: A Double-Blind, Placebo-Controlled, Randomised, Phase 2 Trial. *Eur Urol*. 2009 May 1;55(5):1112–23.
220. Heerspink HJL, Andress DL, Bakris G, Brennan JJ, Correa-Rotter R, Dey J, et al. Rationale and protocol of the Study Of diabetic Nephropathy with AtRasentan (SONAR) trial: A clinical trial design novel to diabetic nephropathy. *Diabetes Obes Metab* [Internet]. 2018 Jun 1 [cited 2022 Jun 7];20(6):1369–76. Available from: <https://pubmed.ncbi.nlm.nih.gov/29405626/>
221. Matsuura T, Miura K, Ebara T, Yukimura T, Yamanaka S, Kim S, et al. Renal vascular effects of the selective endothelin receptor antagonists in anaesthetized rats. *Br J Pharmacol* [Internet]. 1997 [cited 2022 Jun 11];122(1):81–6. Available from: <https://pubmed.ncbi.nlm.nih.gov/9298531/>
222. Johnström P, Fryer TD, Richards HK, Harris NG, Barret O, Clark JC, et al. Positron emission tomography using 18F-labelled endothelin-1 reveals prevention of binding to cardiac receptors owing to tissue-specific clearance by ET B receptors in vivo. *Br J Pharmacol* [Internet]. 2005 Jan [cited 2022 Jun 11];144(1):115–22. Available from: <https://pubmed.ncbi.nlm.nih.gov/15644875/>

223. Ahn D, Ge Y, Stricklett PK, Gill P, Taylor D, Hughes AK, et al. Collecting duct-specific knockout of endothelin-1 causes hypertension and sodium retention. *J Clin Invest* [Internet]. 2004 Aug 16 [cited 2022 Jun 11];114(4):504–11. Available from: <https://pubmed.ncbi.nlm.nih.gov/15314687/>
224. Tashkin DP, Elashoff R, Clements PJ, Roth MD, Furst DE, Silver RM, et al. Effects of 1-Year Treatment with Cyclophosphamide on Outcomes at 2 Years in Scleroderma Lung Disease. *Am J Respir Crit Care Med* [Internet]. 2007 Nov 11 [cited 2022 Jun 7];176(10):1026. Available from: </pmc/articles/PMC2078679/>
225. de Zeeuw D, Coll B, Andress D, Brennan JJ, Tang H, Houser M, et al. The Endothelin Antagonist Atrasentan Lowers Residual Albuminuria in Patients with Type 2 Diabetic Nephropathy. *J Am Soc Nephrol* [Internet]. 2014 Apr 10 [cited 2014 Apr 13];1–11. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/24722445>
226. Carnahan RH, Rokas A, Gaucher EA, Reynolds AB. The molecular evolution of the p120-catenin subfamily and its functional associations. *PLoS One*. 2010 Dec 31;5(12):e15747.
227. McCrea PD, Gottardi CJ. Beyond  $\beta$ -catenin: prospects for a larger catenin network in the nucleus. *Nat Rev Mol Cell Biol*. 2016 Jan;17(1):55–64.
228. Lu Q, Paredes M, Medina M, Zhou J, Cavallo R, Peifer M, et al. delta-catenin, an adhesive junction-associated protein which promotes cell scattering. *J Cell Biol* [Internet]. 1999 Feb 8 [cited 2018 Sep 3];144(3):519–32. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/9971746>
229. Lu Q, Aguilar BJ, Li M, Jiang Y, Chen YH. Genetic alterations of  $\delta$ -catenin/NPRAP/Neurojungin (CTNND2): functional implications in complex human

- diseases. *Hum Genet* [Internet]. 2016 Oct 5 [cited 2018 Sep 2];135(10):1107–16.  
Available from: <http://www.ncbi.nlm.nih.gov/pubmed/27380241>
230. Huang F, Chen J, Wang Z, Lan R, Fu L, Zhang L.  $\delta$ -Catenin promotes tumorigenesis and metastasis of lung adenocarcinoma. *Oncol Rep*. 2017 Dec 8;
231. Huang F, Chen J, Lan R, Wang Z, Chen R, Lin J, et al. Hypoxia induced  $\delta$ -Catenin to enhance mice hepatocellular carcinoma progression via Wnt signaling. *Exp Cell Res*. 2019 Jan 1;374(1):94–103.
232. Wipff J, Dieude P, Avouac J, Tiev K, Hachulla E, Granel B, et al. Association of hypoxia-inducible factor 1A (HIF1A) gene polymorphisms with systemic sclerosis in a French European Caucasian population. <https://doi.org/10.1080/03009740802629432> [Internet]. 2009 [cited 2022 Jun 10];38(4):291–4. Available from: <https://www.tandfonline.com/doi/abs/10.1080/03009740802629432>
233. Jung YS, Park J il. Wnt signaling in cancer: therapeutic targeting of Wnt signaling beyond  $\beta$ -catenin and the destruction complex. *Experimental & Molecular Medicine* 2020 52:2 [Internet]. 2020 Feb 10 [cited 2022 Jun 10];52(2):183–91. Available from: <https://www.nature.com/articles/s12276-020-0380-6>
234. Gillespie J, Ross RL, Corinaldesi C, Esteves F, Derrett-Smith E, McDermott MF, et al. Transforming Growth Factor  $\beta$  Activation Primes Canonical Wnt Signaling Through Down-Regulation of Axin-2. *Arthritis & Rheumatology*. 2018 Jun;70(6):932–42.
235. Lafyatis R, Mantero JC, Gordon J, Kishore N, Carns M, Dittrich H, et al. Inhibition of  $\beta$ -Catenin Signaling in the Skin Rescues Cutaneous Adipogenesis in Systemic Sclerosis: A Randomized, Double-Blind, Placebo-Controlled Trial of C-82. *Journal of Investigative Dermatology*. 2017 Dec;137(12):2473–83.

236. Liu X, McBride J, Zhou Y, Liu Z, Ma JX. Regulation of endothelial progenitor cell release by Wnt signaling in bone marrow. *Invest Ophthalmol Vis Sci*. 2013 Nov 11;54(12):7386–94.
237. Heuberger J, Birchmeier W. Interplay of cadherin-mediated cell adhesion and canonical Wnt signaling. *Cold Spring Harb Perspect Biol*. 2010 Feb;2(2):a002915.
238. Good RB, Gilbane AJ, Trinder SL, Denton CP, Coghlan G, Abraham DJ, et al. Endothelial to Mesenchymal Transition Contributes to Endothelial Dysfunction in Pulmonary Arterial Hypertension. *Am J Pathol*. 2015 Jul 1;185(7):1850–8.
239. Good RW, Trinder SL, Denton CP, Abraham DJ, Holmes AM. Dysfunctional Endothelial Progenitor Cells may Contribute to Vasculopathy in Systemic Sclerosis. *European Journal of Vascular and Endovascular Surgery*. 2014 Jun 1;47(6):694.
240. Lusco MA, Fogo AB, Najafian B, Alpers CE. *AJKD Atlas of Renal Pathology: Thrombotic Microangiopathy*. 2016;
241. FISHER ER, RODNAN GP. Pathologic observations concerning the kidney in progressive systemic sclerosis. *AMA Arch Pathol*. 1958 Jan;65(1):29–39.
242. Smeets MWJ, Bierings R, Meems H, Mul FPJ, Geerts D, Vlaar APJ, et al. Platelet-independent adhesion of calcium-loaded erythrocytes to von Willebrand factor. Schulz C, editor. *PLoS One*. 2017 Mar 1;12(3):e0173077.
243. Yoshimura T, Yuhki N, Moore SK, Appella E, Lerman MI, Leonard EJ. Human monocyte chemoattractant protein-1 (MCP-1). Full-length cDNA cloning, expression in mitogen-stimulated blood mononuclear leukocytes, and sequence similarity to mouse competence gene JE. *FEBS Lett [Internet]*. 1989 Feb 27 [cited 2016 Jan 16];244(2):487–93. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/2465924>

244. Charo IF, Taubman MB. Chemokines in the pathogenesis of vascular disease. *Circ Res* [Internet]. 2004 Oct 29 [cited 2016 Jan 16];95(9):858–66. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/15514167>
245. Yamamoto T, Eckes B, Hartmann K, Krieg T. Expression of monocyte chemoattractant protein-1 in the lesional skin of systemic sclerosis. *J Dermatol Sci* [Internet]. 2001 Jun [cited 2016 Jan 16];26(2):133–9. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/11378330>
246. Schmidt K, Martinez-Gamboa L, Meier S, Witt C, Meisel C, Hanitsch LG, et al. Bronchoalveolar lavage fluid cytokines and chemokines as markers and predictors for the outcome of interstitial lung disease in systemic sclerosis patients. *Arthritis Res Ther* [Internet]. 2009 Jan [cited 2016 Jan 16];11(4):R111. Available from: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2745793&tool=pmcentrez&rendertype=abstract>
247. Watson L, Midgley A, Pilkington C, Tullus K, Marks SD, Holt RCL, et al. Urinary monocyte chemoattractant protein 1 and alpha 1 acid glycoprotein as biomarkers of renal disease activity in juvenile-onset systemic lupus erythematosus. *Lupus* [Internet]. 2012 Apr 6 [cited 2022 Jun 7];21(5):496–501. Available from: <https://journals.sagepub.com/doi/10.1177/0961203311431249>
248. Dong X, Zheng Z, Luo X, Ding J, Y L, Z L, et al. Combined utilization of untimed single urine of MCP-1 and TWEAK as a potential indicator for proteinuria in lupus nephritis: A case-control study. *Medicine (Baltimore)*. 2018 Apr;97:16.
249. Da Y, Akalya K, Murali T, Vathsala A, Tan CS, Low S, et al. Serial Quantification of Urinary Protein Biomarkers to Predict Drug-induced Acute Kidney Injury. *Curr Drug*



- Metab [Internet]. 2019 Sep;20(8):656–64. Available from:  
<https://doi.org/10.2174%2F1389200220666190711114504>
250. Vignon-Zellweger N, Heiden S, Miyauchi T, Emoto N. Endothelin and endothelin receptors in the renal and cardiovascular systems. *Life Sci*. 2012 Oct 15;91(13–14):490–500.
251. Heerspink HJL, Parving HH, Andress DL, Bakris G, Correa-Rotter R, Hou FF, et al. Atrasentan and renal events in patients with type 2 diabetes and chronic kidney disease (SONAR): a double-blind, randomised, placebo-controlled trial. *The Lancet* [Internet]. 2019 Apr [cited 2019 Apr 16];0(0). Available from:  
<https://linkinghub.elsevier.com/retrieve/pii/S014067361930772X>
252. Hanaoka H, Ishigaki S, Takei H, Hiramoto K, Saito S, Kondo Y, et al. Early combination of pulmonary vasodilators prevents chronic kidney disease progression in connective tissue disease-associated pulmonary hypertension. *Int J Rheum Dis*. 2021 Nov;24(11):1419–26.
253. Zibotentan and Dapagliflozin for the Treatment of CKD (ZENITH-CKD Trial) - Full Text View - ClinicalTrials.gov [Internet]. [cited 2022 Jun 10]. Available from:  
<https://clinicaltrials.gov/ct2/show/NCT04724837>