Investigation of novel camel-derived antibodies and V_HH fragments in targeting fibrotic disease pathways

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Declaration

I, Nada Mohamed-Ali, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Nada Mohamed-Ali Date: 27-Sep-2024

Abstract

Systemic sclerosis (SSc) is a chronic, autoimmune disorder characterised by fibrosis of the skin and internal organs. Highest mortality rates are observed in patients with pulmonary comorbidities such as SSc-associated pulmonary fibrosis (SSc-PF), and SSc-associated pulmonary arterial hypertension (SSc PAH). Pro-inflammatory cytokines interleukin-6 (IL-6) and connective tissue growth factor (CTGF) have been implicated in the pathogenesis of SSc. Current therapeutics include monoclonal antibodies (mAbs), such as Tocilizumab, targeting these cytokines and their signalling molecules. Often lack of specificity of mAbs render them more prone to adverse side effects. Studies have suggested that heavy chain only (HcAb) camelid antibodies and fragments of their hypervariable binding regions (V_HH fragments) have advantageous properties. Owing to their smaller size and differences in their tertiary structures, HcAbs and V_HH fragments and have been suggested to have greater stability and higher tissue penetrative ability in comparison to their conventional counterparts. Additionally, it has been suggested that these antibodies may target novel epitopes not recognized by conventional antibodies.

This study identified cytokine targets specific for SSc-PF, SSc-PAH and SSc-NLD (no lung disease) using proteomics. Elevated levels of MCP-1, MCP-3 and MCP-4 were found in SSc-PF, compared to all other groups. No robust discriminatory cytokines were found for SSc-PAH or SSc-NLD. Measurement of IL-6 and MCP-1 serum concentrations by ELISA confirmed proteomics results. Investigation of IL-6 trans signalling molecules found increased pro-inflammatory sIL-6R in the pulmonary groups.

Secondly, the project aimed to set up in vitro tissue culture models to further clarify the fibrotic signalling pathways. In primary skin fibroblasts, IL-6 with the sIL-6R at pharmacological levels was found to induce Type I Collagen expression and phosphorylation of STAT3. Treatment with CTGF was found to induce IL-6 release through phosphorylation of ERK1/2. Increased IL-6 was found to induce production of MCP-1. The most inflammatory treatment in terms of Type I Collagen production was CTGF with sIL-6R.

Finally, this project produced and characterised available, novel, camelid conventional, HcAb and V_HH fragment antibodies targeting sIL-6R and CTGF, to attenuate these fibrotic pathways. Camel-derived anti-sIL-6R conventional and HcAbs were found to significantly reduce the expression of pSTAT3 and pERK1/2 in a similar manner as Tocilizumab. However, anti-sIL-6R V_HH fragment indicated high specificity towards the ERK pathway, significantly dampening pERK1/2 expression but with little effect on pSTAT3. In skin fibroblasts, a combination of anti-CTGF and anti-sIL-6R conventional and HcAbs reduced secretion of IL-6 to a greater extent than Tocilizumab, and depleted MCP-1 expression.

Impact statement

This PhD thesis seeks introduce a novel approach to therapeutic interventions for fibrotic diseases, through the use of camel-derived antibodies. By exploring the use of camel-derived antibodies (HcAbs) and variable domain of heavy chain antibodies (V_HH fragments), this study introduces innovative biological agents with the potential to specifically target and modulate pro-fibrotic signalling pathways. This could lead to the development of more precise and effective treatments for fibrotic diseases.

Additionally, this research addresses significant gaps in the understanding and treatment of inflammatory fibrotic diseases, particularly those related to Systemic Sclerosis (SSc). The use of proteomics to differentiate between SSc-PAH and SSc-PF provides a detailed understanding of disease-specific biomarkers and pathways. This comprehensive profiling has the potential to uncover new diagnostic markers and therapeutic targets, paving the way for personalized medicine approaches. The research also provides valuable insights into the roles of key cytokines in SSc, including IL-6, CTGF, and MCP-1, in the pathogenesis of fibrosis. Understanding these mechanisms at a molecular level enhances our knowledge of disease progression and could identify new molecular targets for therapeutic intervention.

This thesis holds implications for both the scientific community and clinical practice, aiming to revolutionize the therapeutic approaches to managing fibrotic diseases. The innovative use of camel-derived antibodies and V_HH fragments represents a significant step forward in the development of targeted biotherapeutics, offering hope for more effective and personalized treatments.

Acknowledgements

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Common abbreviations

AYA: N-acetylalanine

BMP: Bone morphogenetic protein

BMPR2: Bone morphogenetic protein receptor 2

Ca2+: Calcium

CAb: Conventional antibody

CTGF: Connective tissue growth factor

CVD: Cardiovascular disease

FBS: Foetal bovine serum

HcAb: Heavy-chain only antibody

HepG2: Hepatoblastoma cell line

IFN: Interferon

IgG: Immunoglobulin

IL: Interleukin

IPAH: Idiopathic pulmonary arterial hypertension

IPF: Idiopathic pulmonary fibrosis

JC: Jurkat cell

MAb: Monoclonal antibody

MCP: Monocyte chemoattractant protein

MMP: Matrix metalloproteinase

mPAP: Mean pulmonary arterial pressure

NAFLD: Non-alcoholic fatty liver disease

NASH: Non-alcoholic steatohepatitis

NLD: No lung disease

NO: Nitric oxide

NOS: Nitric oxide synthase

PAEC: Pulmonary arterial endothelial cell

PAH: Pulmonary arterial hypertension

PASMC: Pulmonary arterial smooth muscle

PBS: Phosphate buffered saline

PDGF: Platelet-derived growth factor

PF: Pulmonary fibrosis

PH: Pulmonary hypertension

PVR: Pulmonary vascular resistance

QUIN: Quinolinate

RT-qPCR: Reverse transcription-quantitative polymerase chain reaction

SDMA + ADMA: Dimethylarginine

SDS-PAGE: Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

SEM: Standard error of the mean

SF: Skin fibroblast

SSc: Systemic sclerosis

T2DM: Type 2 diabetes mellitus

T6A: N6-carbamoylthreonyladenosine

TCZ: Tocilizumab

TGFβ: Transforming growth factor beta

TNFα: Tumour necrosis factor alpha

V_HH: Variable Heavy domain of Heavy chain

VLA: Vanillactate

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Publications arising from this thesis

Articles

- Acquaah V. and Mohamed-Ali N., et al., (2024) 'Specific, systemic biomarkers of Systemic sclerosis-associated Pulmonary Artery Hypertension and Systemic sclerosis-associated Pulmonary Fibrosis: discovery of discriminators with omics' (Frontiers in Immunology) (To be published)

Abstracts presented at conferences

- Poster presentation, 17th International Workshop on Scleroderma Research, 'Proteomic biomarkers of scleroderma-associated pulmonary arterial hypertension and scleroderma-associated pulmonary fibrosis' (Boston, 2022)
- Presentation, 10th Annual ADLQ Symposium, 'Investigation of camel derived V_HH fragments in inflammatory fibrotic signalling' (Doha, 2023)

Added value publications

- Al-Nesf, A., Mohamed-Ali, N., Acquaah, V., Al-Jaber, M., Al-Nesf, M., Yassin, M.A., Orie, N.N., Voss, S.C., Georgakopoulos, C., Bhatt, R. and Beotra, A., 2022. Untargeted Metabolomics Identifies a Novel Panel of Markers for Autologous Blood Transfusion. *Metabolites*, *12*(5), p.425.
- Orie, N.N., Raees, A., Aljaber, M.Y., Mohamed-Ali, N., Bensmail, H., Hamza, M.M., Al-Ansari, N., Beotra, A., Mohamed-Ali, V. and Almaadheed, M., 2021. 20-Hydroxyecdysone dilates muscle arterioles in a nitric oxide-dependent, estrogen ER-β receptor-independent manner. *Phytomedicine Plus*, *1 (*3), p.100078.

Chapter 1

Introduction

1.1 Fibrotic disease

Fibrotic diseases represent a diverse group of chronic, progressive disorders characterized by the excessive accumulation of fibrous connective tissue in affected organs. This aberrant tissue remodelling process, driven by dysregulated wound healing responses, leads to tissue scarring, loss of function, and organ dysfunction. Globally, the clinical significance of fibrotic diseases is profound, contributing significantly to morbidity, mortality, and healthcare burden. Despite their substantial impact, however, these diseases remain poorly understood.

Fibrotic diseases encompass a wide range of medical conditions affecting various organs, including but not limited to, the liver, endomyocardium, bone marrow, lung, kidney, gastro intestinal (GI) tract, and skin (Zeisberg & Kalluri, 2013) (**Figure 1.1**). Liver fibrosis exemplifies this process. It is initially reversible, but may progress to irreversible stages such as cirrhosis, and often lead to liver cancer and end-stage disease (Ginès et al., 2021; Ismail & Pinzani, 2009). Various factors contribute to fibrosis, including infections such as Hepatitis B Virus (HBV) and Hepatitis C Virus (HCV), obesity, alcoholism, and exposure to aflatoxin, thereby presenting both opportunities and challenges for prevention efforts. While some fibrotic conditions arise from the chronic production of enzymes, cytokines, and growth factors triggered by radiation or toxins, others may have congenital or autoimmune origins. Despite the diverse aetiologies, the point of irreversibility and the underlying molecular mechanisms remain poorly defined across all types of fibrosis, ultimately culminating in organ failure as the end-result of uncontrolled fibrotic processes (Wynn, 2008).

Fibrotic diseases are widespread globally, however they have a disproportionate impact on minority communities and populations in developing nations worldwide. This disparity is likely attributable to a combination of socioeconomical and genetic factors, as well as implicit bias in healthcare systems and technologies . From 1990 to 2010, global annual deaths from cirrhosis and liver cancer increased from 1.25 million to 1.75 million, mirroring a similar trend in the US where fatalities rose from 44,000 to 69,000 during the same period (Mutsaers et al., 2023). Liver cancer now ranks as the second leading cause of cancer-related deaths globally, with approximately 750,000 annual fatalities and a mortality rate surpassing 90%, accounting for over 9% of all cancer-related deaths. In the US alone, liver cancer claims around 20,000 lives each year, and its mortality rate has been found to be escalating faster than other forms of cancer (Oh & Jun, 2023).

Despite these alarming statistics, reliable registries tracking worldwide incidence and prevalence rates of most fibrotic diseases are lacking, and effective treatments for many fibrotic diseases remain limited. This highlights the urgent need for improved understanding of the underlying pathogenesis, biomarkers for early detection and monitoring, and novel therapeutic interventions. While certain aspects of aetiology, clinical manifestations, affected organs, prevention, and management may vary, fibrotic diseases share common pathogenic mechanisms including inflammation, tissue injury, and dysregulated fibrogenesis. Identifying both commonalities and disparities among organ-specific findings could offer valuable insights into shared and distinct pathways, informing more targeted and efficacious interventions and treatments.

Diseases such as Idiopathic Pulmonary Fibrosis (IPF), Non-alcoholic Steatohepatitis (NASH) and Systemic Sclerosis (SSc) represent prominent examples of fibrotic disorders with substantial morbidity, mortality and economic impact globally. IPF is a chronic and progressive Interstitial Lung Disease (ILD), characterized by fibrosis of the lung tissue. While IPF has no identifiable trigger or cause, several risk factors and potential contributing factors have been acknowledged, such as genetic predisposition, environmental exposures (such as cigarette smoke, occupational dusts, and certain viral infections), and Cardiovascular Disease (CVD) (Barratt et al., 2018; Vancheri et al., 2015). IPF is

the most common, severe and progressive ILD subtype, with a varied worldwide morality rate from ~0.5 to ~12 per 100000 population per year (Kaul et al., 2021; Zheng et al., 2022). In the United Kingdom (UK), current estimates are ~4.6 per 100 000 population per year. It has been suggested that the incidence of IPF is on the rise in the UK, with over >5000 new cases diagnosed per year (Navaratnam et al., 2011)

NASH is a type of Non-alcoholic Fatty Liver Disease (NAFLD) characterized by chronic inflammation and accumulation of fat in the liver tissue, causing liver cell damage, which can progress to fibrosis, cirrhosis, and eventually liver failure (Fraile et al., 2021). Unlike alcoholic liver disease, NASH may occur in individuals without consumption of significant amounts of alcohol, and is closely associated with obesity, insulin resistance, type 2 diabetes (T2DM), high cholesterol, and metabolic syndrome (Z. M. Younossi et al., 2019). The prevalence of NASH is estimated to be between 3-5% globally, up to 30% in high-risk populations such as those with obesity or T2DM, and over 35% in severely obese patients with T2DM. Additionally, NASH is the most common indication for liver transplantation in The United States (US) after chronic hepatitis C (Anstee et al., 2013; Z. Younossi et al., 2017).

SSc is a heterogenous, chronic, autoimmune disease characterised by fibrosis of the skin and internal organs, including the heart, kidney and lungs (Sobolewski et al., 2019). The SSc prevalence in the UK has been estimated to be 2.02 per 100,000 persons per year (García Rodríguez et al., 2019). Although uncommon, SSc has a high morbidity and mortality, mostly arising from pulmonary comorbidities (Volkmann & Fischer, 2021). The leading complications linked to mortality in SSc are SSc-associated pulmonary arterial hypertension (SSc-PAH) and SSc-associated pulmonary fibrosis (SSc-PF) (Chaisson & Hassoun, 2013; Herzog et al., 2014).

IPF, NASH, and SSc represent distinct diseases with diverse clinical presentations, with commonalities in their underlying pathogenic mechanisms involving fibrosis and inflammation. In all three conditions, dysregulated wound healing responses lead to excessive deposition of fibrous connective tissue, resulting in tissue scarring and organ dysfunction. The intricate interplay between

inflammation and fibrosis drives disease progression, contributing to the morbidity and mortality associated with IPF, NASH, and SSc. These diseases impose significant burdens on global health, and effective treatments are limited, underscoring the urgent need for targeted therapeutic interventions. Investigating pathways underlying fibrosis and inflammation may uncover novel therapeutic targets and treatment strategies applicable across multiple fibrotic diseases. By elucidating the molecular mechanisms driving fibrosis and inflammation in these conditions, research can aim to improve patient outcomes, enhance quality of life, and ultimately alleviate the burden of fibrotic diseases on individuals and society as a whole.

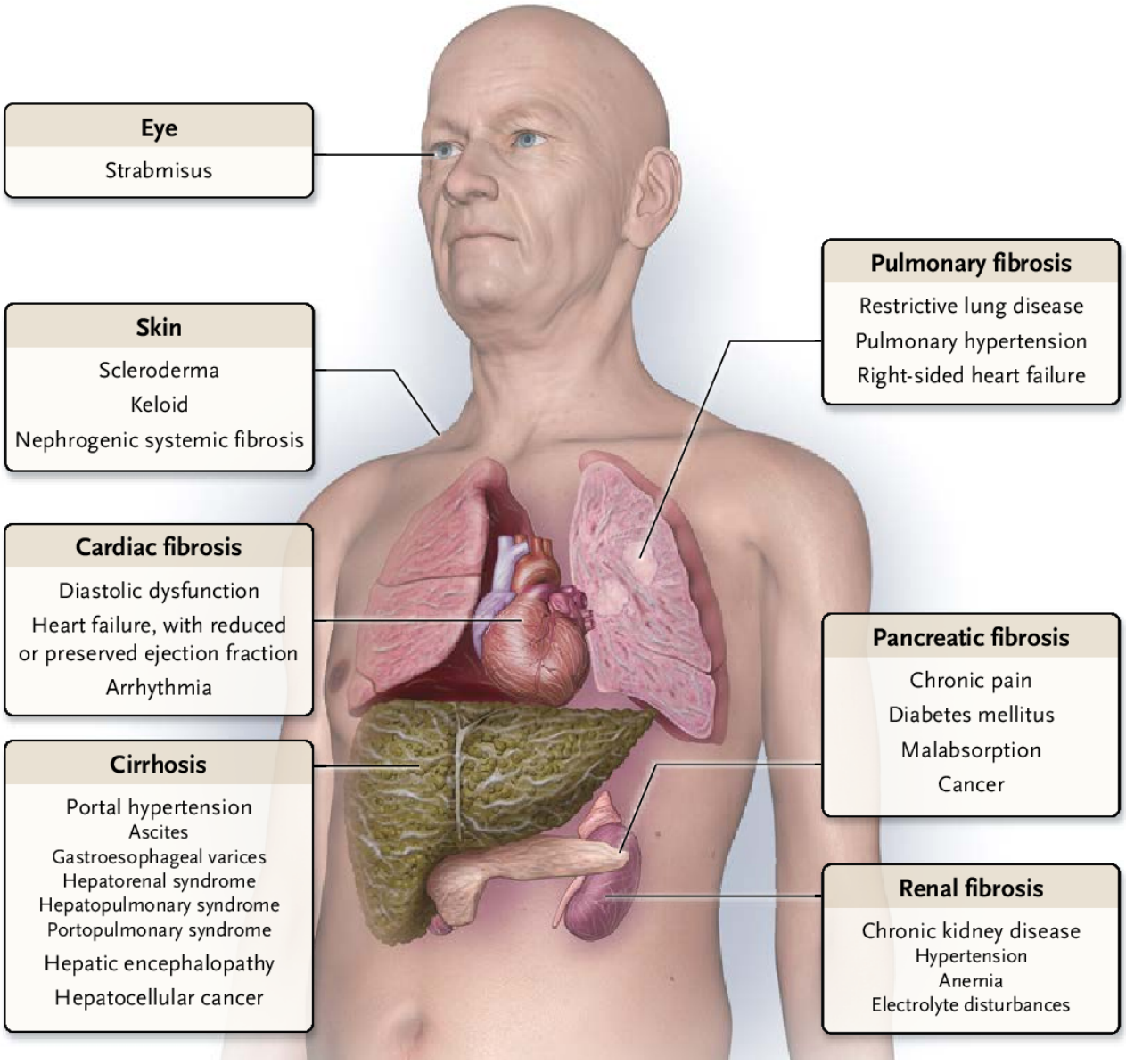


Figure 1.1: Fibrotic diseases in major organ systems. From Rockey, D.; Bell, P.; Hill, J.; Fibrosis--a common pathway to organ injury and failure. New England Journal of Medicine. 2015, 1138-1149, 372 (12) (Rockey et al., 2015).

1.2 Inflammation and fibrosis

Inflammation is a complex and dynamic biological response triggered by the body's immune system in response to injury, infection, or tissue damage. It represents a fundamental component of the body's defence mechanisms, serving to eliminate harmful stimuli, initiate tissue repair processes, and restore homeostasis. The inflammatory response is characterized by a cascade of events involving various immune cells, signalling molecules, and biochemical mediators, orchestrated to contain and neutralize the offending agent, promote tissue healing, and resolve the inflammatory process (**Figure 1.2**).

Acute inflammation is a rapid and short-lived response that occurs immediately following tissue injury or infection. It is characterized by hallmark signs such as redness, heat, swelling, pain, and loss of function, which serve as clinical indicators of the inflammatory process. Acute inflammation is orchestrated by the innate immune system, which comprises cellular components such as neutrophils, macrophages, and dendritic cells, as well as soluble mediators such as cytokines, chemokines, and acute-phase proteins. Key events in acute inflammation include vasodilation and increased vascular permeability, allowing for the recruitment of immune cells and plasma proteins to the site of injury or infection. Neutrophils are the primary effector cells of acute inflammation, tasked with phagocytosing pathogens and debris, releasing antimicrobial agents, and promoting tissue repair. The resolution of acute inflammation is tightly regulated by anti-inflammatory signals and specialized pro-resolving mediators, leading to the restoration of tissue homeostasis and the return to baseline physiological function (Hannoodee & Nasuruddin, 2022; Sherwood & Toliver-Kinsky, 2004).

In contrast to acute inflammation, chronic inflammation is a prolonged and persistent response characterized by sustained tissue damage, aberrant immune activation, and dysregulated repair processes. Chronic inflammation can arise from unresolved acute inflammation, repeated exposure to low-grade irritants or pathogens, autoimmune disorders, or metabolic dysregulation. Unlike acute inflammation, which is predominantly mediated by neutrophils, chronic inflammation involves the recruitment and activation of a diverse array of immune cells, including macrophages, lymphocytes, and fibroblasts, as well as the

sustained production of pro-inflammatory cytokines and chemokines (Murakami & Hirano, 2012; Sherwood & Toliver-Kinsky, 2004). Chronic inflammation can lead to progressive tissue damage, fibrosis, and organ dysfunction, contributing to the pathogenesis of various chronic diseases including Cardiovascular Disease (CVD), obesity, T2DM and SSc (Donath & Shoelson, 2011; Lafyatis & York, 2009; Yudkin et al., 2000b). Chronic inflammation differs from its acute counterpart in that there is a failure to resolve the inflammatory response. This constitutive pro-inflammatory environment can oftentimes lead to tissue/organ malfunction via various mechanisms, including fibrosis (Mack, 2018).

Fibrosis is a key process which maintains normal body function, as it plays a critical role in wound healing, tissue repair, and preserving tissue integrity. Upon stress, injury or infection the body responds by producing collagen and other extracellular matrix (ECM) components to repair the damaged area. This process helps to seal the wound, restore tissue structure, and prevent further damage. Fibrosis helps replace damaged tissue with scar tissue, allowing for the restoration of structural integrity. Without fibrosis, the body's ability to heal and recover from injuries or surgical procedures would be compromised. Additionally, In many organs, fibrous tissue provides structural support, collagen fibres in the skin help maintain its strength and elasticity, while collagen in blood vessels gives them resilience and structure (Wells, 2013).

Fibrosis can become pathological, in instances where mechanisms regulating the resolution of fibrosis have been impaired. This leads to excessive accumulation of fibrous tissue, which disrupts the normal architecture of the affected organs, leading to tissue scarring, stiffness, and impaired function. Over time, progressive fibrosis can lead to organ dysfunction and failure, contributing to significant morbidity and mortality, and is a common feature of chronic diseases and conditions, including IPF, NASH and SSc (Barratt et al., 2018; Fraile et al., 2021; Y. Y. Ho et al., 2014). Understanding the mechanisms underlying fibrosis and developing effective therapies to prevent or reverse fibrotic tissue remodelling are areas of active research and clinical investigation.

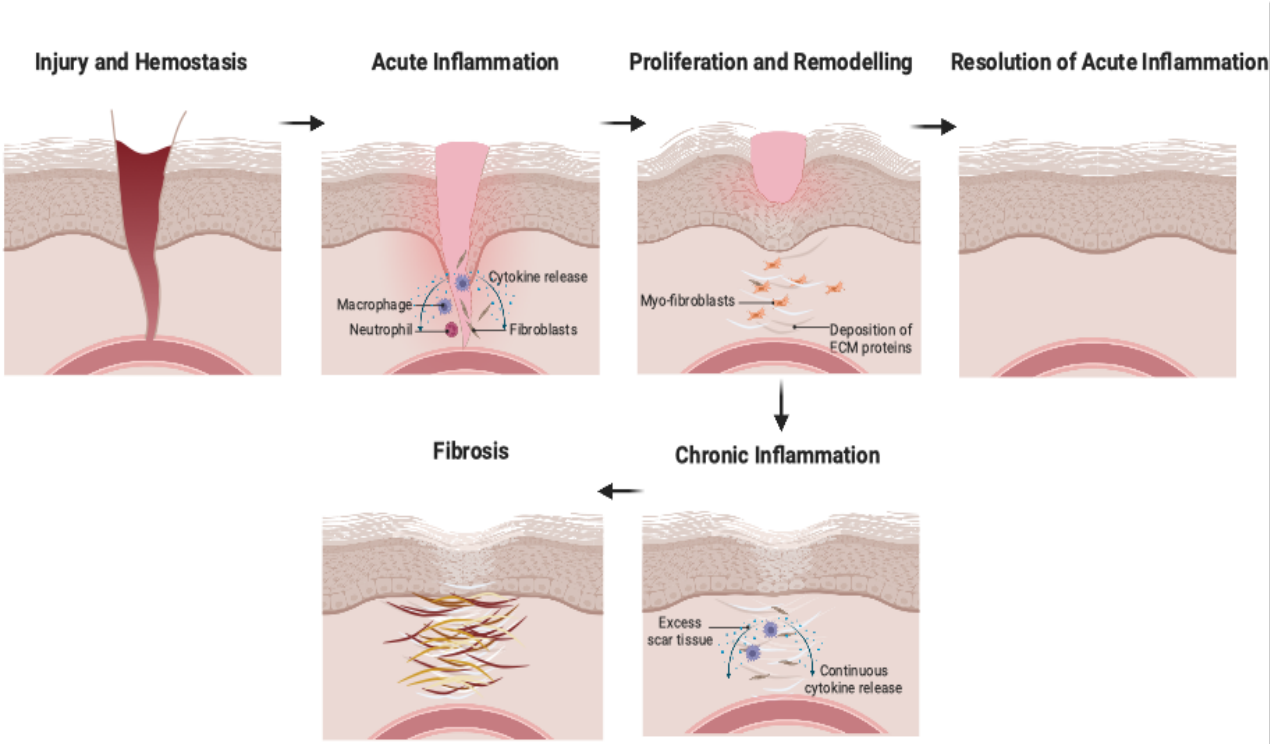


Figure 1.2: Schema of the acute and chronic inflammatory and fibrotic process. Produced using BioRender.

1.3 Mediators of fibrosis

Cytokines are a broad category of small proteins that are secreted by cells and have a specific effect on the interactions and communications between cells. They play a crucial role in the immune system, as well as in the regulation of biological processes such as inflammation, cell proliferation, and differentiation. Cytokines are released by various cell types and exert their effects by binding to specific receptors on the surface of target cells, initiating a cascade of intracellular signalling pathways that alter cell behaviour and function. The mechanism of action of cytokines can be autocrine (acting on the cells that secrete them), paracrine (affecting nearby cells) or endocrine (acting systemically) (Holtmann & Resch, 1995).

Within the normal functions of the immune system, different cytokines can have various effects. Transforming Growth Factor-beta (TGF-β), Interleukin-6 (IL-6), and Platelet-Derived Growth Factor (PDGF) can act in a pro-inflammatory manner, activating fibroblasts and leading to deposition of ECM (Roth et al., 1995; Sanjabi et al., 2009). In other cases, interleukin (IL)-1 receptor antagonist (IL-

1RA), IL-6, IL-10, can be key in resolving inflammation and have anti-fibrotic properties (Opal & DePalo, 2000). Cytokines such as Vascular Endothelial Growth Factor (VEGF) regulate angiogenesis, the formation of new blood vessels, while chemokines such as Monocyte Chemoattractant Proteins (MCPs) direct the migration of immune cells to sites of inflammation or injury (Holtmann & Resch, 1995). However, in the context of fibrotic diseases such as IPF, NASH and SSc, dysregulation of cytokines is a common feature, and drugs targeting cytokines or their receptors can be used to treat various inflammatory, fibrotic and autoimmune conditions.

Fibrosis is characterized by the appearance of activated fibroblasts (myo-fibroblasts), which synthesize and deposit large amounts of ECM at the site of injury/infection. The source of myofibroblasts may be resident stromal cells, such as fibroblasts, which have been activated by surrounding immune cells or circulating bone marrow-derived progenitor cells known as fibrocytes (Conway & Hughes, 2012; Hinz et al., 2012). Myofibroblasts may also be produced from transdifferentiated epithelial and endothelial cells which adopt properties of myofibroblasts through a process known as epithelium to mesenchymal transition (EMT) and endothelium to mesenchymal transition (EndoMT) (Lovisa, 2021; Piera-Velazquez et al., 2016). Various cell types play crucial roles in initiating and perpetuating the fibrotic process. At the site of tissue damage or pathogen entry, damage- and pathogen-associated molecular patterns (DAMPs or PAMPs) are released by necrotic cells and/or microorganisms, respectively. DAMPs and PAMPs are recognised by pattern recognition receptors (PRR) expressed on innate immune cells, which initiate the inflammatory process (Henderson et al., 2020).

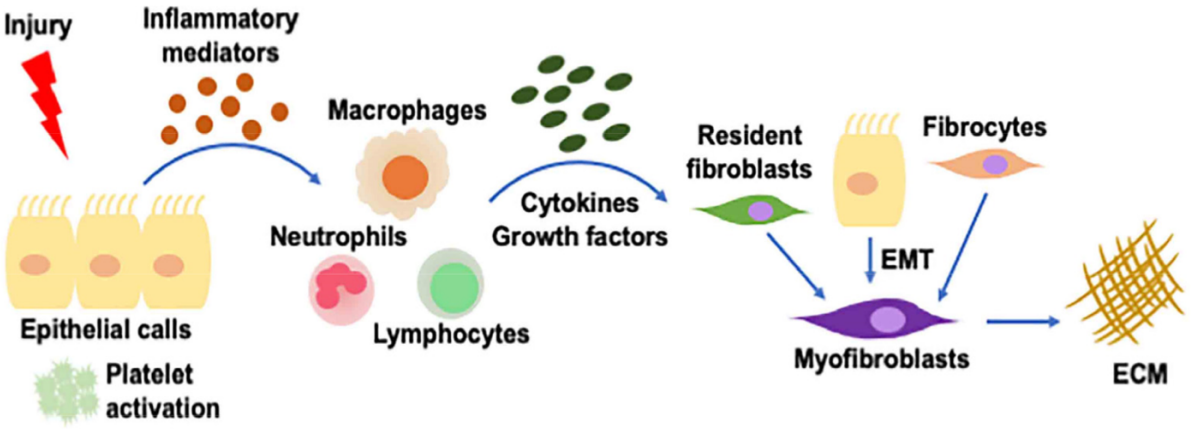
The role of immune cells in the initiation and perpetuation of fibrosis is multifaceted, involving the release of inflammatory mediators, cytokines, growth factors, and interactions with other cell types in the tissue microenvironment (**Figure 1.3**). The sequential influx of neutrophils and macrophages upon injury is a hallmark of acute tissue damage. Macrophages become activated in response to DAMPs/PAMPs causing a shift towards a pro-inflammatory (M1) phenotype and secretion of pro-inflammatory cytokines such as tumour necrosis factor alpha (TNFα), interleukin 1 (IL-1), interleukin 6 (IL-6), interleukin 8 (IL-8),

and interleukin 12 (IL-12) (Lech & Anders, 2013; X. Zhang & Mosser, 2008). Tissue resident macrophages also play a role in recruiting neutrophils to the inflammatory site through release of chemoattractants such as CXC motif ligands CXCL1 and CXCL2, and monocyte chemoattractant protein 1 (MCP-1) (Herrero-Cervera et al., 2022). Once at the site of inflammation, these neutrophils heighten the immune response by further recruiting monocytes and shaping monocyte differentiation and macrophage polarization. During the resolution phase of the inflammatory process, macrophages are needed to remove those neutrophils that undergo NETosis or apoptosis. The phagocytosis of apoptotic cells is a central element that changes the macrophage M1 phenotype into M2, and subsequently promotes the resolution of inflammation. M2 macrophages promote tissue repair and fibrosis by stimulating fibroblast activation, collagen synthesis, and ECM deposition (E. Huang et al., 2020). In the context of fibrotic diseases, it may be that this mechanism is impaired, resulting in more M1-type macrophages, more pro-inflammatory cytokine release, and more deposition of ECM proteins.

T and B lymphocytes also play a role in initiating and resolving the inflammatory process. The Th1 subtype of $CD4^+$ helper T cells serve to recruit and activate macrophages through the release of interferon gamma (IFN-γ). Th2 cells however, are involved in the antibody-mediated immune response, stimulating B cells to produce antibodies to destroy pathogens (Fowell, 2016; Hao et al., 2020). The Th1/Th2 balance is crucial for the development of fibrosis as interleukin 4 (IL-4) producing Th2 cells induce fibrosis while IFN-γ producing Th1 cells inhibit fibrosis in experimental models of fibrosis (Sakkas & Simopoulou, 2023). A functional imbalance of the Th1/Th2 immune response has been thought to play a crucial role in IPF pathogenesis. Additionally $CD8^+$ cytotoxic T cells, which work to eliminate infected or damaged cells, have been found to be significantly increased in the lung tissue and bronchial lavage fluid of patients with IPF (Deng et al., 2023).

While essential for normal wound healing and tissue repair in maintaining health, research has shown that the dysregulation of cellular and cytokine activity is correlated with excessive fibrosis in conditions such as IPF and SSc, and can culminate in tissue damage and organ failure. In one study, higher circulating levels of IL-14 was found to be associated with increased risk of IPF (Jia et al.,

2023). Additionally, the IL-17 family of cytokines has been implicated in the progression of IPF from inflammation to fibrosis (Nie et al., 2022). In patients with SSc, TGF-β, IL-6 have been shown to play roles in the pathogenesis of the disease (Shima, 2021). Cytokines, therefore, represent a significant focus for research and therapeutic intervention in these conditions.



i, Injury	ii, Inflammation	iii, Tissue repair
• Epithelial/endothelial cell damage • Platelet activation • Clotting and coagulation	• Cytokine, chemokine, growth factor release • Fibroblast migration, proliferation, and activation • EMT • Fibrocyte recruitment	• Wound contraction • Re-epithelialization • Tissue remodeling

Figure 1.3: Schema detailing the roles of fibrotic mediators. From Ishida, Y.; Kuninaka, Y.; Mukaida, N.; Kondo, T. Immune Mechanisms of Pulmonary Fibrosis with Bleomycin. Int. J. Mol. Sci. **2023**, 24, 3149. https://doi.org/10.3390/ijms24043149 (Mancini et al., 2023)

1.4 Systemic Sclerosis

Systemic Sclerosis (SSc), is a chronic, autoimmune disease characterised by fibrosis of the skin and visceral organs. This occurs via dysregulation of the immune system, vasculopathy and connective tissue remodelling as a result of a heightened, chronic inflammatory response (Denton & Khanna, 2017). SSc is considered a rare disease, with an estimated prevalence of 50 to 300 cases per million individuals worldwide. SSc predominantly affects women, at a ratio of 4:1, and typically presents in adulthood (Mayes, 2003). While not fully understood, the

aetiology of SSc is believed to result from a complex interplay of genetic, environmental, and immunological factors.

SSc presents with a wide spectrum of clinical manifestations that can be categorized into limited cutaneous systemic sclerosis (lcSSc) and diffuse cutaneous systemic sclerosis (dcSSc), based on the extent of skin involvement. In lcSSc skin involvement is restricted to the hands, forearms, face, and neck. Patients often exhibit features of CREST syndrome, which includes Calcinosis, Raynaud's phenomenon, Esophageal dysmotility, Sclerodactyly, and Telangiectasia (Denton & Black, 2004). DcSSc involves widespread skin thickening, including the trunk and proximal extremities. This subtype is associated with a higher risk of internal organ involvement, such as interstitial lung disease, scleroderma renal crisis, and cardiac dysfunction, leading to a worse prognosis compared to lcSSc (Masi, 1980). Another important clinical feature of SSc is the presence of autoantibodies, which have proven useful in the diagnosis of affected patients, and in predicting their prognosis. Anti-centromere antibodies (ACA) are the most common auto-antibodies in SSc, and are highly specific in distinguishing SSc patients from healthy controls or subjects with other connective tissue diseases. Anti-topoisomerase (ATA) antibodies also show high specificity for SSc, however they are also strong biomarkers for patients with SSc-associated pulmonary fibrosis (Nihtyanova & Denton, 2010).

In SSc, the highest morbidity and mortality rates have been observed among patients with cardiopulmonary conditions, specifically SSc-PAH and SSc-PF (Elhai et al., 2017). The pathogenesis of these diseases is complex, involving both genetic and environmental triggers, which lead to changes in protein and metabolite expression. These changes are associated with inflammation, fibrosis and vascular remodelling (Sobolewski et al., 2019b).

1.4.1 SSc-associated Pulmonary Arterial Hypertension (SSc-PAH)

Pulmonary Arterial Hypertension (PAH) is a type of hypertension affecting the pulmonary arteries and arterioles, and is clinically classified by a sustained elevation of mean pulmonary arterial pressure (mPAP) of >25 mmHg at rest or to >30 mmHg during physical activity (**Figure 1.4**) (Schermuly et al., 2011). When occurring as a secondary complication of SSc, this is defined as SSc-PAH.

SSc-PAH occurs due to extensive vascular remodelling within the pulmonary arteries as a result of the chronic inflammatory processes attributed to SSc. This vascular remodelling results in a narrowed arterial lumen, and thus increased pulmonary arterial pressure. Additionally, the increased resistance to blood flow through the lungs causes strain on the right ventricle of the heart, resulting in right ventricular hypertrophy and, eventually, right heart failure. SSc-PAH occurs in approximately 12% of SSc patients, with a 50% mortality rate within 3 years of diagnosis. In comparison to patients with idiopathic forms of PAH (IPAH), SSc-PAH patients have a later diagnosis, poorer response to treatment, and a threefold increased risk of death (Chaisson & Hassoun, 2013b).

The causes of SSc-PAH are complex, however evidence suggests a genetic link between mutations in the bone morphogenetic protein receptor type II (BMPR2), involved in osteogenesis and cell differentiation. BMPR2 mutations are correlated with increased susceptibility to develop SSc-PAH (Xu et al., 2021). This may play a role in instigating the cellular changes such as increased apoptosis, endothelial cell dysfunction, recruitment of inflammatory cells and fibrosis in all layers of the vessels (Matucci Cerinic et al., 2003; Sgonc et al., 2000). Additionally, Chronic inflammation in SSc-PAH may lead to dysregulated endothelial cell proliferation through activation of endothelial-to-mesenchymal transition (Endo-MT) pathways (Stenmark et al., 2016). The transition of endothelial cells to a mesenchymal-like phenotype contributes to endothelial dysfunction, through elevation of proinflammatory cytokines, including IL-6, IL-8 and TNF-α (Good et al., 2015). SSc-PAH patients also exhibit distinctive, unfavourable metabolic profiles which can contribute to the pathogenesis of the disease and are attributable to mitochondrial dysfunction (Paulin & Michelakis, 2014).

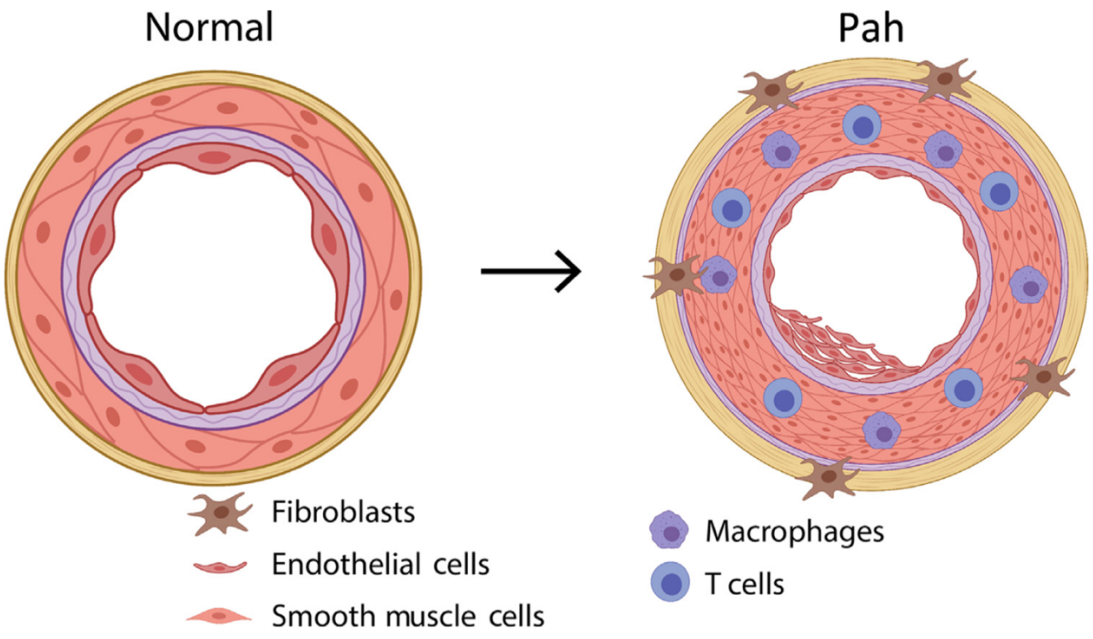


Figure 1.4: Schematic of the pathophysiology of PAH. Progression of an artery from normal to PAH, including a narrowed arterial lumen, recruitment of immune cells and vascular remodelling. From Rajagopal, S., Yu, Y., The Pathobiology of Pulmonary Arterial Hypertension. Cardiology Clinics. **2022**, 1-12, 40(1). DOI 10.1016/J.CCL.2021.08.001 *(Rajagopal & Yu, 2022)*.

1.4.2 SSc-associated Pulmonary Fibrosis (SSc-PF)

Pulmonary Fibrosis (PF) refers to a group of heterogeneous, chronic and often progressive lung conditions that primarily affect the lung interstitium. Inflammation and fibrosis within the interstitial space lead to impaired gas exchange leading to eventual respiratory failure and death (Koudstaal et al., 2023). PF can ensue secondary to another condition such as Rheumatoid Arthritis (RA) or SSc, or appear spontaneously as in idiopathic PF (IPF).

SSc-PF is an interstitial lung disease (ILD), characterized by inflammation, fibrosis, and deposition of ECM proteins around the alveoli, leading to diminished gas exchange and decreased vital lung capacity (**Figure 1.5**). The prevalence of PF in patients with SSc varies, but it is a common and serious complication. Studies indicate that about 30% to 50% of SSc patients develop interstitial lung disease (ILD), which includes pulmonary fibrosis (Martín-López & Carreira, 2023).

SSc-PF is associated with endothelial dysfunction, epithelial damage and vascular injury resulting in the release of pro-inflammatory and pro-fibrotic factors (Schoenfeld & Castelino, 2015). The fibrotic process is mediated by fibroblasts and myofibroblasts, which are responsible for the synthesis and deposition of ECM components such as collagen. TGF-β plays a central role in the activation and differentiation of fibroblasts into myofibroblasts, while other profibrotic factors, such as IL-6 and CTGF, serve to aggravate the fibrotic process (Hasegawa, Sato, Ihn, et al., 1999; Munger et al., 1999; Sato et al., 2000).

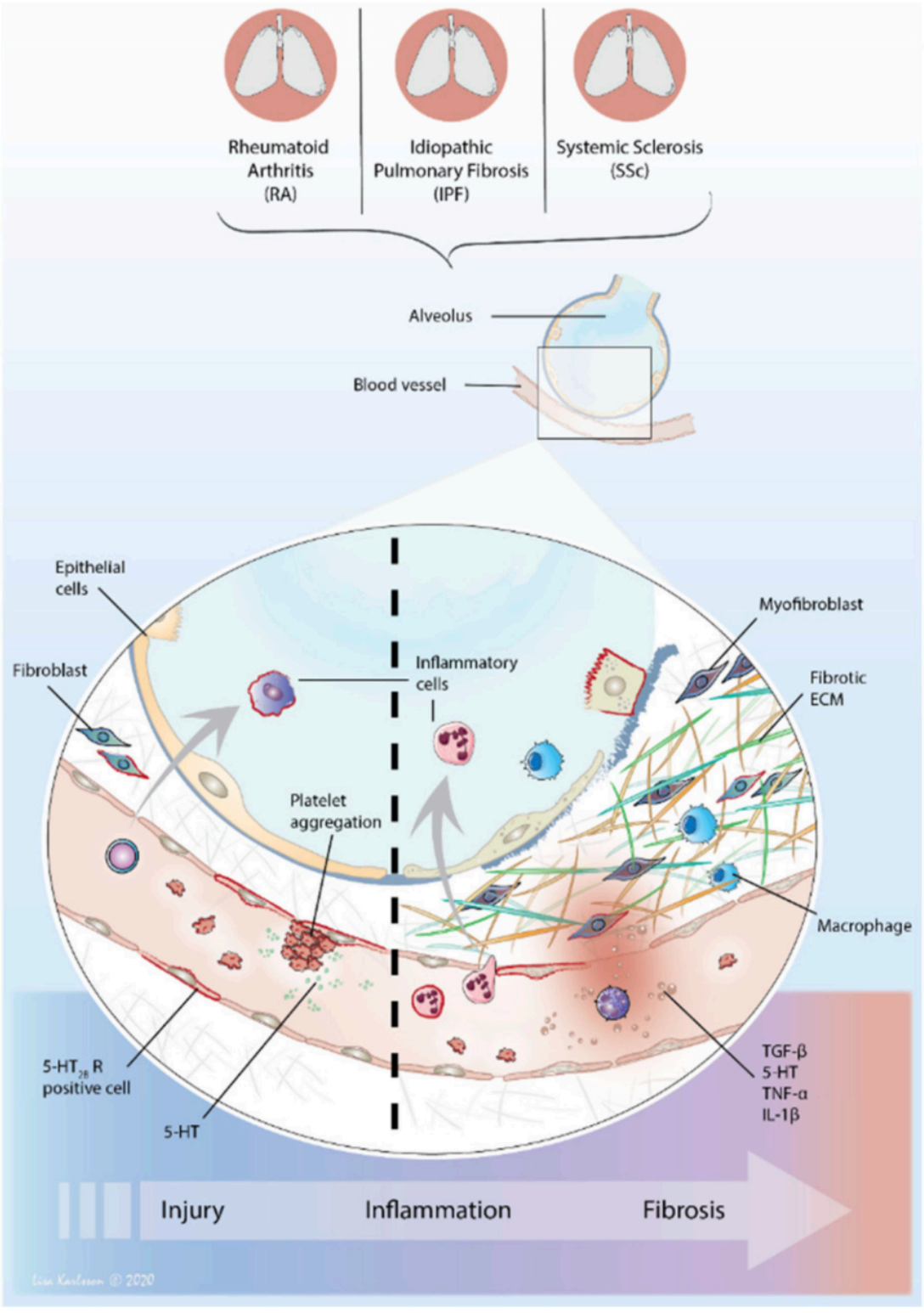


Figure 1.5: Converging pathogenic pathways linking interstitial lung diseases. From Martín-López, M., Carreira, P. E., The Impact of Progressive Pulmonary Fibrosis in Systemic Sclerosis–Associated Interstitial Lung Disease, Journal of Clinical Medicine, **2023**, 6680, 12(20). DOI 10.3390/JCM12206680 *(Martín-López & Carreira, 2023)*

1.4.3 Cytokine mediators of Systemic Sclerosis

Although the exact pathogenesis of SSc remains unknown, cytokine production and release are crucial events which have been found to be correlated to disease progression. The majority of cytokines involved in SSc pathogenesis are derived from and/or have a direct effect on immune cells. One of the most prominent cytokines involved in SSc is transforming growth factor-beta (TGF-β).

TGF-β belongs to a super family which also includes BMPs, activins and other related proteins which affect cell proliferation, differentiation and migration. TGF-β is widely recognized as a master regulator of the fibrotic process, influencing proliferation, differentiation, apoptosis, tissue homeostasis and regeneration (Ayers et al., 2017). However, TGF-β has also been suggested to play an anti-inflammatory role in some instances (Sanjabi et al., 2024). The cytokine exerts its effects through binding to TGF-β receptors type I and II (TGF-βRI and TGF-βRII), initiating a cascade of intracellular signalling events. Upon ligand binding, TGF-βRII phosphorylates TGF-βRI, which in turn activates receptor-regulated SMAD proteins (R-SMADs), primarily SMAD2 and SMAD3. These R-SMADs form a complex with the common-mediator SMAD (co-SMAD), SMAD4, and translocate to the nucleus, where they regulate the transcription of target genes involved in ECM production, cell proliferation, and differentiation. TGF-β signalling also intersects with non-SMAD pathways, including the MAPK, PI3K/AKT, and Rho-like GTPase pathways, thereby integrating various signals that contribute to cytoskeletal reorganization, cell migration, and survival (Derynck & Zhang, 2003).

In SSc, expression of TGF-β related genes in the skin and lungs have been found to positively correlate with disease severity, indicating the importance of this cytokine in SSc pathogenesis (Sargent et al., 2010). Additionally, over expression in TGF-β receptors have been identified in SSc patients. Interestingly, however, little evidence has suggested an increase in circulating levels, as serum concentration of TGF-β in SSc patients have been found to be similar, if not marginally lower, than those of healthy controls (Van Caam et al., 2020; Vanneaux et al., 2013). This altered TGF-β signalling in SSc may lead to persistent myofibroblast activation, and be complicit in driving the pathological

accumulation of ECM components, resulting in the characteristic tissue fibrosis and internal organ dysfunction.

TGF-β significantly influences the expression and activity of various cytokines, thereby orchestrating a complex network that underlies the pathogenesis of fibrotic diseases such as SSc (**Figure 1.6**). In fibroblasts, TGF-β1 has been found to induce expression of ECM proteins such as CTGF, fibronectin and alpha-smooth muscle actin (α-SMA). In the same study, knocking down the CTGF gene significantly diminished TGF-β1-induced expression of CTGF, fibronectin and α-SMA. This interplay between TGF-β1 and downstream mediators such as CTGF may amplify the fibrotic response by promoting excessive fibroblast proliferation and ECM production (Tsai et al., 2018).

Additionally, TGF-β plays a role in modulating the expression of interleukins such as IL-13. IL-13 can promote tissue fibrosis by directly activating fibroblasts, or indirectly via TGF-β stimulation. In lymphocytes from healthy donors, TGF-β significantly decreased IL-13 mRNA and protein expression, however the inverse was true in patients with SSc, further accentuating the idea that impaired TGF-β downstream signalling cascades are key contributors to the pathogenesis of SSc (Baraut et al., 2011).

Another key cytokine in the pathogenesis of SSc that is directly linked to TGF-β activity is IL-6. IL-6 is a pleotropic cytokine involved in the promotion of chronic inflammation. Dysregulation of IL-6 activity or of its signalling cascade has been implicated in the pathogenesis of a number of fibrotic diseases, including cardiac, renal and pulmonary fibrosis, as well as SSc (Y. Chen et al., 2023). Studies have identified the role of TGF-β in inducing IL-6 mRNA and protein expression and, in turn, IL-6 has also been shown to enhance TGF-β signalling, creating a feedback loop that perpetuates fibrosis (Turner et al., 1990; Xiao et al., 2005). It is clear that the interplay between TGF-β and these cytokines highlights the intricate and multifaceted role of TGF-β in modulating the cytokine milieu, which is pivotal in driving the progression of SSc and other fibrotic diseases.

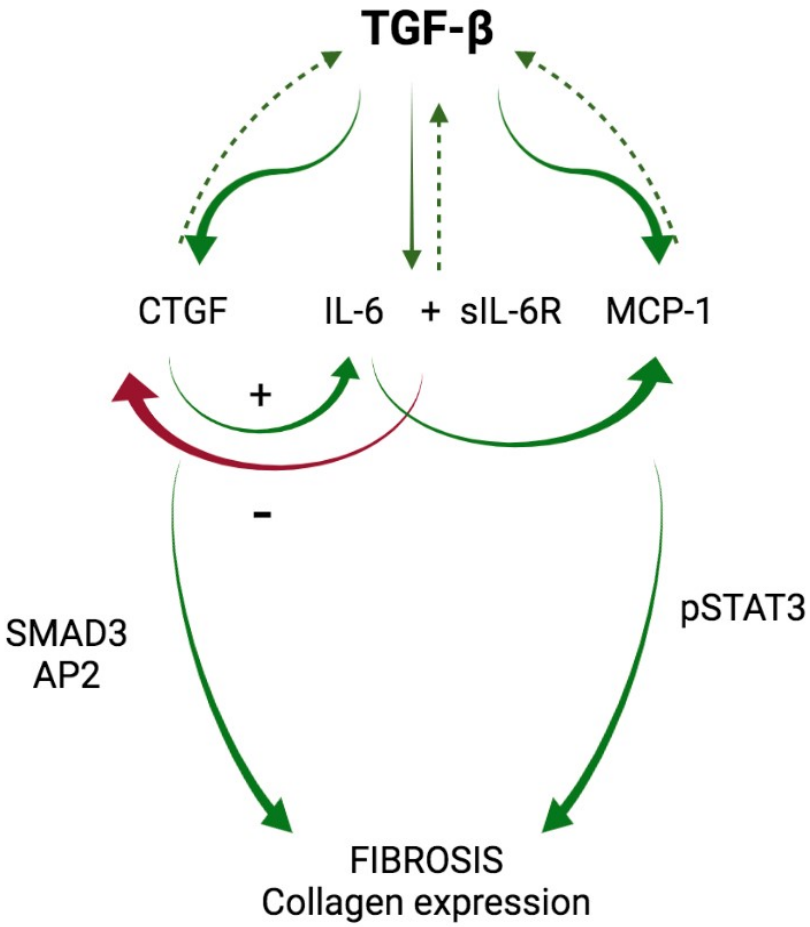


Figure 1.6: TGF-β fibrotic signalling cascades. Downstream effects of TGF-β on CTGF, IL-6, sIL-6R and MCP-1. Produced using BioRender.

Accounting for TGF-β's multi-faceted roles, it is apparent why the cytokine and its signalling pathways have become an attractive target for developing therapeutics (Akhurst, 2017). The multi-target drug Pirfenidone, which includes downregulation of TGF-β as part of its mechanism of action, has been approved as a treatment for IPF. Pirfenidone has also been shown to inhibit TGF-β-induced tumour progression, fibrosis, and inflammatory response in colorectal cancer (Jamialahmadi et al., 2023; Shah et al., 2021). Despite this, few drugs that specifically target TGF-β have achieved therapeutic success. One of these, Fresolimumab, a monoclonal antibody inhibitor of TGF-β, is currently under clinical investigation in osteogenesis imperfecta and non-small cell lung cancer, however it's efficacy is yet to be determined (Eser & Jänne, 2018; I. W. Song et al., 2022). While Fresolimumab has been suggested to improve clinical symptoms such as skin score in patients with SSc, it is unclear whether this has an effect on visceral fibrosis, and subsequently morbidity and mortality in this patient population (Rice et al., 2015).

Targeting TGF-β as a therapeutic strategy for SSc has faced significant challenges, resulting in limited success to date. Additionally, the complexity and ubiquity of TGF-β's roles in various physiological processes contribute to the difficulty of effectively targeting this pathway, leading to unintended systemic effects, including impaired wound healing, increased susceptibility to infections, and potential tumorigenesis, due to TGF-β's tumour-suppressive functions in certain contexts (Derynck et al., 2001). Moreover, TGF-β signalling is intricately intertwined with other signalling pathways, making it difficult to achieve a selective blockade that only targets the pathological aspects of its activity without disrupting its normal physiological functions. Additionally, the heterogeneity of SSc itself poses a significant hurdle. The variability in disease presentation, progression, and response to treatment among patients necessitates a highly personalized approach, which is challenging to implement with broad-spectrum TGF-β inhibitors. Research into therapies for SSc, therefore, may need to focus on downstream signalling pathways, for more selective modulation of fibrosis in order to mitigate the risks while enhancing therapeutic efficacy.

1.4.3.1 Interleukin-6 (IL-6)

Interleukin-6 (IL-6) is a pleiotropic and polyfunctional cytokine that plays both pro- and anti-inflammatory roles in the human body. It was first identified by several separate research groups between 1986 and 1987, each investigating various, seemingly unrelated biological processes. These groups initially labelled this protein differently based on the characteristics they identified. Consequently, IL-6 is known by a variety of names, including B Cell Stimulatory Factor 2 (BSF2), Hybridoma Growth Factor (HGF), Hepatocyte Stimulating Factor (HSF), and Interferon-Beta 2 (IFN-β2). The identification of IL-6 in these diverse biological contexts underscores its immense importance in both health and disease, and highlights its versatile role in numerous physiological processes (Scheller et al., 2011; Snick, 1990). IL-6 is made up of 212 amino acids, including a 28-amino-acid signal peptide. The structure of IL-6 includes four helix bundles, which are arranged in an up-up-down-down topology, and three loops (two long loops, A–B and C–D, and a short loop, B–C). The protein contains three binding sites, with site I binding to the IL-6 receptor (IL-6R) and sites II and III binding to the signal transducer glycoprotein 130 (gp130) (Tanaka et al., 2013).

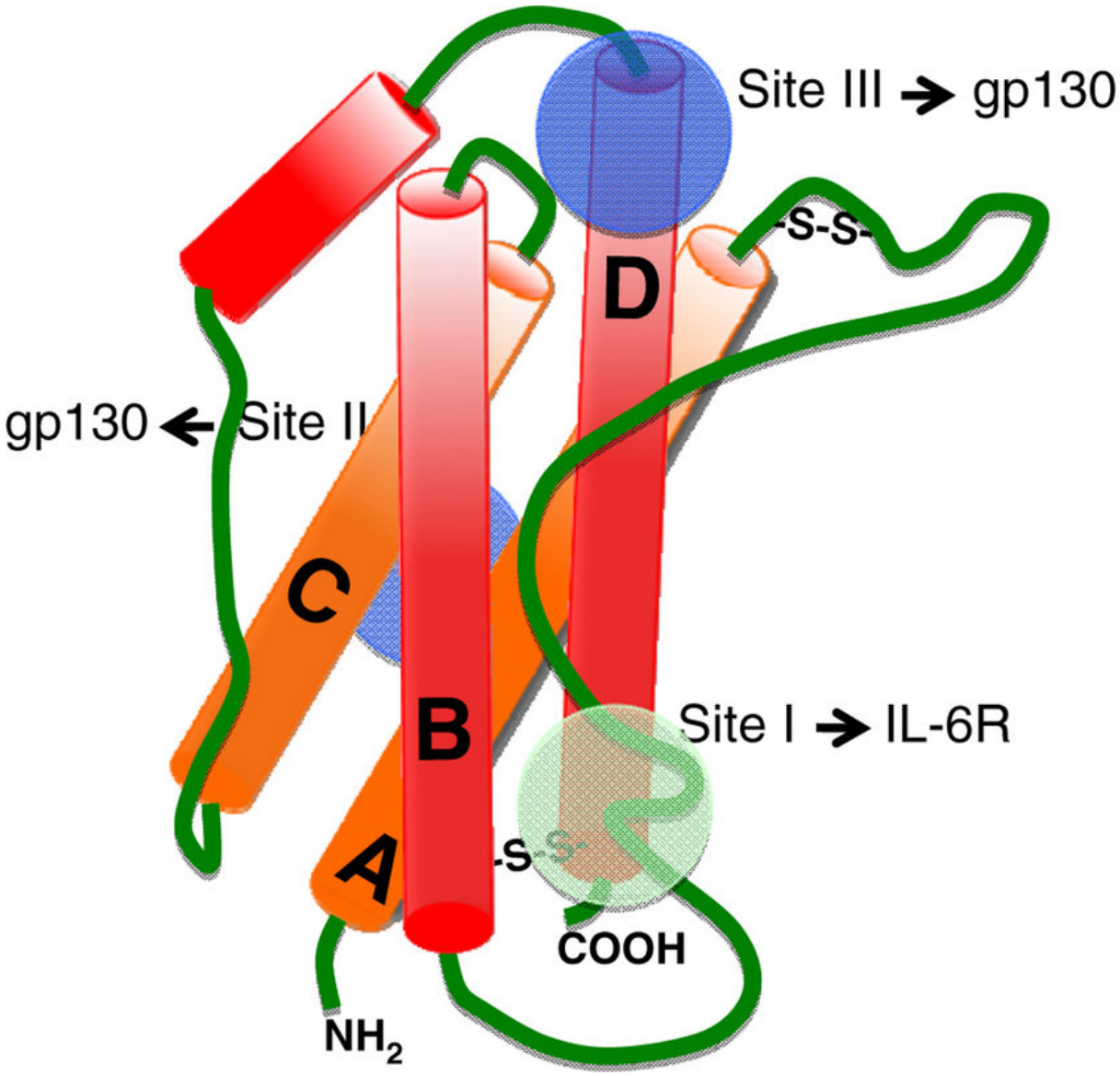


Figure 1.7: IL-6 protein structure. The structure of IL-6 includes four helix bundles A-D, three loops and three binding sites. From Tanaka, T., Ogata, A., and Narazaki, M., (2013). Tocilizumab: An Updated Review of Its Use in the Treatment of Rheumatoid Arthritis and Its Application for Other Immune-Mediated Diseases. Clinical Medicine Insights: Therapeutics. 5. 33-52. 10.4137/CMT.S9282 (Tanaka et al., 2013).

IL-6 is released by many different cell types, including hepatocytes, adipocytes, smooth muscle cells, and leukocytes. As an endocrine cytokine, IL-6 is secreted into the circulation and acts systemically, affecting various tissues and organs throughout the body. It is a well-known regulator of the immune response, playing roles in both chronic and acute inflammation. The dual role of IL-6 in both promoting and resolving inflammation highlights its context-dependent functions. Upon injury and infection, IL-6 aids in pathogen clearance and tissue repair, acting as a crucial mediator that orchestrates the immune response to infection or injury. In this context, IL-6 helps to mobilize immune cells to the site of inflammation and supports the healing process by promoting tissue regeneration and repair. However, in chronic inflammation, the persistent expression of IL-6 can contribute to pathological conditions such as autoimmune diseases, chronic inflammatory diseases, and cancer. The chronic elevation of IL-6 levels can lead to sustained immune activation and tissue damage, underpinning its role in the progression of these disorders (Scheller et al., 2011).

1.4.3.1.1 IL-6 in Acute Inflammation

Acute inflammation is a fundamental, immediate response of the body's immune system to tissue injury, infection, or harmful stimuli, aimed at restoring homeostasis and promoting healing. Characterized by rapid onset and short duration, acute inflammation involves a well-coordinated series of events that include the recognition of pathogens or damage by pattern recognition receptors (PRRs) on immune cells, such as macrophages and dendritic cells. This recognition triggers the release of pro-inflammatory cytokines like TNF-α, IL-1, and IL-6, which orchestrate the recruitment and activation of leukocytes, particularly neutrophils, to the site of injury (Mogensen, 2009). The vascular response, marked by vasodilation and increased permeability, allows immune cells, plasma proteins, and nutrients to migrate into the affected tissue, facilitating the clearance of pathogens, removal of debris, and initiation of tissue repair processes (Landén et al., 2016). Acute inflammation also activates the complement system and generates reactive oxygen species (ROS) to enhance microbial killing (Mittal et al., 2014). The resolution phase is equally crucial, involving the cessation of pro-inflammatory signalling, apoptosis of neutrophils, and the subsequent phagocytosis of apoptotic cells and debris by macrophages,

which switch to an anti-inflammatory phenotype to promote tissue repair and regeneration. Dysregulation of these tightly controlled processes can lead to chronic inflammation or excessive tissue damage, underscoring the delicate balance required for effective acute inflammatory responses.

IL-6 plays a pivotal role in the orchestration of acute inflammation, acting as both a pro-inflammatory and anti-inflammatory cytokine. Upon tissue injury or infection, IL-6 is rapidly produced by a variety of cells, including macrophages, dendritic cells, endothelial cells, and fibroblasts, in response to stimuli such as microbial products, other cytokines (e.g., IL-1 and TNF-α), and Toll-like receptor (TLR) activation (Tanaka & Kishimoto, 2014). IL-6 exerts its effects primarily through binding to its receptor complex, comprising the IL-6 receptor (IL-6R) and the signal-transducing component gp130, subsequently activating the JAK/STAT3 signaling pathway. This activation leads to the transcription of numerous target genes involved in the acute phase response, including the induction of acute-phase proteins such as C-reactive protein (CRP) and serum amyloid A (SAA) in the liver. These proteins enhance the opsonization and clearance of pathogens, thus contributing to the innate immune response Additionally, IL-6 facilitates the differentiation of naive T cells into effector T cells, particularly Th17 cells, which play a role in pathogen defence and inflammation. IL-6 also promotes the proliferation and survival of B cells, aiding in the adaptive immune response (Korn & Hiltensperger, 2021). Despite its crucial role in promoting inflammation and immune responses, IL-6 also has anti-inflammatory functions by stimulating the production of IL-10 and inhibiting TNF-α and IL-1, thus contributing to the resolution phase of inflammation. The dual role of IL-6 in acute inflammation highlights its importance in balancing the immune response to ensure effective pathogen clearance while preventing excessive tissue damage (Tanaka & Kishimoto, 2014).

IL-6 is a key conductor of the acute phase response, playing a pivotal role in initiating and regulating the inflammatory process. Immediately after injury, macrophages are activated and release IL-6 at the site of inflammation. IL-6 acts on hepatocytes, eliciting a change in serum levels of acute phase proteins (APPs). Positive APPs, whose concentrations increase in response to IL-6, include C-reactive protein (CRP), serum amyloid A (SAA), haptoglobin, α1-

antichymotrypsin, and fibrinogen. These proteins play essential roles in the inflammatory response by promoting fever, acting as anti-proteinases, marking foreign antigens for phagocytosis, and initiating complement activation and clotting cascades (Akira & Kishimoto, 1992).

IL-6 also downregulates negative APPs, including fibronectin, albumin, and transferrin, to fine-tune the inflammatory response. The modulation of APPs by IL-6 ensures a balanced and effective response to injury and infection, preventing excessive tissue damage while promoting repair (Tanaka & Kishimoto, 2014). The cytokine mediating properties of IL-6 are of great importance in acute inflammation. Studies involving IL-6 gene knockout mice have demonstrated that IL-6 suppresses the release of pro-inflammatory cytokines without affecting anti-inflammatory cytokines, highlighting its dual role in controlling inflammation (Xing et al., 1998a). Moreover, IL-6 stimulates the production of anti-inflammatory cytokines such as IL-1 receptor antagonist, further protecting the host from potential cytokine storms and excessive inflammatory responses (Tilg et al., 1994).

Despite its significant role in mediating host defence, research has shown that sustained, sub-clinical elevation of IL-6 can lead to chronic inflammatory complications. Chronic low-grade inflammation is associated with various pathological conditions, including autoimmune diseases, cardiovascular diseases, and metabolic disorders. The persistent presence of IL-6 in circulation can contribute to the continuous activation of inflammatory pathways, leading to tissue damage and disease progression. Understanding the delicate balance of IL-6 in acute and chronic inflammation is crucial for developing therapeutic strategies aimed at modulating its activity. Targeting IL-6 signalling pathways has shown promise in treating chronic inflammatory diseases and preventing the detrimental effects of prolonged IL-6 elevation.

1.4.3.1.2 IL-6 in Chronic Inflammation

Chronic inflammation is a prolonged and dysregulated immune response that persists beyond the initial injury or infection, often leading to tissue damage and the development of various chronic diseases. Unlike acute inflammation, which is characterized by a rapid and transient response aimed at resolving injury and

eliminating pathogens, chronic inflammation involves a sustained influx of immune cells, predominantly macrophages, lymphocytes, and plasma cells, which continuously release pro-inflammatory cytokines, chemokines, and reactive oxygen species (ROS). This persistent inflammatory milieu results in ongoing tissue destruction and repair, leading to fibrosis and alterations in tissue architecture. The aetiology of chronic inflammation is multifactorial, including unresolved infections, autoimmune reactions, prolonged exposure to irritants (such as pollutants or industrial chemicals), and metabolic dysregulation as seen in obesity and type 2 diabetes. Key molecular players in chronic inflammation include cytokines like IL-6, TNF-α, and IL-1β, as well as signaling pathways such as NF-κB and JAK/STAT. These mediators perpetuate a state of low-grade inflammation that can contribute to the pathogenesis of numerous chronic conditions, including cardiovascular diseases, cancer, neurodegenerative disorders, and autoimmune diseases such as rheumatoid arthritis and systemic sclerosis (SSc) (L. Chen et al., 2017). Understanding the mechanisms underlying chronic inflammation is crucial for developing therapeutic strategies aimed at modulating the immune response to prevent tissue damage and improve outcomes in chronic inflammatory diseases.

IL-6 is a central cytokine in the orchestration and perpetuation of chronic inflammation, contributing to the pathogenesis and progression of numerous chronic inflammatory diseases. Unlike its transient role in acute inflammation, IL-6 remains persistently elevated in chronic inflammatory states, driving continuous immune activation and tissue damage. IL-6 promotes the differentiation of naive T cells into Th17 cells, which produce pro-inflammatory cytokines and sustain the inflammatory response (Kimura & Kishimoto, 2010). It also supports the production, proliferation and survival of B cells in proinflammatory auto immune conditions (Maeda et al., 2010). Furthermore, IL-6 stimulates hepatocytes to produce acute-phase proteins such as C-reactive protein (CRP) and serum amyloid A (SAA), which are biomarkers and mediators of chronic inflammation (Gulhar et al., 2023). Through the JAK/STAT3 signaling pathway, IL-6 induces the expression of genes involved in cell survival, proliferation, and differentiation, contributing to the chronic activation of immune cells and fibroblasts. This persistent activation leads to the production of extracellular matrix proteins and subsequent tissue fibrosis, as seen in diseases like systemic sclerosis (SSc).

Additionally, IL-6 can induce metabolic changes that exacerbate inflammatory responses, such as promoting insulin resistance in obesity-related inflammation (Rehman & Akash, 2016). The multifaceted role of IL-6 in chronic inflammation makes it a critical target for therapeutic intervention, with IL-6 inhibitors showing promise in mitigating the deleterious effects of chronic inflammation and improving clinical outcomes in conditions such as rheumatoid arthritis and SSc (Yip & Yim, 2019). In patients with Systemic-Onset Juvenile Chronic Arthritis (S-JCA), polymorphisms in the IL-6 gene have been found to contribute to disease pathogenesis, highlighting the genetic basis of IL-6 dysregulation (Fishman et al., 1998). Furthermore, adipose tissue, which is a significant source of IL-6, contributes to chronic inflammation in obesity. The constitutive release of IL-6 from adipose tissue advances the progression of obesity and its associated comorbidities. In Type II Diabetes and Coronary Heart Disease (CHD), IL-6 plays a pro-fibrotic role, promoting tissue scarring through increased fibrinogen levels, which underscores its involvement in both metabolic and cardiovascular diseases (Yudkin et al., 2000b).

The transition from acute to chronic inflammation involves the recruitment of monocytes to the site of infection or injury. IL-6, in complex with its receptor, stimulates endothelial cells to secrete IL-8 and monocyte chemoattractant protein 1 (MCP-1), attracting monocytes to the inflamed area. This recruitment is crucial for sustaining chronic inflammation, as monocytes differentiate into macrophages that perpetuate the inflammatory response (Gabay, 2006). Additionally, a significant reduction in neutrophil populations at the site of inflammation may influence the onset of chronic inflammation, as neutrophils typically play a role in resolving acute inflammation (Butterfield et al., 2006).

IL-6 exhibits two conflicting inflammatory profiles. In acute inflammation, IL-6 exerts anti-inflammatory effects by suppressing pro-inflammatory cytokines and stimulating the release of IL-1 receptor antagonist, thereby contributing to the resolution of inflammation. However, in chronic inflammatory diseases, IL-6 assumes a pro-inflammatory role, maintaining and exacerbating the inflammatory state. The dual nature of IL-6's actions can be attributed to its cellular and tissue origins, the inducing signals, and its unique signaling pathways. For instance, IL-6 signaling through the classic pathway (via membrane-bound IL-6R) typically

exerts regenerative or anti-inflammatory effects, whereas signaling through the trans-signaling pathway (via soluble IL-6R) is associated with pro-inflammatory responses.

Understanding the molecular mechanisms that govern IL-6's dual roles is essential for developing targeted therapies aimed at modulating its activity. The ability to selectively inhibit the pro-inflammatory effects of IL-6 while preserving its beneficial functions could revolutionize the treatment of chronic inflammatory diseases. Research into the specific contexts and regulatory mechanisms of IL-6 action will provide deeper insights into its complex roles in inflammation and offer new avenues for therapeutic intervention.

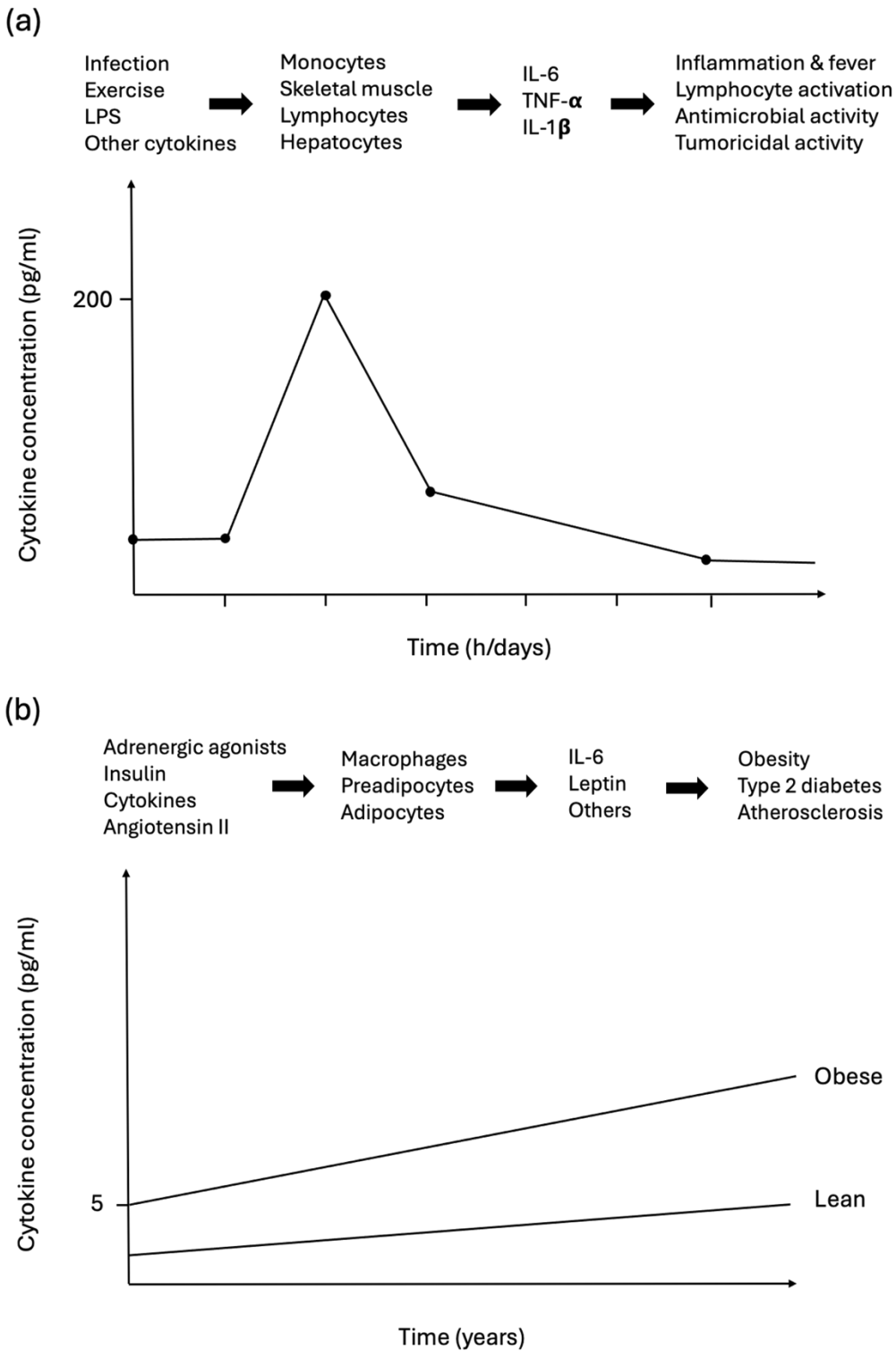


Figure 1.8: IL-6 inducers, cellular origins, effects and systemic concentrations. IL-6 cytokine concentration over time in acute inflammation (A) and chronic inflammation (B). From Yudkin, J., Kumari, M., Humphries, S., Mohamed-Ali, V., Inflammation, obesity, stress and coronary heart disease: is interleukin-6 the link?, Atherosclerosis, Volume 148, Issue 2, 2000, Pages 209-214, ISSN 0021-9150, https://doi.org/10.1016/S0021-9150(99)00463-3. (Yudkin et al., 2000).

1.4.3.1.3 IL-6 Signalling

IL-6 exerts its effects by binding to its specific receptor, IL-6R. The IL-6R is made up of two-subunits. The α subunit (IL-6Rα) is the portion that binds to the IL-6 protein and can be found in both membrane-bound and soluble forms (Mohamed-ali et al., 1999).. The β subunit (IL-6Rβ) is another terminology for the signal transducer gp130 present on the surface of most cells. IL-6, upon binding to IL-6Rα, forms a complex. The IL-6/IL-6Rα complex then associates with gp130, leading to the dimerization and activation of gp130. This activation triggers intracellular signalling cascades, which ultimately result in the transcription of target genes involved in inflammation, immune response, and cell survival. The binding of IL-6 to the IL-6 receptor components is detailed in **Figure 1.9**.

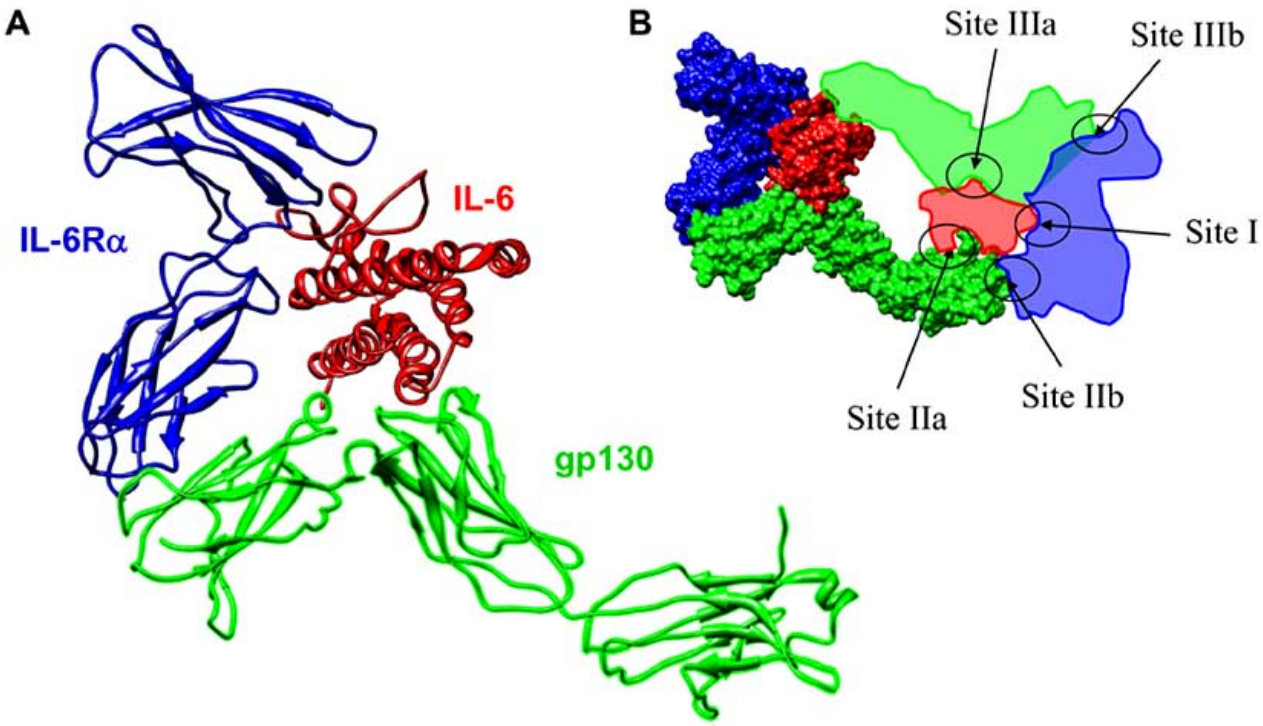


Figure 1.9: Structure and binding of the trimeric IL-6/IL-6Rα/gp130 complex. (A) Schematic view of the IL-6 protein (red) binding to IL-6Rα (blue) and gp130 (green). (B) Partial space-filling model of the IL-6/IL-6Rα/gp130 complex with the three conserved binding sites (I, IIa and IIb, IIIa and IIIb). From Abeywardena, M., Leifert, W., Warnes, K., Varghese, J., & Head, R., (2009). Cardiovascular Biology of Interleukin-6. Current pharmaceutical design. 15. 1809-21. 10.2174/138161209788186290. (Abeywardena et al., 2009)

While it is unclear exactly how IL-6 produces both pro- and anti-inflammatory responses, recent research suggests that these effects occur through two distinct pathways: classical signalling and trans-signalling. Classical signalling has been proposed to activate anti-inflammatory genes, whereas trans-signalling has been implicated in pro-inflammatory responses (Reeh et al., 2019).

Classical signalling occurs when IL-6, released into the circulation, binds to its membrane-bound receptor (mIL-6R). This receptor is not ubiquitously expressed; it is primarily found on hepatocytes, neutrophils, monocytes, and CD4+ T-cells (Gauldie et al., 1987; Oberg et al., 2006). The IL-6/mIL-6R complex then interacts with the gp130 domain, which is present on the surface of most cells. This interaction initiates the Janus kinase/signal transducers and activators of transcription (JAK/STAT) signaling pathway. Upon binding, the JAK kinases associated with gp130 become activated through autophosphorylation, creating docking sites for proteins with SH2 domains. Signal transducers and activators of transcription (STATs) bind to these sites and are subsequently phosphorylated, leading to their dissociation from the receptor, dimerization, and translocation to the nucleus where they induce the transcription of anti-inflammatory genes (Schaper & Rose-John, 2015).

Additionally, the IL-6/mIL-6R/gp130 complex can also activate the Mitogen-Activated Protein Kinase (MAPK) pathway. ERK1/2 pathway. This occurs through the recruitment and activation of Src homology region 2 domain-containing phosphatase-2 (SHP2). SHP2 is recruited to the phosphorylated tyrosine residues on the membrane bound gp130. Activated SHP2 subsequently induces activation of the Ras/Raf/MEK/ERK pathway. This pathway leads to the phosphorylation and activation of extracellular signal-regulated kinases 1 and 2 (ERK 1/2), which translocate to the nucleus and phosphorylate various transcription factors and other nuclear proteins, leading to changes in gene expression and activation of anti-inflammatory pathways (Costa-Pereira, 2014).

IL-6 trans-signalling is initiated when IL-6 binds to soluble IL-6R (sIL-6R), forming a complex that can interact with the ubiquitously expressed glycoprotein 130 (gp130). This interaction also leads to the activation of downstream signaling cascades, including STAT and ERK1/2 pathways, in cells that do not express the

membrane-bound IL-6R. Upon binding of the IL-6/sIL-6R complex to gp130, associated Janus kinases (JAKs), predominantly JAK1 and sometimes JAK2, are activated. These kinases phosphorylate tyrosine residues on the cytoplasmic tail of gp130, creating docking sites for STAT proteins, primarily STAT3. Phosphorylated STAT3 then translocate to the nucleus, where it regulates the transcription of target genes involved in inflammation, cell proliferation, and anti-apoptotic processes. The activation of STAT3 in IL-6 trans-signalling is pivotal for mediating pro-inflammatory responses and cellular survival mechanisms. Similarly, the trans signalling pathway activates the ERK1/2 via SHP2 activation of the Ras/Raf/MEK/ERK pathway. This activation leads to regulation of pro-inflammatory genes (Rose-John et al., 2023).

The activation of STAT and ERK1/2 pathways by IL-6 trans-signaling has significant biological implications in both physiological and pathological contexts. Enhanced IL-6 trans-signaling is associated with chronic inflammatory conditions, autoimmune diseases, and cancer progression, highlighting its role in disease pathogenesis. Conversely, targeted inhibition of IL-6 trans-signaling pathways has emerged as a therapeutic strategy for mitigating excessive inflammation and controlling disease progression. There is evidence to suggest that an imbalance away from the MAPK pathway via removal of regulation by suppressor of cytokine signaling 3 (SOCS3) towards the pro-inflammatory STAT3 signaling pathway contributes to auto-immune disease (Tanaka & Kishimoto, 2014). Despite the limited expression of mIL-6R, a soluble form of the receptor (sIL-6R) is present in the circulation and has been found to exist in molar excess compared to the membrane-bound form (Mohamed-ali et al., 1999). This soluble receptor is generated either by proteolytic cleavage of mIL-6R or by alternative splicing of its mRNA (Lust et al., 1992a; Mülberg et al., 1993). When IL-6 binds to sIL-6R, the complex can still engage gp130, leading to the activation of the JAK/STAT and ERK1/2 pathways, but this time promoting the transcription of pro-inflammatory genes. This mechanism, known as trans-signalling, allows IL-6 to exert its effects on cells that do not express mIL-6R, thereby expanding the range of its inflammatory actions.

The dual pathways of IL-6 signalling illustrate its complex role in regulating immune responses. Classical signalling is typically associated with regenerative

or protective responses, crucial in resolving inflammation and promoting tissue repair. In contrast, trans-signalling is often linked to chronic inflammation and autoimmune pathology, driving sustained inflammatory responses that can contribute to disease progression. The balance between these two pathways determines the overall outcome of IL-6 activity in various physiological and pathological contexts.

Understanding the differential roles of IL-6 signalling pathways has significant therapeutic implications. Targeting IL-6 trans-signaling specifically, while sparing classical signalling, could provide a means to attenuate chronic inflammation without compromising the beneficial effects of IL-6 in tissue regeneration and immune regulation. Therapeutic agents, such as selective IL-6 trans-signalling inhibitors, are being explored to achieve this balance. The development of such targeted therapies holds promise for treating chronic inflammatory diseases, autoimmune disorders, and even certain cancers where IL-6 plays a critical role.

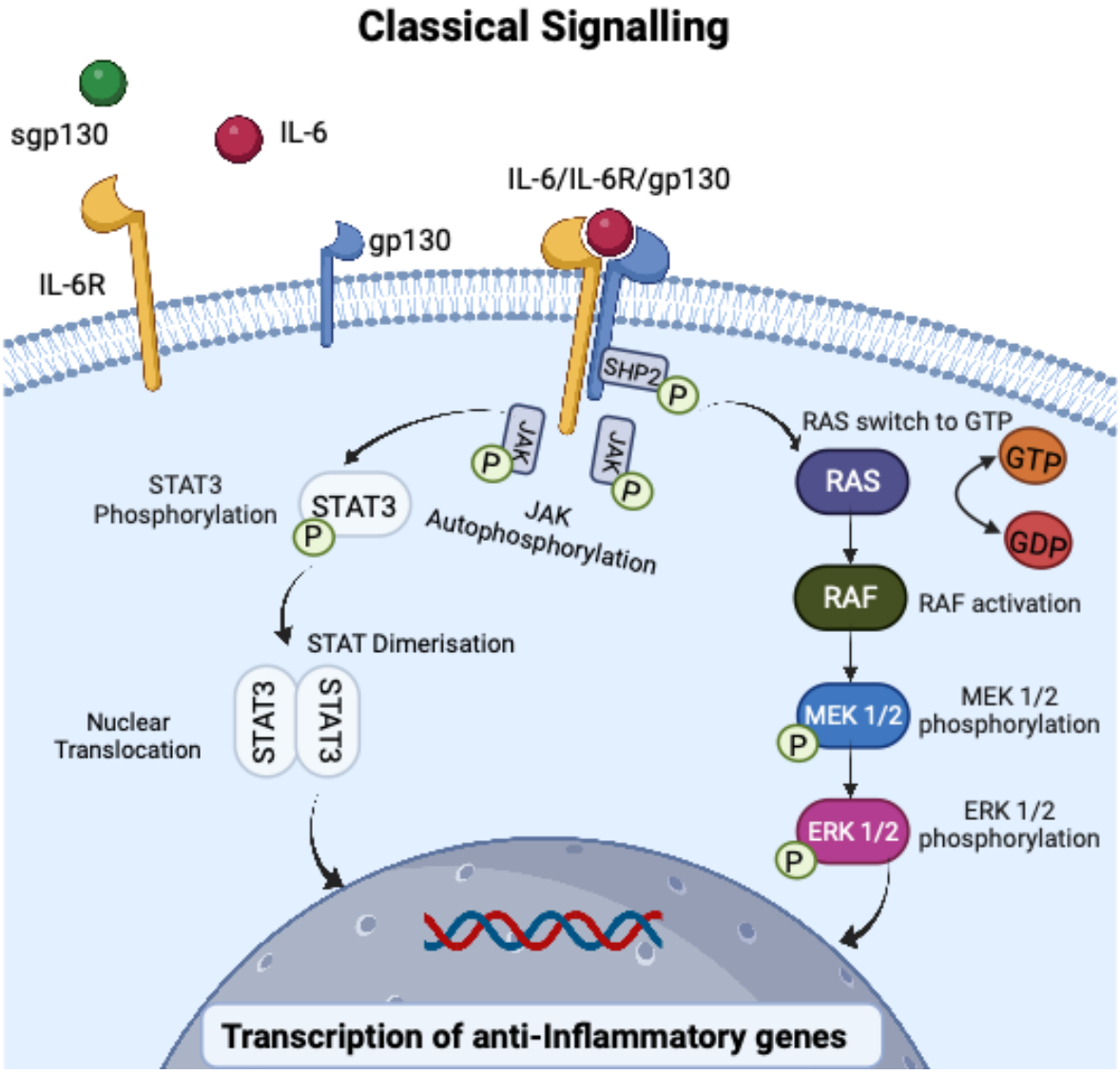


Figure 1.10: IL-6 Classical signalling cascade. IL-6/IL-6R/gp130 complex leading to STAT3 phosphorylation through the JAK/STAT signalling pathway and ERK1/2 phosphorylation through the RAS/RAF/MEK/ERK pathway. Both pathways lead to the transcription of anti-inflammatory genes (produced using BioRender).

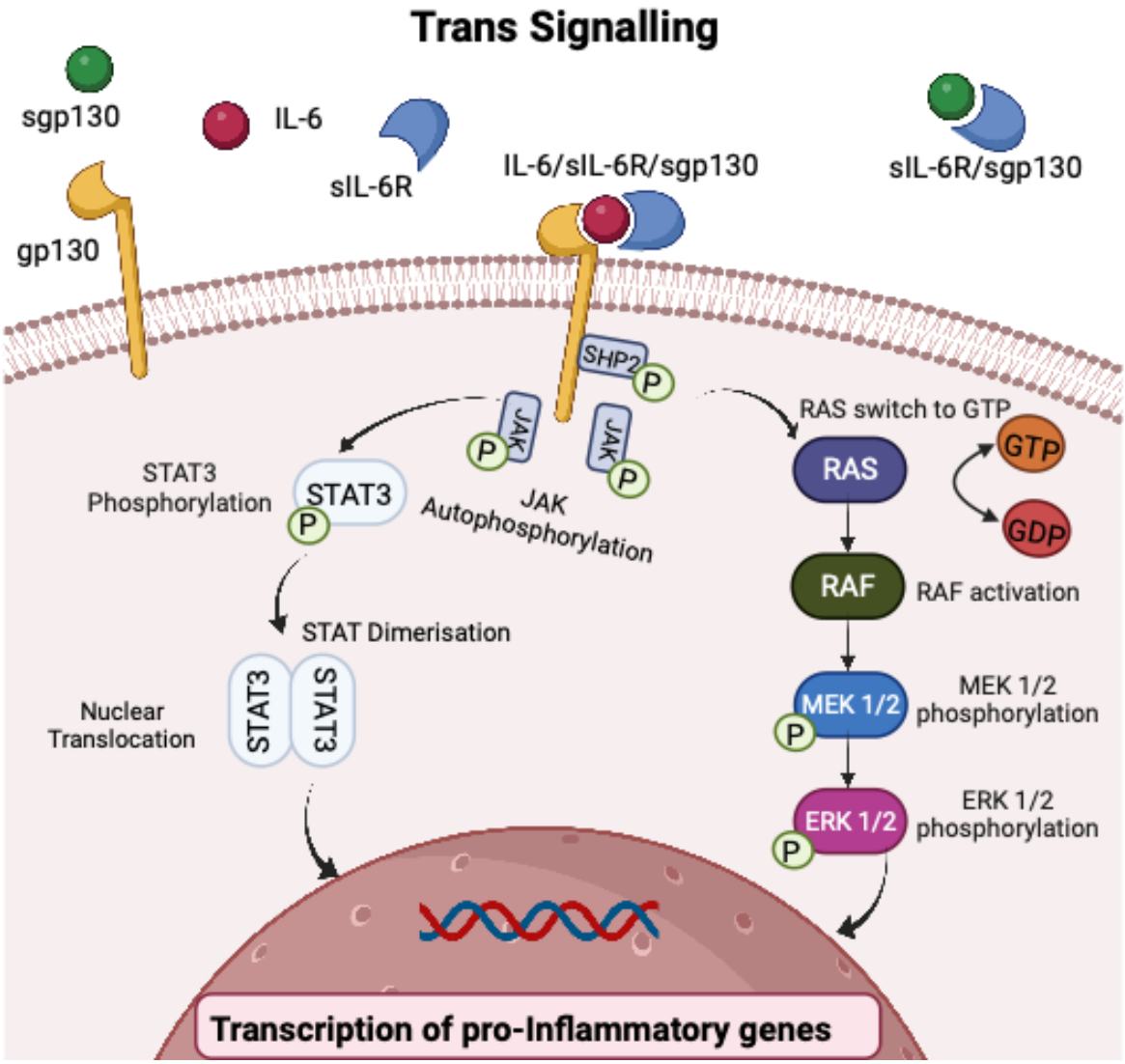


Figure 1.11: IL-6 Trans signalling cascade. IL-6/sIL-6R/gp130 complex leading to STAT3 phosphorylation through the JAK/STAT signalling pathway and ERK1/2 phosphorylation through the RAS/RAF/MEK/ERK pathway. Both pathways lead to the transcription of pro-inflammatory genes (produced using BioRender).

In the context of SSc, IL-6 is a key cytokine implicated in the disease's pathogenesis. Elevated levels of IL-6 have been found in the serum and affected tissues of SSc patients, correlating with disease severity and progression (Hasegawa, Sato, Ihn1, et al., 1999; Khan et al., 2012). IL-6 promotes the differentiation of naive T cells into Th17 cells, which are involved in autoimmunity and chronic inflammation (Singh et al., 2014). Additionally, IL-6 stimulates fibroblasts to produce collagen and other extracellular matrix components, contributing to fibrosis. The fibrotic process in SSc involves the continuous activation of fibroblasts and the excessive deposition of extracellular matrix proteins, leading to tissue stiffening and organ dysfunction. IL-6 thus plays a central role in both the immune dysregulation and fibrotic processes that characterize SSc.

Therapeutically, targeting IL-6 and its signaling pathways offers a promising approach for managing SSc. IL-6 blockade with monoclonal antibodies, such as tocilizumab, has shown efficacy in reducing inflammation and slowing disease progression in clinical trials (Khanna et al., 2022). By inhibiting IL-6 signaling, these therapies can potentially mitigate the inflammatory and fibrotic processes that drive SSc, offering relief from symptoms and improving patient outcomes. Understanding the precise mechanisms by which IL-6 contributes to SSc is essential for developing targeted therapies that can effectively mitigate the detrimental effects of this cytokine while preserving its beneficial functions. This nuanced understanding will facilitate the design of more precise and effective treatment strategies, potentially improving the quality of life for patients with SSc.

While some IL-6 signaling-targeted biologics, such as Tocilizumab, have demonstrated efficacy in treating various inflammatory diseases, there are currently no therapeutics available that can selectively inhibit the pro-inflammatory trans-signaling pathway while preserving the anti-inflammatory effects of classical signaling. This specificity is crucial because it allows for the attenuation of harmful chronic inflammation without disrupting the beneficial regenerative and immune-regulatory roles of IL-6. The challenge lies in achieving this selective inhibition.

A soluble form of the signal transducing subunit gp130 (sgp130) is present systemically and has been proposed as the natural inhibitor of the IL-6 trans-signaling pathway. Sgp130 binds to the IL-6/sIL-6R complex, preventing it from interacting with membrane-bound gp130 and thus inhibiting trans-signaling. However, studies have shown that this inhibition is non-specific *in vivo*. Under inflammatory conditions, sgp130 binds to all free IL-6 molecules within IL-6/sIL-6R/sgp130 complexes, leading to the inhibition of both classical and trans-signalling pathways (Garbers et al., 2011). This non-specific binding reduces the therapeutic potential of sgp130 as a selective inhibitor, as it compromises the protective and reparative functions mediated by classical IL-6 signalling.

Given these limitations, there is a strong justification for developing novel biologics that can selectively inhibit the trans-signalling pathway. Such therapeutics would mitigate the fibrotic and chronic inflammatory effects of IL-6 without impairing its critical roles in maintaining health. One promising approach involves the use of anti-sIL-6R V_HH fragments, also known as nanobodies. These small, single-domain antibodies derived from camelid heavy-chain antibodies can be engineered to selectively target sIL-6R, thereby blocking the trans-signalling pathway without affecting classical signalling. This selectivity is achieved through the high affinity and specificity of V_HH fragments for sIL-6R, allowing them to neutralize the pro-inflammatory effects of IL-6 trans-signalling while preserving its anti-inflammatory actions.

1.4.3.2 Connective Tissue Growth Factor (CTGF)

Connective Tissue Growth Factor (CTGF) is a small secreted protein and is a member of the CCN family of peptides. The CCN family of proteins consists of six multifunctional members designated CCN1 to CCN6, with CTGF also being denoted as CCN2. The CCN family are secreted ECM-associated proteins and have been shown to have a diverse range biological functions. Some of the functions of the CCN family include adhesion, signalling and migration within the ECM. They can also inhibit cell apoptosis, regulate cell proliferation and promote wound healing (Holbourn et al., 2008a).

The common feature of this family of proteins is their structure, consisting of distinct modules that are similar in structure to other functional proteins. CTGF's structure is no different, comprising of four distinct modular domains arranged in a 'U-shape'. The modules include an insulin-like growth factor-binding protein (IGFBP) domain, a von Willebrand factor type C (VWC) domain, a thrombospondin type 1 repeat (TSR) domain, and a C-terminal (CT) domain (**Figure 1.12**). Each domain contributes to its diverse interactions with other proteins and cellular components, thereby influencing a wide array of biological activities. The modular nature of CTGF allows it to participate in the regulation of cellular behaviours and tissue remodelling, particularly through its interaction with ECM components such as fibronectin, integrins, and collagen.

The discovery of CTGF in the early 1990s as a product of human umbilical vein endothelial cells stimulated by platelet-derived growth factor (PDGF) marked the beginning of extensive research into its functions and mechanisms. CTGF is now recognized for its ability to mediate the effects of multiple growth factors, including transforming growth factor-beta (TGF-β), bone morphogenetic proteins (BMPs), and vascular endothelial growth factor (VEGF), making it a central mediator in a complex network of signaling pathways.

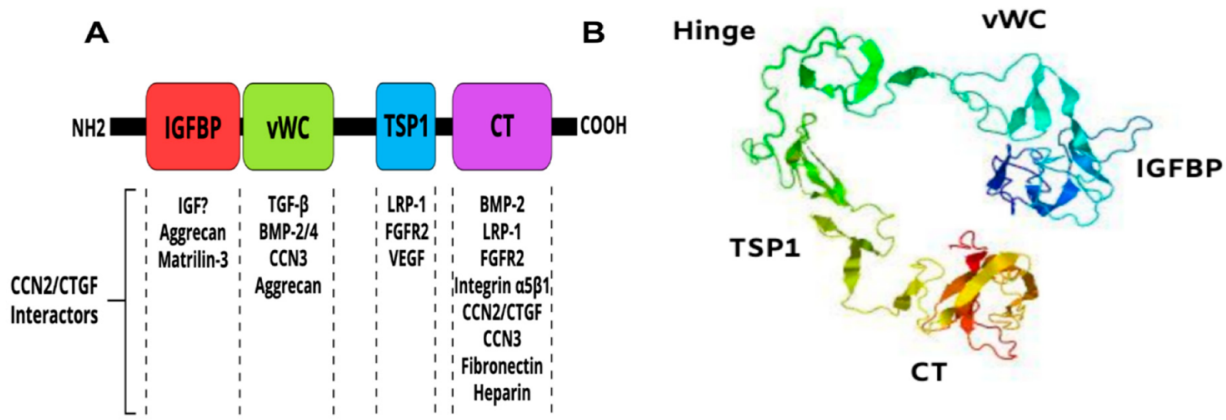


Figure 1.12: Multi-modular structure of the CCN2/CTGF protein. (A) Schematic diagram of the constitutive domains of the CTGF protein and potential interactors (B) Predicted three-dimensional model of the CCN2/CTGF protein with a wide “U”-shaped arrangement of its domains. From Chaqour B, Karrasch C. Eyeing the Extracellular Matrix in Vascular Development and Microvascular Diseases and Bridging the Divide between Vascular Mechanics and Function. International Journal of Molecular Sciences. 2020; 21(10):3487. https://doi.org/10.3390/ijms21103487 (Chaqour & Karrasch, 2020)

CTGF is a matricellular protein, meaning it is non-structural, yet dynamically expressed within the ECM and plays a critical role in various physiological and pathological processes, including the regulation of cell proliferation, differentiation, adhesion, migration, and extracellular matrix (ECM) production (Z. Chen et al., 2020a). CTGF is ubiquitously expressed in multiple cells types such as fibroblasts epithelial cells, neuronal cells, endothelial cells, smooth muscle cells, and chondrocytes, and has significant implications in developmental biology, wound healing, fibrosis, and cancer (Puglisi & Vancheri, 2018).

1.4.3.2.1 CTGF in inflammation

In acute inflammation, CTGF contributes to the initial immune response and subsequent tissue repair. It promotes the recruitment and activation of immune cells, such as macrophages and neutrophils, to the site of injury. These immune cells release cytokines and growth factors that stimulate fibroblasts and endothelial cells, leading to tissue repair and angiogenesis. CTGF's involvement in these processes underscores its importance in orchestrating a coordinated response to tissue damage. Chronic inflammation, characterized by prolonged immune cell activation and persistent tissue damage, often results in fibrosis,

where excessive ECM deposition leads to tissue scarring and organ dysfunction. CTGF is a key player in the pathogenesis of fibrosis, driving the activation of fibroblasts to myofibroblasts, which are responsible for the excessive production of ECM components.

CTGF has been linked to various fibrotic diseases, with correlation between its expression and the severity of fibrosis and disease progression. CTGF levels are upregulated in cardiac, renal, and hepatic fibrosis (Kodama et al., 2011; Shi-Wen et al., 2007; Toda et al., 2018). Furthermore, in fibroblasts isolated from SSc patients, there was a significant overexpression of CTGF (Makino et al., 2017). In mouse models, neutralizing antibodies against CTGF attenuated fibrosis (Tam et al., 2021a). In addition, selective deletion of the CTGF gene reduced experimentally induced pulmonary fibrosis and pulmonary arterial hypertension (Ponticos, 2013). CTGF expression is upregulated in response to pro-inflammatory cytokines such as TGF-β, IL-1β, and TNF-α, which are key mediators in inflammatory pathways. This upregulation contributes to tissue remodeling and fibrosis, hallmark features of chronic inflammatory conditions like rheumatoid arthritis, systemic sclerosis, and inflammatory bowel disease. In these diseases, CTGF acts by promoting fibroblast proliferation, enhancing ECM deposition, and modulating the activity of other growth factors, thereby exacerbating tissue damage and fibrosis. Moreover, CTGF's role in angiogenesis and endothelial cell function further implicates it in the perpetuation of inflammatory responses. Understanding the molecular mechanisms underlying CTGF regulation and function in inflammation provides valuable insights into potential therapeutic targets, suggesting that modulating CTGF activity could ameliorate fibrosis and inflammation, offering new avenues for the treatment of inflammatory diseases.

The dual role of CTGF in promoting both inflammation and tissue repair presents a complex therapeutic target. On one hand, inhibiting CTGF activity could mitigate chronic inflammation and fibrosis, offering potential treatment for fibrotic diseases. On the other hand, enhancing CTGF function might improve tissue repair and regeneration in acute inflammatory conditions. Therefore, a nuanced understanding of CTGF’s regulatory mechanisms in inflammation is essential for designing targeted therapeutic interventions.

1.4.3.2.2 CTGF signalling

As a matricellular protein, CTGF interacts with a wide array of cell surface receptors, growth factors, and extracellular matrix (ECM) components, orchestrating a complex network of signaling events.

The TGF-β signaling pathway is one of the most well-characterized pathways involving CTGF. TGF-β is a potent inducer of CTGF expression, and the interaction between these two molecules plays a critical role in fibrosis and tissue remodeling. TGF-β binds to its receptors (TGFBR1 and TGFBR2) on the cell surface, leading to the phosphorylation of receptor-regulated Smads (R-Smads), particularly Smad2 and Smad3. These phosphorylated Smads form complexes with the common-mediator Smad (Smad4), which then translocate to the nucleus to regulate the transcription of target genes, including CTGF (Biernacka et al., 2011).

Once expressed, CTGF can act in an autocrine and paracrine manner, reinforcing TGF-β signaling by stabilizing the Smad complexes and enhancing their transcriptional activity. This feedback loop amplifies the fibrotic response, leading to the activation of fibroblasts and their differentiation into myofibroblasts, which are responsible for excessive ECM production and tissue scarring in conditions such as cardiac fibrosis (M. M. Chen et al., 2000).

The MAPK/ERK pathway is a key signaling cascade that regulates cell growth, differentiation, and survival. CTGF can activate this pathway through various mechanisms, including integrin-mediated signaling and interactions with growth factor receptors. Binding of CTGF to its receptors leads to the activation of Ras, which subsequently activates the RAF-MEK-ERK kinase cascade. Activated ERK translocates to the nucleus, where it phosphorylates and activates transcription factors such as ELK1 and AP-1, leading to the expression of genes involved in cell proliferation and survival. The MAPK/ERK pathway also cross-talks with other signaling pathways, including TGF-β and Wnt, highlighting the integrative role of CTGF in cellular signaling networks (Z.-M. Song et al., 2018).

Integrins are transmembrane receptors that mediate cell-ECM interactions and play a crucial role in CTGF signaling. CTGF contains integrin-binding motifs with

its cysteine knot region (**Figure 1.12**) that allow it to interact with various integrin receptors, such as αvβ3, αvβ5, and α6β1. These interactions trigger intracellular signaling cascades that regulate cell adhesion, migration, and survival (Chaqour & Karrasch, 2020). Binding of CTGF to integrins activates focal adhesion kinase (FAK) and Src family kinases, which phosphorylate downstream targets, including paxillin and p130Cas. This leads to the activation of the Ras-ERK and PI3K-Akt pathways, promoting cell proliferation and survival. Additionally, integrin-mediated signaling can modulate the organization of the actin cytoskeleton, influencing cell shape and motility (M. C. Brown et al., 2005).

The Wnt/β-catenin signaling pathway is another critical pathway influenced by CTGF. Wnt proteins bind to Frizzled receptors and co-receptors LRP5/6 on the cell surface, leading to the inhibition of glycogen synthase kinase 3β (GSK-3β). This inhibition prevents the phosphorylation and subsequent degradation of β-catenin, allowing it to accumulate in the cytoplasm and translocate to the nucleus, where it activates target gene transcription. CTGF is a downstream target of the Wnt/β-catenin pathway, and its expression can be induced by Wnt signaling. Conversely, CTGF can also modulate Wnt signaling by binding to Wnt receptors or co-receptors, thereby influencing β-catenin stability and activity. This bidirectional interaction plays a role in tissue regeneration, cancer progression, and fibrosis (Mercurio et al., 2004).

Currently, very little is understood about CTGF's signalling pathways, and it has no known receptors. However, the pro-inflammatory, pro-fibrotic effects of CTGF may occur through its stimulation by, and modulation of, various cytokines and growth factors. CTGF is induced primarily by TGFβ, via activation of the SMAD pathway, however, vascular endothelial growth factors (VEGF) and integrins have also been implicated (Ayers et al., 2017; Holmes et al., 2001; Z.-M. Song et al., 2018). Additionally, there is some evidence to suggest that CTGF directly increases IL-6 and MCP-1 expression in human synovial fibroblasts (S. C. Liu et al., 2012; S.-C. Liu et al., 2013). CTGF may also participate in vascular and ECM remodelling through regulation of VEGFs, fibroblast growth factor 2 (FGF-2), bone morphogenic protein 4 (BMP4) (Ramazani et al., 2018).

Consequently, CTGF can be considered an attractive, albeit poorly understood, therapeutic target. Novel anti-CTGF heavy chain only antibodies and V_HH fragments may provide further insight into CTGF's molecular mechanisms as well as mitigate its pro-fibrotic effects. In addition, studying the synergistic effects of IL-6 and CTGF signalling in a fibrotic model may offer even greater comprehension of their roles *in vivo.*

1.5 Biologics and Therapeutic Antibodies

Biologics and therapeutic antibodies have revolutionized the landscape of medicine by offering targeted and potent therapies for a wide array of diseases. These molecules, derived from living organisms or their components, include monoclonal antibodies, cytokines, growth factors, and fusion proteins, among others. The specificity of therapeutic antibodies, in particular, allows for precise targeting of disease-causing molecules or cells, minimizing off-target effects and enhancing therapeutic efficacy. The development and optimization of biologics involve sophisticated engineering techniques to improve stability, specificity, and pharmacokinetic properties. Their applications span oncology, immunology, infectious diseases, and chronic inflammatory conditions, showcasing their versatility in clinical settings. Furthermore, biologics often offer novel therapeutic options where traditional small molecule drugs fall short, such as in diseases with complex molecular mechanisms or when targeting specific cell types within the immune system. As research advances in biotechnology and immunology, the future of biologics and therapeutic antibodies holds promise for personalized medicine and tailored treatments that cater to individual patient needs, paving the way for enhanced therapeutic outcomes and improved patient care.

In 1976, Köhler and Milstein first introduced hybridoma technology. An antibody-producing B-cell, fused with an immortal myeloma cell (hybridoma) was created, allowing for mass production of monoclonal antibodies (Köhler & Milstein, 1976). More recently, phage display technology offered an alternative to the traditional hybridoma technique. With this method, antibodies displayed on the surface of bacteriophages can be quickly and easily isolated and screened (McCafferty et al., 1990). Inspired by these technological advances, therapeutic monoclonal antibodies (mAbs) against many cytokines and their receptors emerged. Currently, there are over 80 mAbs granted approval for use in various indications (Castelli et al., 2019). The applicability mAbs in the last 5 decades is reflective of their increasing market share (**Figure 1.13**).

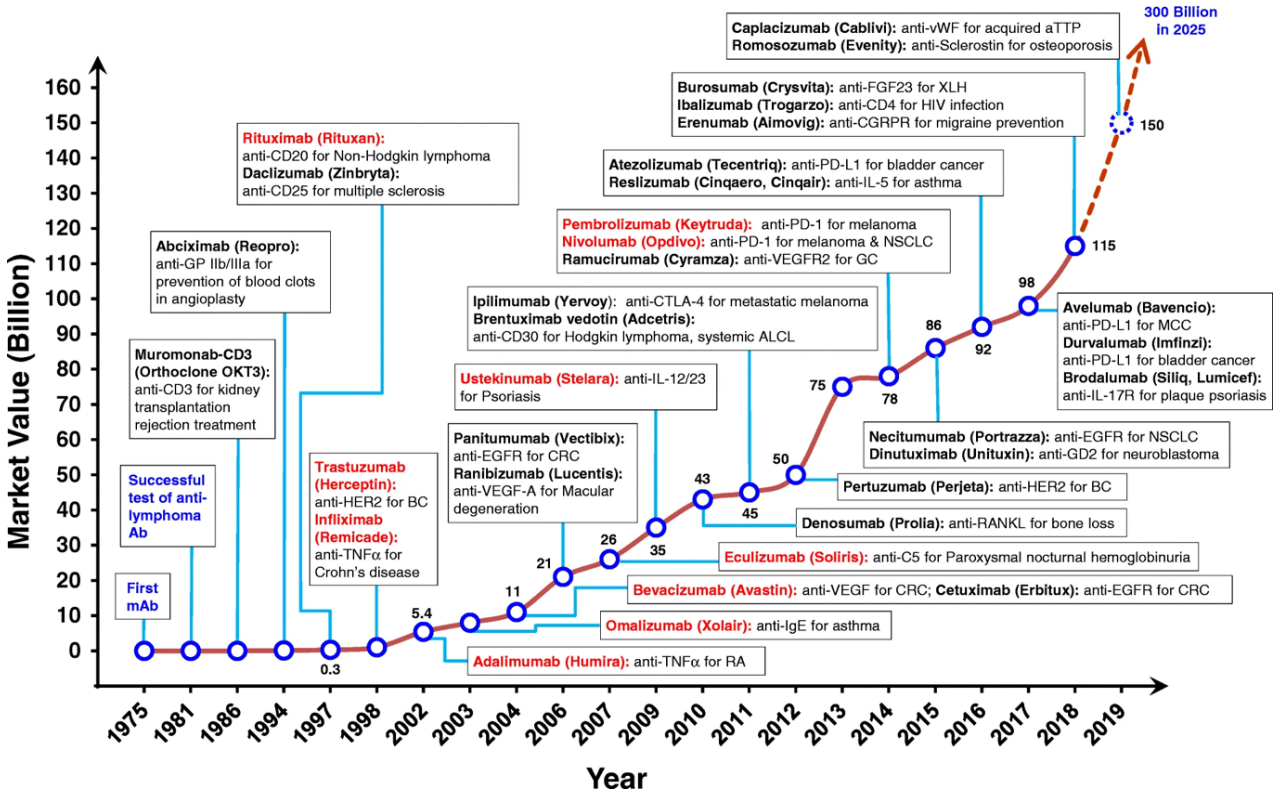


Figure 1.13: Timeline showing therapeutic applications of mAbs and their market value. Production of mAbs from first application in 1975 until 2019 with projected market value of $300 Billion in 2025. From Lu, RM., Hwang, YC., Liu, IJ. et al. Development of therapeutic antibodies for the treatment of diseases. J Biomed Sci **27**, 1 (2020). https://doi.org/10.1186/s12929-019-0592-z (Lu et al., 2020)

Some mAbs have proven to be effective in the treatment of inflammatory conditions. Tocilizumab, an anti-IL-6 receptor (IL-6R) mAb is used routinely to treat Rheumatoid Arthritis and has shown preliminary efficacy in treating SSc in clinical trials (Dhillon, 2014; Khanna et al., 2020). Furthermore, in mouse models, neutralizing anti-CTGF mAbs ameliorated TGF-β-induced fibrosis (Ikawa et al., 2008). However, current mAb therapies are not specific against pro-fibrotic pathways, and this may contribute to their significant side effects (Hansel et al., 2010). Thus, further investigation of novel alternative mAbs is considerable. This report will explore the use of camelid antibodies as a novel alternative therapy, to elucidate inflammatory fibrotic signalling.

1.6 Camelid Antibodies

Camelid antibodies, including single-domain antibodies derived from camelids such as llamas and camels, represent a unique class of biologics that offer

distinct advantages over conventional antibodies. These antibodies, also known as nanobodies or V_HH (variable domain of heavy chain of heavy-chain antibody) antibodies, are characterized by their small size (approximately 15 kDa) and exceptional stability, making them well-suited for therapeutic and diagnostic applications. Their single-domain nature allows them to access epitopes that are typically inaccessible to conventional antibodies, including clefts, grooves, and active sites on target molecules. This attribute enhances their binding affinity and specificity, facilitating potent neutralization of pathogens or targeting of disease-associated proteins with high precision. Furthermore, camelid antibodies exhibit superior solubility, thermal stability, and resistance to proteases, which are advantageous for manufacturing and storage. The development of camelid antibodies involves innovative techniques such as phage display and immunization strategies tailored to exploit the unique immune system of camelids. Their versatility extends to imaging, drug delivery, and therapeutic interventions across various disease areas, positioning camelid antibodies as promising candidates for advancing personalized medicine and addressing unmet clinical needs. Continued research into their structural characteristics, engineering strategies, and therapeutic efficacy promises to unlock further potential for camelid antibodies in biotechnology and medicine.

Commonly, mammalian immunoglobulin-γ (IgG) antibodies follow a conventional configuration, consisting of two identical heavy (H) chains and two identical light (L) chain polypeptides, linked together by disulphide bonds (**Figure 1.10**). The H chain consists of one variable (VH) domain followed by a constant domain (CH1), a hinge region for added flexibility, and two more constant (CH2 and CH3) domains. The L chain has two successive domains, a constant (CL) and variable (VL) region. The IgG antibody is shaped in a Y structure, with the two forks containing the antigen binding fragments (Fab), and the bottom delineating the cell binding constant portion (Fc region) (Muyldermans et al., 2009).

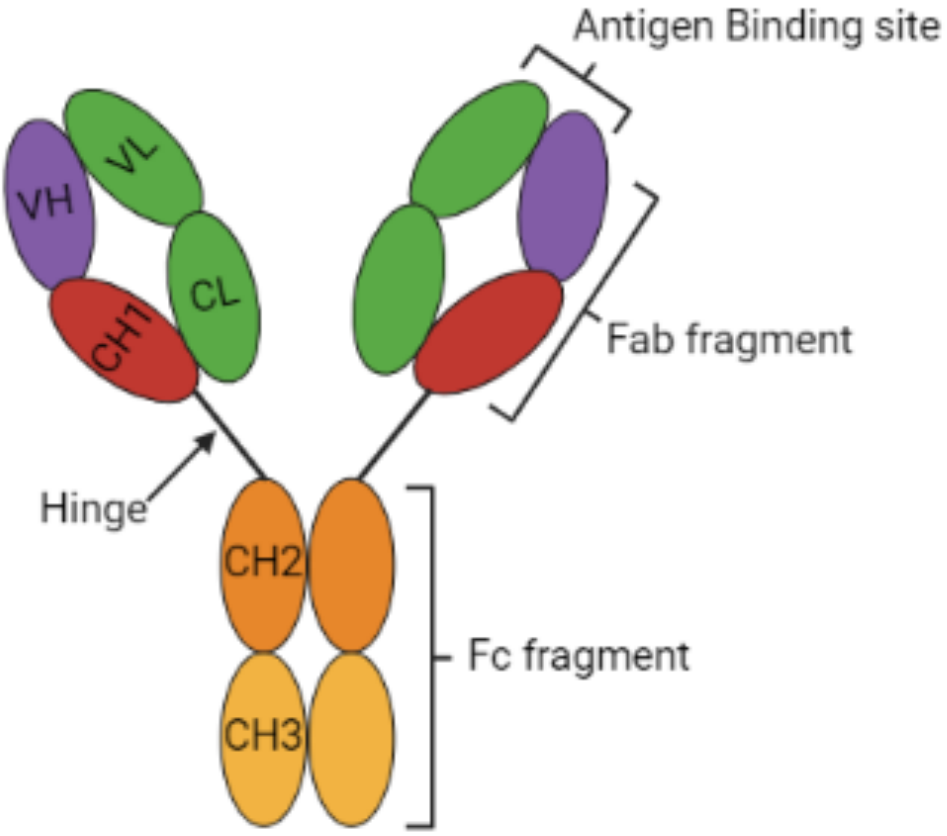


Figure 1.14: Structure of Conventional IgG. Conventional antibody with variable heavy (VH), and light chains (VL), and constant heavy (CH) and light chains (CL), antigen binding fragment (Fab) and cell binding fragment (Fc). Produced using BioRender.

One peculiar exception to the conventional mammalian IgG structure is present in the species of Camelidae (Hamers-Casterman et al., 1993). This species naturally produces conventional antibodies, as well as a considerable quantity of heavy chain only antibodies (HcAbs). HcAbs lack the two light chains and are thus smaller and more penetrative than their conventional counterparts (Muyldermans, 2013). Reports on the ratio of HcAbs to conventional antibodies in camels is varied, however it is estimated that HcAbs make up between 50-80% of a camels' natural antibody repertoire (Vincke & Muyldermans, 2012). Moreover, the hyper variable region (V_HH) of these antibodies can be synthesised *in vitro*. These recombinant V_HH fragments have become an attractive research target.

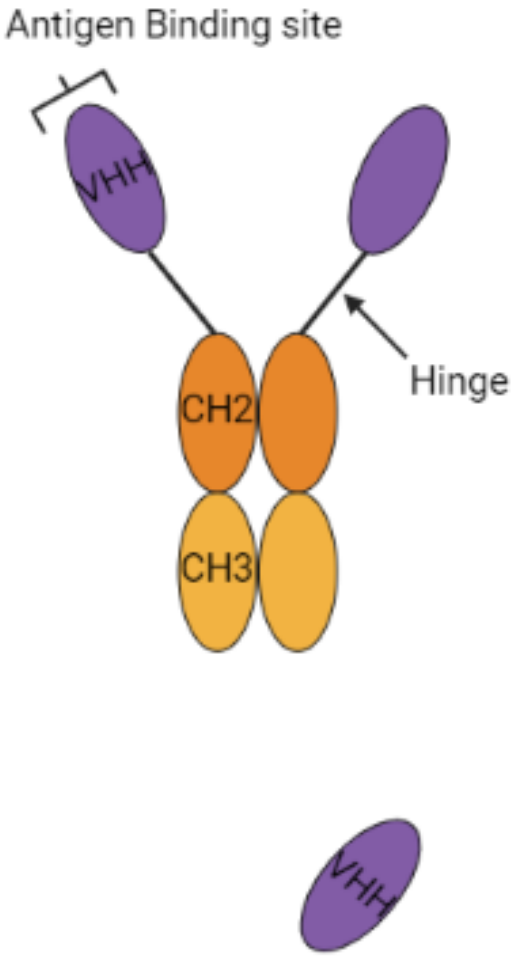


Figure 1.15: Heavy chain only antibody (HcAb) IgG structure and hypervariable region (V_HH fragment). HcAb, containing antigen binding site and constant region of the heavy chain (CH), and V_HH fragment. Produced using BioRender.

V_HH fragments possess unique qualities that make them advantageous in comparison to conventional antibodies. They are antigenic, while maintaining the diversity of the camel's natural repertoire, allowing for a vast number of clones to be produced against one antigen. Their small 15kD size, in comparison to that of the 80kD conventional antibody, allows for higher tissue penetration, and their ability to cross the blood-brain barrier has been suggested (Arbabi-Ghahroudi et al., 2005; Pothin et al., 2020). Moreover, V_HH fragments can be cloned and expressed in bacterial models such as *Escherichia coli*, which is economical and can generate a soluble, non-aggregating product (Dumoulin et al., 2002). Finally,

V_HH fragments have been reported to possess astonishing stability, surviving both chemical and thermal denaturation. This has been attributed to their ability to refold efficiently, allowing them to regain functionality (Van Der Linden et al., 1999; Wesolowski et al., 2009).

HcAbs and V_HH fragments are also favourable because of their unusual binding profiles. Conventional antibodies bind to an antigen in a concave manner, attaching to extruding epitopes on the antigen. HcAbs and V_HH fragments have the capacity to bind convexly. They form thin, finger-like projections that can extend into the cavities of the antigen. This gives them an exceptional ability to access hidden epitopes, otherwise undetected by conventional antibodies (Roovers et al., 2007).

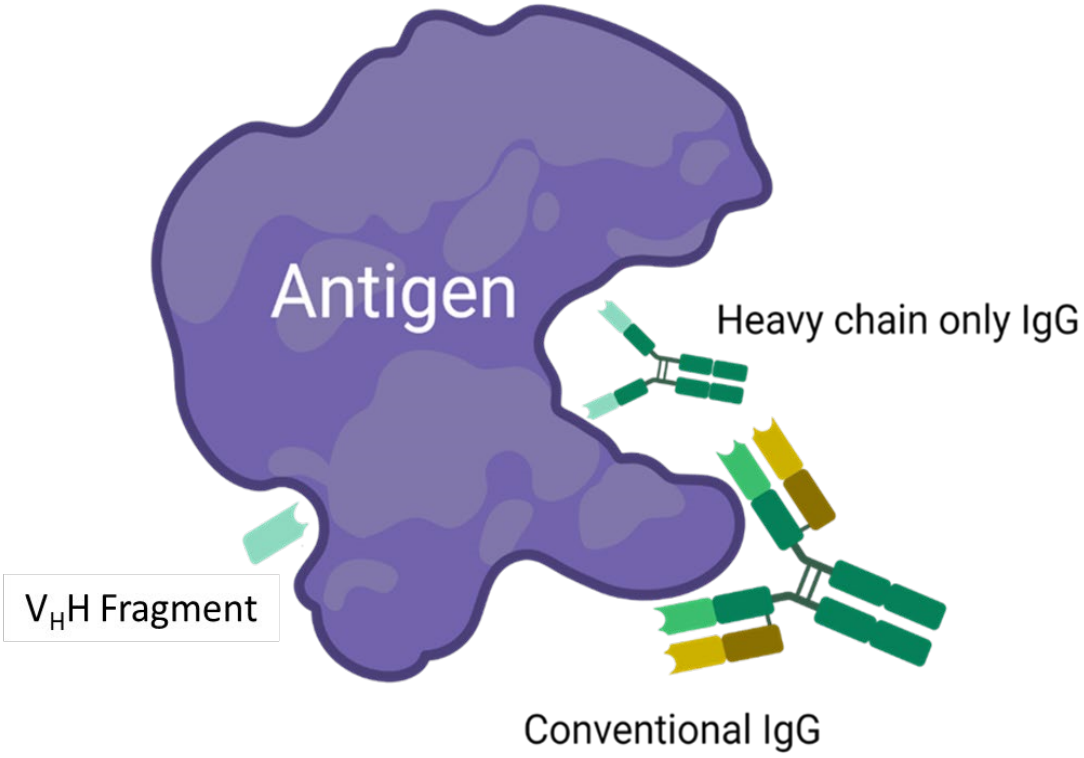


Figure 1.16: Concave conventional IgG, and convex heavy chain only IgG/V_HH binding profiles. Illustration of antigen with conventional IgG, HcAbs and V_HH fragment binding. Produced using BioRender.

1.6.1 V_HH Fragments in Therapeutics

Considering the benefits, it's comprehensible why the role of HcAbs and V_HH fragments in therapeutics and diagnostics has become an area of intense research activity, with the recombinant proteins being trialled in a variety of conditions. The anti-von Willebrand factor (vWF) V_HH fragment Caplacizumab (Cablivi), produced by Ablynx (Sanofi), was the first successful therapeutic implementation of a V_HH fragment (Callewaert et al., 2012). Cablivi was granted FDA approval for use in adults with acquired thrombotic thrombocytopenic purpura (TTP) in 2019.

Since, several other V_HH fragments have been permitted for use globally across various fields. Ozoralizumab, an anti-TNF-α V_HH fragment was approved for treatment of Rheumatoid Arthritis in Japan in 2022 (Keam, 2023a). LCAR-B38M, a dual epitope-binding CAR T cell therapy directed against two B cell maturation antigen epitopes, approved in patients with multiple myeloma (W. H. Zhao et al., 2018). The anti-programmed death ligand 1 (PD-L1) V_HH fragment Envafolimab has been approved for use in adults with advanced solid tumours in China (Markham, 2022). As a cancer diagnostic tool, V_HH fragments targeting human epidermal growth factor receptor 2 (HER2) have proven promising and are undergoing phase I and II clinical trials (D'Huyvetter et al., 2021a; Gondry et al., 2024a; Keyaerts et al., 2016).

Similar to traditional monoclonal antibody therapeutics, the applications of V_HH fragments appear to be on the rise, with a growing pipeline of antibodies at various phases of the clinical trial process. There are currently over 50 V_HH fragments under investigation for various indications (B. K. Jin et al., 2023) (Steeland et al., 2016) (**Table 1.1**). Despite their multiple benefits, V_HH fragments are not without their criticisms. Monomeric nanobodies are quickly metabolized in the body and are excreted as nanobodies rapidly. This means that frequent injections may be required for successful therapeutic use. Strategies have been employed to increase the half-life of V_HH fragments in the circulation, such as the production of multivalent, usually trivalent fragments, increasing their molecular weight to 45 KDa. Additionally, coupling the V_HH fragments to anti-Human Serum Albumin (HSA) antibodies has been proven successful.

Table 1.1: Selection of V_HH fragments used in multiple therapeutic areas (B. K. Jin et al., 2023) (Steeland et al., 2016)

Product name	Indication	Target Antigen	Clinical Trial Status	References
68-GaNOTA-Anti-HER2-VHH1	Breast carcinoma	HER2	Phase 2	(Gondry et al., 2024b)
Ciltacabtagene autoleucel, LCAR-B38M	Refractory/relapsed multiple myeloma	B-cell maturation antigen	Approved	(Chekol Abebe et al., 2022)
1311-GMIB-Anti-HER2-VHH1	Breast carcinoma	HER2	Phase 1	(D'Huyvetter et al., 2021b)
Calplacizumab	Acquired thrombotic thrombocytopenic purpura	vWF	Approved (USA, EU)	(Duggan, 2018)
Ozoralizumab	Rheumatoid arthritis	TNF-α	Approved (Japan)	(Keam, 2023b)
Vobarilizumab (ALX-0061)	Rheumatoid arthritis, systemic lupus erythematosus	IL-6R	Phase 2	(B. K. Jin et al., 2023; Van Roy et al., 2015)
Sonelokimab	Psoriasis	IL-17A/F	Phase 2	(Papp et al., 2021)
Gefurulimab (ALXN1720)	Myasthenia Gravis	ACH receptor autoantibodies	Phase 3	(Jindal et al., 2024)
M6495	Osteoarthritis	A Disintegrin and MMP	Phase 1	(Bihlet et al., 2024)
Nb V565	Crohn's Disease	TNF	Phase 2	(B. K. Jin et al., 2023; Nurbhai et al., 2019)
ARP1, VHH batch 203027	Diarrhea	Rotavirus	Phase 2	(Sarker et al., 2013)
ALX-0171	Lower respiratory tract infection	Respiratory syncytial virus	Phase 2	(Cunningham et al., 2021)
LMN-101	Campylobacteriosis	*Campylobacter jejuni*	Phase 2	(B. K. Jin et al., 2023)

1.7 Aims and Hypothesis

This study aims to investigate the therapeutic potential of novel camelid antibodies targeting pro-fibrotic signalling molecules sIL-6R and CTGF. Specifically, this project seeks to:

1. Utilize proteomics to uncover systemic cytokines and growth factors that are specific to Systemic Sclerosis-associated Pulmonary Arterial Hypertension (SSc-PAH) and Systemic Sclerosis-associated Pulmonary Fibrosis (SSc-PF) in patients attending the same clinical centre.
2. Identify the roles of IL-6, sIL-6R and CTGF in signalling pathways leading to fibrosis *in vitro*.
3. Test the efficacy of camel-derived conventional, heavy chain antibodies (HcAbs) and variable domain of heavy chain antibodies (V_HH fragments) against sIL-6R and CTGF to alleviate fibrosis *in vitro*.

The central hypothesis of this project posits that the pro-inflammatory cytokines sIL-6R and CTGF interact synergistically to create an inflammatory environment conducive to fibrosis by activation of the STAT3 and ERK1/2 pathways, resulting in the production of collagen type 1. Camelid antibodies can be synthesized, targeting these molecules and can be used to ameliorate this fibrosis. This hypothesis will be explored through the following sub-hypotheses:

1. Proteomic analyses will reveal distinct systemic cytokine and growth factor profiles specific to SSc-PAH and SSc-PF, allowing for the identification of early disease pathways and potential therapeutic targets.
2. Elevated levels of IL-6, sIL-6R and CTGF contribute significantly to the fibrotic processes observed in SSc-PAH and SSc-PF.
3. Targeting these sIL-6R and CTGF with camel-derived conventional, HcAbs and V_HH fragments effectively reduces fibrosis in models of fibrotic disease.

This study will provide critical insights into the molecular mechanisms driving fibrosis in SSc and evaluate the therapeutic potential of novel antibody-based interventions.

Chapter 2

Materials and methods

2.1 Ethical approval

Patient studies were approved by a UK registered ethical committee (REC ref. 6398) and the experiments conducted in accordance with national research guidelines. All animal experiments conducted were carried out at Anti-Doping Lab Qatar (ADLQ) in compliance with the Animals Scientific Procedures Act (1986) and University College London Research guidelines.

2.2 Patient recruitment and inclusion criteria

128 European Caucasian participants were recruited from the Royal Free Scleroderma Centre and categorized into four groups: SSc-PAH (systemic sclerosis and pulmonary arterial hypertension; n=32), SSc-PF (systemic sclerosis and pulmonary fibrosis; n=32), SSc-NLD (systemic sclerosis with no lung disease; n=32) and HC (healthy control; n=32) subjects.

All SSc patients were diagnosed according to the EULAR 2013 classification (van den Hoogen et al., 2013). Stringent clinical criteria were used to ensure the four study groups were clearly defined. Autoantibody profiles were also used as selection criteria, as 95% of SSc patients have detectable autoantibodies which are specific for the disease (K. T. Ho & Reveille, 2003a).

SSc-PAH patients were identified as those with SSc, and a mean pulmonary artery pressure (mPAP) >25 millimetre of mercury (mmHg). A pulmonary artery wedge pressure (PAWP) of ≤15mmHg and a pulmonary vascular resistance (PVR) of >3.0 wood units measured using a right heart catheter confirmed the presence of PAH. Within this group, only subjects with an absence of significant pulmonary fibrosis and positive anti-centromere antibodies (ACA) were recruited.

SSc-PF participants were classified as SSc patients with >30% fibrosis of the lung when measured using high resolution computed tomography (HRCT) or 10-30% fibrosis of the lung with a forced vital capacity (FVC) <70%. For this group, anti-topoisomerase (ATA) positive patients with an absence of significant pulmonary hypertension (measured on an echocardiogram using the tricuspid regurgitation velocity (TR vel) were selected (TR vel<2.8).

Patients included in the SSc-NLD group were either ATA or ACA positive, with a minimum of 5 years since their initial diagnosis, as this is the time it would take to develop pulmonary complications. Healthy control criteria included no SSc, nor family history of SSc, or any other known disease. Figure 2.1 outlines the recruitment and study design.

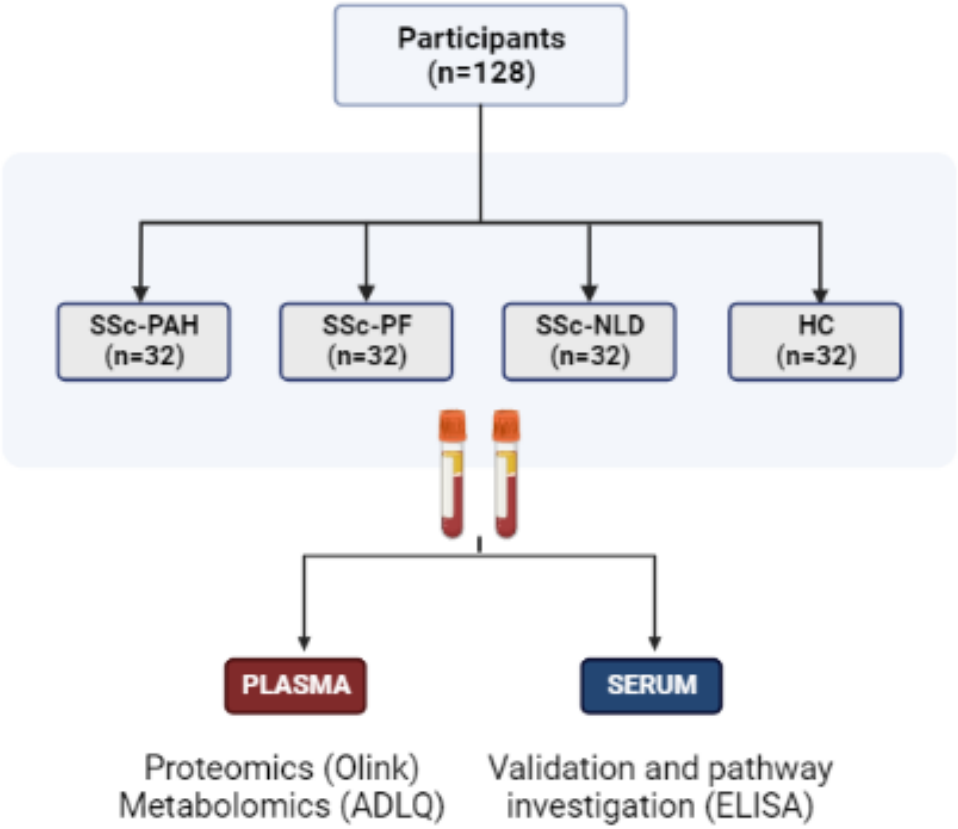


Figure 2.1: SSc proteomics and metabolomics study design. Flow chart detailing participant classification into the four study groups and the experimental design of the project. Produced using BioRender.

2.3 Patient blood sampling and processing

A total of 25ml whole blood was taken from consenting participants using a 20-gauge needle. Two 10ml vacuum tubes containing Ethylenediaminetetraacetic acid (EDTA) were used to prevent clotting for plasma extraction. One 5ml vacuum tube without EDTA was used for serum extraction. Serum and plasma were extracted by centrifugation at 3000rpm for 10min at room temperature and stored at -20ºC. Plasma was used for proteomic and metabolomic analyses, while serum samples were used for investigation of systemic cytokines by ELISA.

2.4 Proteomics

Plasma protein levels were measured using the Olink platform (Olink proteomics AB, Uppsala Sweden), Cardiovascular II and Immuno-oncology 96 plex immunoassay panels. Analytes duplicated on the two panels were removed from the Immuno-oncology panel, leaving a total of 167 analytes measured (appendix I). Protein levels were reported in Olink's arbitrary unit, normalized protein expression (NPX). NPX values were calculated from Ct values and data was pre-processed to minimize inter and intra assay variation. A high NPX value correlated with a high protein concentration (*What Is NPX? - Olink*, n.d.).

Statistical analysis utilizing R Programming software (R v.4.1.3, R studio build 485) was carried out with Dr Vanessa Acquaah. As NPX values are normally distributed, a one-way ANOVA was utilized. For pairwise comparisons between the groups, Tukey's multiple comparisons test was used as the group sizes were of equal number. For correlations, Pearson's correlations were used as the data was normally distributed. A p-value of <0.05 was considered significant throughout. Protein-protein interaction networks were generated using the STRING database. Pathway enrichment was conducted using the Enrichr tool of the gene ontology (GO) database.

2.5 Metabolomics

Sample preparation, processing and QC generation were completed at the Anti-Doping Lab Qatar (ADLQ, Doha, Qatar) while final QC review and curation were completed by Metabolon (Metabolon Inc, Durham, NC, USA). Metabolomics

profiling was performed via Ultra-high Performance Liquid Chromatography-Tandem Mass Spectroscopy (UPLC-MS/MS) using established protocols. Raw data were extracted, peak-identified and quality control processed using Metabolon's hardware and software. Compounds were compared to a library of >3300 purified standard compounds. Unknown compounds were also recorded and may be identified in future by acquisition of a matching purified standard or by classical structural analysis.

As the metabolite levels were normally distributed, they were compared pairwise between using an unpaired t-test and correlated with each other within groups using Pearson's correlation test, with Dr Vanessa Acquaah. For pairwise comparisons, a stringent p-value of <0.0001 was used to identify the most significantly different metabolites between groups. For correlations, a p-value of <0.05 was used. Volcano plots were generated using R software. Selected metabolites were compared in all groups a using one-way ANOVA with Tukey's multiple comparisons test with as the data was normally distributed. PLS-DA analysis was conducted using the mixOmics R package. Pathway analysis was performed using the MetaboAnalyst 5.0 software.

2.6 Systemic concentrations of selected cytokines

Commercially available Quantikine Enzyme-Linked Immunosorbent Assay (ELISA) kits were used to measure serum levels of IL-6 (High sensitivity), sIL-6R, sgp130 and MCP-1 (R&D Systems, Biotechne) in all groups. A DuoSet ELISA kit (R&D Systems, Biotechne), along with Ancillary Kit 2 (R&D Systems, Biotechne) was used to measure serum concentrations of CTGF. All kits had inter and intra assay CVs of <11%. The kits were used in accordance with the manufacturer's instructions.

For the IL-6 (HS) kit, all SSc serum samples were diluted 1:5 in reagent diluent, while healthy control samples were used neat. For sIL-6R, sgp130 and CTGF, all samples were diluted 1:100. For MCP-1, all samples were diluted 1:2. Absorbance was measured at 570nm, and the concentrations were calculated by linear regression, considering all dilutions above.

Statistical analysis of ELISAs was conducted using SPSS software (v.28), and graphs plotted on GraphPad Prism. Concentrations of cytokines in circulation have a large amount of variability, and therefore the data was not found to be normally distributed. For this reason, an Independent-samples Kruskal-Wallis test was used to analyse the difference in cytokine concentration between groups. For bivariate correlations, Spearman's Rank correlation coefficients and statistical test were used on SPSS software (v.28). A p-value of <0.05 was considered significant for all.

2.7 Cell culture

2.7.1 Primary skin fibroblasts

Primary, healthy control skin fibroblasts were isolated from consenting volunteers by punch biopsy (Dr Shiwen). The characteristics of the volunteers are denoted in appendix 3. The cells were cultured in sterile, 75 cm^2 (T-75) cell culture flasks (Corning) at 37°C in a humidified atmosphere with 5% CO_2. Cell medium used was 10ml Dulbecco's Modified Eagle's Medium (DMEM; Life Technologies), supplemented with 10% foetal bovine serum (FBS; Life Technologies), the antibiotic penicillin (100 U/ml; GibcoTM) and the antifungal agent streptomycin (100 µg/ml; GibcoTM).100 U/ml penicillin and 100 µg/ml streptomycin are collectively denoted as 1% PenStrep in this thesis.

Culture media was replaced every 2-3 days in a to ensure adequate nutrition for cell growth. All cell culture steps utilised sterilised equipment and a fume hood to eliminate contaminants. Once at 80-90% confluency, adherent skin fibroblasts were liberated from the cell culture flasks for further passaging or cryopreservation by trypsinisation with 0.25% Trypsin-EDTA (TE; GibcoTM). Cells were washed twice with 1X Dulbecco's Phosphate Buffered Saline (DPBS; GibcoTM) and incubated with TE at 37°C for 2 minutes or until the cells detached. TE was neutralised with DMEM with 10% FBS, and the cells were pelleted by centrifugation at 220 x g. The supernatant was discarded to remove TE and the cell pellet was re-suspended in 2 ml of DMEM with 10% FBS.

For cell counting, 10µl of the cell suspension was mixed with an equal volume of the vital stain Trypan Blue (0.4%; Sigma-Aldrich). The mixture was pipetted into a C-chip disposable haemocytometer with the Neubauer improved grid

(NanoEnTeK). Only cells which excluded the dye and remained unstained were counted.

For experimentation, cells were seeded onto 12-well plates at a density of $2X10^4$ cells per well. Serum starvation using 1ml per well DMEM, supplemented with 0.1% bovine serum albumin (BSA; Sigma-Aldrich), 1% PenStrep was used to ensure minimal concentration of cytokines and growth factors in the media. Once grown to 90% confluency, cells were incubated in starving media for 24hrs prior to treatment.

For cryopreservation, fibroblasts were re-suspended in freezing media containing 90% FBS, 10% DMSO at a density of $2.5X10^5$ – $5X10^5$ cells per ml and transferred to 1.2 ml cryogenic vials (cryovials; Nalgene). Cryovials were placed in a Mr Frosty freezing containers (Nalgene) and stored at -80°C overnight. The cryovials were then transferred to liquid nitrogen for long-term storage.

2.7.2 Human Coronary Arterial Endothelial Cells (HCAECs)

HCAECs were purchased from PromoCell and cultured in sterile, T-75 flasks at 37°C in a humidified atmosphere with 5% CO_2. Prior, the flasks were coated with 2ml 1X Attachment Factor (AF; GibcoTM) and incubated at 37°C, 5% CO_2 for 30 minutes. AF mimics the ECM and provides a surface upon which endothelial cells can adhere to and grow. AF was then removed, and the plate washed briefly with 2ml DPBS. Cryopreserved HCAECs were revived and cultured in Endothelial Cell Growth Medium MV (ECGM; PromoCell), supplemented with the corresponding Supplement Mix (PromoCell) and 1% PenStrep. ECGM was replaced every 2-3 days until 80-90% confluency was reached. Confluent HCAECs were trypsinised, counted, passaged and cryopreserved as described for primary skin fibroblasts.

For experimentation, 12-well plates were coated with 0.5ml AF per well for 30 minutes. This was removed and washed with 0.5ml DPBS, and the cells seeded at a density of $2X10^4$ per well. For serum starvation, HCAECs were bathed in Endothelial Cell Basal Medium (ECBM; PromoCell), with 0.1% BSA and 1% PenStrep for 24 hours prior to treatment.

2.7.3 Jurkat cells

Commercial Jurkat cells (ATCC) were cultured in T-75 flasks at 37°C, 5% CO_2 in Roswell Park Memorial Institute 1640 Medium (RPMI; Life Technologies), 10% FBS, 1% P/S. Initially, the cells were cultured in 15ml media, with 5ml added every 2-3 days to ensure sufficient nutrition. To further expand the cells, 1ml of the cell suspension was removed, and added to a new T-75 containing 15ml fresh media. As Jurkat cells grow in suspension, the flasks were stored in a vertical position to minimise adhesion and provide space for effective gas exchange. Cell counting and cryopreservation for Jurkat cells was completed as described for skin fibroblasts and HCAECs.

For experimentation, cells were centrifuged at 220 x g and resuspended in starving medium (RPMI, 0.1% BSA, 1% PenStrep). The cells were pipetted onto 12-well plates at a density of $2X10^4$ cells per well and serum starved for 24 hrs prior to treatment.

2.7.4 Human Hepatoma (HepG2) Cells

HepG2 cells (ATCC), were cultured in DMEM; Life Technologies, supplemented with 10% FBS,1% PenStrep, and grown in an incubator at 37°C, 5% CO_2. Before all experiments, cells were grown in T75cm^2 flasks, taking care to pass the cells through at 20-guage needle to avoid clumping. At 80-90% confluency, these were then removed using the trypsinization protocol described above and seeded onto 12-well plates at a density of $2X10^4$ cells/well. Cryopreservation was achieved as detailed prior.

2.8 Western blotting

2.8.1 Protein extraction and quantification

To extract the proteins from adherent cells (skin fibroblasts and HCAECs), media was aspirated, and the cells washed twice with 1ml PBS per well. 50µl ice-cold radioimmunoprecipitation assay (RIPA) buffer (Sigma-Aldrich) supplemented with cOmplete™ protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktails 2 (Sigma-Aldrich) and 3 (Sigma-Aldrich) was added to each well and incubated for 5 minutes. The cells were then scraped off the wells using a sterile

pipette tip. As Jurkat cells were grown in suspension, the cells were centrifuged at 220 x g for 5 mins, before being resuspended in 1ml PBS and centrifuged once more. 50µl of ice-cold RIPA lysis buffer was then added to the pellet and incubated for 5 mins. The lysates were centrifuged at 4°C, 13,000 rpm for 10 minutes and the supernatant taken.

Pierce™ bicinchoninic acid (BCA) Assay (Thermofisher Scientific) was used to determine the total protein concentrations of the cell lysates. The assay utilises a biuret reaction, in which peptide bonds within a protein can reduce light-blue divalent copper (Cu^{2+}) to monovalent copper (Cu^{1+}) in an alkaline environment. Cu^{1+} then reacts with bicinchoninic acid to produce a violet or purple product, which exhibits absorbance at 562 nm. The intensity of the colour is directly proportional to the concentration of peptide bonds in the sample. The concentration of protein can be estimated by comparing the absorbance of the sample with a standard curve generated from known concentrations.

The BCA working reagent was prepared according to the manufacturers' instructions. To generate a standard curve, pre-diluted BSA standards (Thermofisher Scientific) ranging from 125-2000 µg/mL were used. RIPA buffer was used as the 0 standard. 10µL of each standard, along with 200µL working reagent were pipetted in duplicate into 96-well plates. Similarly, in the same plate, 10µL of cell lysates with 200µL of BCA working reagent were introduced. The plate was incubated at 37°C for 30 minutes. Absorbance at 562 nm was measured using a Mithras LB 940 Multimode Microplate Reader (Berthold Technologies). Absorbance values were averaged and then plotted against the concentrations of the BSA standards. Linear regression was applied to create a line of best fit. The equation of the line was then used to determine the concentration of the cell lysates. Samples were stored at -20°C.

2.8.2 SDS-PAGE and protein transfer

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate the proteins within the cell lysates by molecular weight. The cell lysates were denatured to separate the proteins, by heating with 1X NuPAGE LDS Sample Buffer (Invitrogen) and 1X NuPAGE Sample Reducing Agent (Invitrogen) at 70°C for 10 minutes. The sample buffer and reducing agent act to

confer an overall negative charge on the proteins, allowing them to migrate down the gel towards the positively charged electrode.

Equal quantities (20µg) of protein were loaded into the wells of a pre-cast polyacrylamide hydrogel (NuPAGE 4-12% Bis-Tris Protein Gels; Invitrogen), alongside a SeeBlue Plus2 Pre-stained Protein Standard (Invitrogen), to estimate the molecular weight of the proteins. The gel was run in NuPAGE MOPS SDS Running Buffer (1X; Invitrogen) at 180 V for 1 hour.

Following gel electrophoresis, the proteins were wet transferred onto a nitrocellulose membrane of 0.2 µm pore size (GE Healthcare Life Sciences). The hydrogels, nitrocellulose membranes, filter paper and sponges were sandwiched into a cast containing NuPAGE Transfer Buffer (1X; Invitrogen) with 20% methanol at 30 V for 3 hours, with water on the outside of the cast to prevent overheating. Successful transfer was confirmed using Ponceau S solution (Sigma-Aldrich), a reversible stain used to detect protein bands on nitrocellulose membranes. Ponceau S staining was removed by washing the membrane with 1X PBS with 0.1% (v/v) Tween 20 (Sigma-Aldrich) for 5 minutes on a rocker.

2.8.3 Blocking and antibody incubation

Following successful protein transfer, the nitrocellulose membranes were blocked for 1 hour at room temperature with gentle agitation to reduce non-specific binding. One of two blocking buffers, 1X Casein Blocking Buffer (Sigma-Aldrich) and 1X TBST (Cell Signalling) with 5% (w/v) skimmed milk (Marvel) were used, depending on the primary antibody. After blocking, the membranes were incubated with the appropriate primary antibody in blocking buffer overnight at 4°C with rolling. The primary antibody binds to the protein of interest on the nitrocellulose membrane. A list of primary antibodies used, their species, dilutions and purchasing details are outlined in Table 2.1.

Table 2.1: Primary Antibodies for Western Blots

Primary Antibody	Species	Dilution	Company	Cat. No
CTGF	Rabbit	1:1000	Abcam	ab6992
Collagen Type I	Goat	1:3000	Merck Millipore	AB758
IL-6R	Rabbit	1:2000	Abcam	ab128008
STAT3	Mouse	1:200	R&D Systems	MAB1799

pSTAT3 (Tyr705)	Rabbit	1:1000	Cell Signalling	9131
ERK 1/2	Mouse	1:200	R&D Systems	MAB1576
pERK 1/2 (Thr202/Tyr204)	Rabbit	1:1000	Cell Signalling	9101
GAPDH	Mouse	1:10000	Abcam	ab8245
β-tubulin	Rabbit	1:5000	Abcam	ab15568

After overnight incubation, membranes were washed thrice with 1X PBST for 5 minutes each to remove any non-bound primary antibody. Membranes were then incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody in the same blocking buffer for 1 hour at room temperature with rolling. The secondary antibody binds to the primary antibody, already bound to the protein of interest. A list of secondary antibodies used, their species, dilutions and purchasing details are outlined in Table 2.2.

Table 2.2: Secondary Antibodies for Western Blots

Secondary Antibody	Species	Dilution	Company	Cat. No
Rabbit IgG; HRP linked	Goat	1:2000	Cell Signalling	7074S
Mouse IgG; HRP linked	Horse	1:2000	Cell Signalling	7076S
Goat IgG; HRP linked	Rabbit	1:2000	Agilent Dako	P044901-2

After secondary antibody incubation, membranes were washed thrice with 1X PBST for 5 minutes each to remove the non-bound secondary antibody. The HRP-conjugated secondary antibody allows for detection of the specific protein by enhanced chemiluminescence (ECL). HRP is an enzyme which oxidises luminol in the detection reagents to 3-aminophthalate, which emits light in the presence of phenols. The phenols act to increase the light output and prolong the duration of emission. A higher concentration of protein is directly correlated with a larger amount of HRP bound to the membrane. Similarly, higher concentrations of HRP correlate to more light emitted by the reagents. Equal volumes of Amersham ECL Western blotting Detection Reagents 1 and 2 (GE Healthcare) were mixed according to the manufacturers' instructions and applied to the membranes. This was left for 2 minutes before any excess reagent was removed.

2.8.4 Analysis

The emitted light was detected using the iBright™ CL750 Imaging System (Invitrogen), which captures the emitted photons. The signal was recorded and quantified by densitometric analysis using ImageJ (v2.9.0) (Schneider et al., 2012), to determine the relative abundance of the target protein in the original

sample. Relative protein expression was estimated by optical density (OD) value and normalised by dividing the sample OD by the housekeeping protein OD.

2.9 Camelid antibody production and screening

2.9.1 Camel immunisation and immune response investigation

Immunisation and blood sampling was carried out by Dr Babiker (ADLQ). Prior to commencing immunisations, a 5ml jugular vein sample of whole blood from adult, male camels were collected in PAXgene RNA tubes (Qiagen). 2.5ml was used for RNA isolation and 2.5ml for serum extraction. Camels were immunised with 200µg of either sIL-6R (ProspecBio) or Truncated CTGF (PeproTech) in Complete Freund's Adjuvant (FCA; Sigma) for a total injection volume of 1.2ml (600µl of antigen + 600µl of FCA), injected subcutaneously at 4 different sites. 4 weeks post initial (1st) immunisation, 5ml whole blood was collected, and a second injection of sIL-6R (200µg) or CTGF (200µg) in Incomplete Freund's Adjuvant (Sigma) was delivered (total injection volume of 1.2ml). 4 weeks from the 2nd dose, 5ml whole blood was collected. A 3rd immunisation was completed (8 weeks post-initial injection) with sIL-6R or CTGF in Incomplete Freund's Adjuvant (200µg, total injection volume 1.2ml), and a further 5ml whole blood sample taken.

Serum was separated from pre- and post-immune blood samples by centrifugation at 2500 rpm for 15 minutes. The strength of the immune response to the antigen was measured by ELISA. Blood samples determined to be at the plateau phase of antigen response were used for heavy chain only antibody and V_HH fragment production.

To investigate the immune response, Nunc Maxisorp (Thermo Fisher Scientific) plated were coated with 100µl of 5µg/ml CTGF or sIL-6R in a carbonate-bicarbonate coating buffer (15mM Sodium carbonate, 10mM sodium bicarbonate, pH 9.6) overnight at 4°C. The wells were decanted and washed with 300µl phosphate buffered saline (PBS) 0.05% w/v Tween20 (PBST) twice. This was then blocked with 300µl 3% BSA in PBS for 1 hour at 37°C, and further washed with PBST as stated above. 100 µl of pre-immune and post-immune sera in different dilutions (1:4, then 6-10 4-fold serial dilutions were used). Incubation was performed at room temperature for 1 hour, and the plate washed 5 times with

PBST. 100µl of anti-camel HRP (Alpha Diagnostics International) was used as a secondary antibody at 1:2000 dilution. This was incubated for 1 hour at room temperature and washed with PBST. 100µl of TMB substrate solution (5 mg TMB tablet (Sigma Aldrich), dissolved in 5ml DMSO (Sigma Aldrich) in 0.05M Phosphate-citrate buffer, pH 5.0) was added and observed until colour change developed (3-4 min). The reaction was quenched with 2M Sulfuric acid, and the absorbance was measured at 450nm using a spectrophotometer.

2.9.2 Production and purification of polyclonal camelid antibodies

Anti-CTGF conventional (IgG1) and HcAbs (IgG2/IgG3) were purified from the immune sera using Protein A and Protein G chromatography (**Figure 2.2**). Purification of all IgGs was achieved using Cytiva HiTrap Protein G and Protein A column chromatography in accordance with manufacturer's instructions. The conventional IgG1 antibody, with both heavy and light chains. IgG2 and IgG3 are heavy chain only, however IgG3 is characterized by a shorter hinge region (12 amino acids in length, compared to 35 amino acids for IgG2). IgG1 and IgG3 both show adsorption onto Protein G columns, likely because of their similar amino acid hinge region sequence (Fig 5). They were differentially eluted with buffers of different pH. Buffer A (NaCl + acetic acid buffer-pH 3.5) was used to elute IgG3, and Buffer B (Glycine buffer-pH 2.7) was used to elute IgG1. Since IgG2 does not adsorb onto the Protein G column, the flow through was collected and run on a Protein A column. IgG2 was eluted from the Protein A column using Buffer A.

Flow through, wash and elution fractions were collected and a spectrophotometer was used to measure absorbance of each fraction at 280nm. The eluted fractions were collected and the concentration of antibody subclass was determined by BCA assay. The purity of these fractions was assessed by SDS PAGE and Western blot, and their antigenicity was assessed by ELISA.

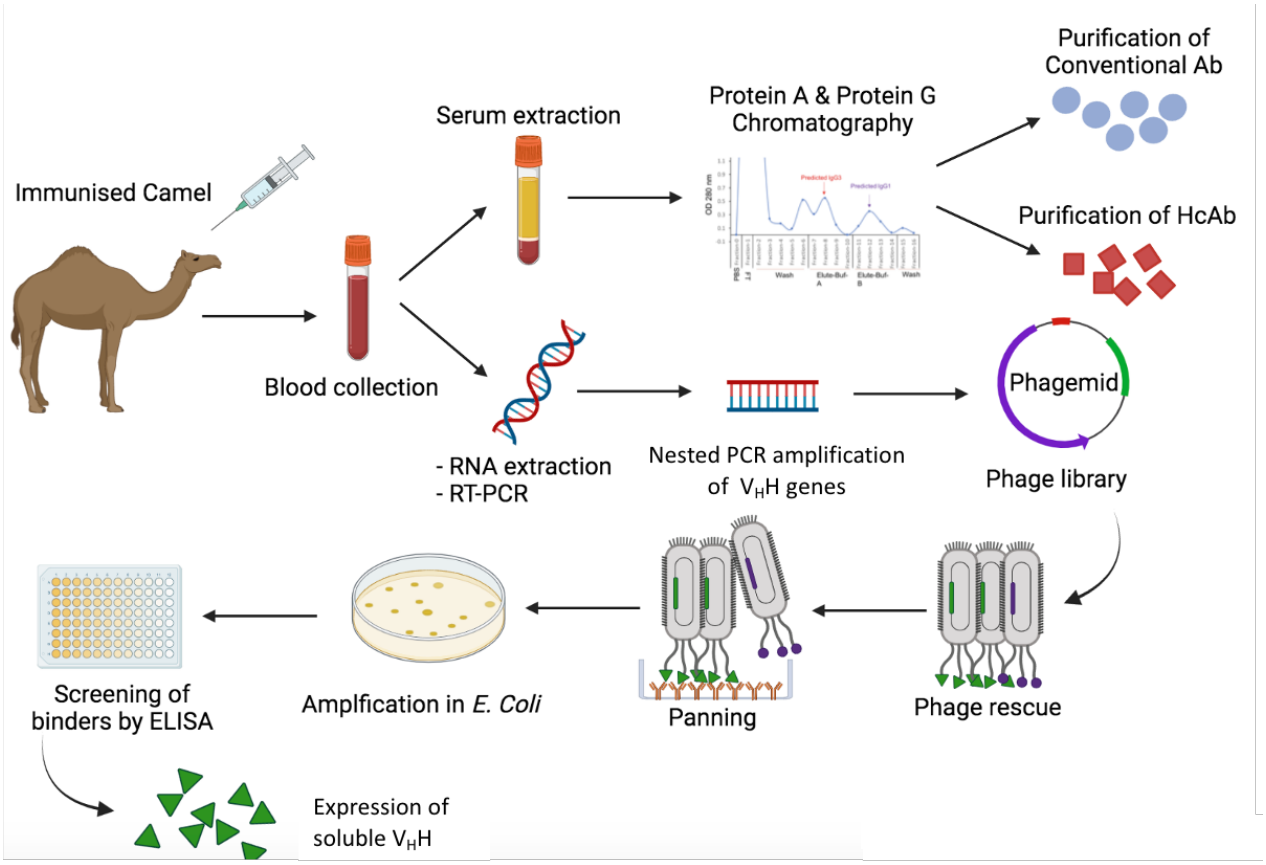


Figure 2.2: Production of conventional antibodies, HcAbs and V_HH fragments. Schematic detailing methodology from immunisation to purification and expression. Produced using BioRender.

2.9.3 V_HH fragment antibody production

Anti-sIL-6R and anti-CTGF V_HH fragment antibodies were produced by phage display technology using methods outlined previously (Pardon et al., 2014) (**Figure 2.2**). Production and sequencing of clones was completed prior to commencing the project by ADLQ. The process involved blood sampling from immunised camels at the plateau phase of their immune response. RNA was extracted from these samples and used to amplify the V_HH transcripts by nested PCR. These transcripts were then expressed in antigen presenting bacteriophages, and panning was used to purify and select specific binders. These clones were amplified in *E. Coli* before purifying soluble V_HH fragments.

2.9.3.1 RNA Isolation and cDNA Synthesis

Isolation of RNA was achieved using the total RNA extraction protocol from the PAXgene Blood RNA kit (Qiagen). Concentration and purity were assessed using

the Nanodrop 2000 Spectrophotometer. Measurements of optical density (OD) at 260 nm and 280 nm were taken, and the purity of the extracted RNA was evaluated as a ratio of these, in which a reading between 1.7 and 2.0 was considered acceptable purity.

Further analysis of RNA quality was conducted using Agilent 2100 Bioanalyzer. RNA was evaluated as a RIN (RNA Integrity Number), showing the ratio of 18S to 28S ribosomal subunits. A pure, high-quality sample shows two clear bands on the electropherogram, corresponding to those subunits. A RIN ≥ 5-6 and a clear 2-peak gel images were considered non-degraded RNA and were stored at -80°C. Extracted RNA was converted into cDNA using the High-Capacity cDNA Reverse Transcription (RT) Kit (Thermofisher Scientific), according to manufacturer's instructions under specific cycling conditions (**Table 2.3**).

Table 2.3: PCR Thermocycling Conditions for Reverse Transcription

	Step 1	Step 2	Step 3	Step 4
Temperature (°C)	25	37	85	4
Time (min)	10	120	5	∞

2.9.3.2 Amplification of V_HH Genes

A nested PCR was used for the amplification of transcripts for the immune library, using cDNA synthesised from camels immunised with sIL-6R. The DNA sequences coding for the heavy chain only antibodies were amplified initially, using gene specific primers, CALL001 (forward) and CALL002 (reverse), under specific cycling conditions (**Table 2.4**).

Table 2.4: PCR Thermocycling Conditions for HcAbs Amplification

	Step 1	Step 2			Step 3	Step 4
No. of cycles	1		2-31		32	-
Temperature (°C)	95	94	57	72	72	4
Time (min)	3min	1min	1min	45sec	7min	∞

The PCR products were analysed by gel electrophoresis on a 1% agarose gel containing ethidium bromide, at 30V for 1 hour, run alongside a 100bp DNA ladder (Sigma Aldrich). Two DNA fragments were amplified in the gel. One of ~1000bp, representing the conventional antibodies, and another of ~700bp,

corresponding to the heavy chain only antibody repertoire. The smaller, 700bp band was then cut out of the gel and purified using the QIAquick Gel Extraction Kit (Qiagen).

The second PCR amplified the V_HH fragments from the previously purified heavy chain only antibody transcripts. The forward (ADL2 sense and ADLR), and the reverse (ADL Anti-sense with ADL2 Anti-sense) primers were used to generate a fragment ~400 bps, corresponding to the V_HH fragment, under specific cycling conditions (**Table 2.5**). The purity of the product was confirmed by gel electrophoresis, as detailed above.

Table 2.5: PCR Thermocycling Conditions for V_HH Amplification

	Step 1	Step 2			Step 3	Step 4
No. of cycles	1		2-16		17	-
Temperature (°C)	94	94	55	72	72	4
Time (min)	5min	30sec	30sec	30sec	7min	∞

2.9.3.3 Vector Digestion and Test Ligation

The vector used for bacteriophage expression was the pADL™- 22c Phagemid Vector (Antibody Design Laboratories). The vector contained a Poly-histidine (HIS) tag, a Hemagglutinin (HA) tag and an ampicillin resistance gene (**Figure 2.3).** This was digested with restriction enzymes and ligated with the insert, before being transformed in bacterial cells.

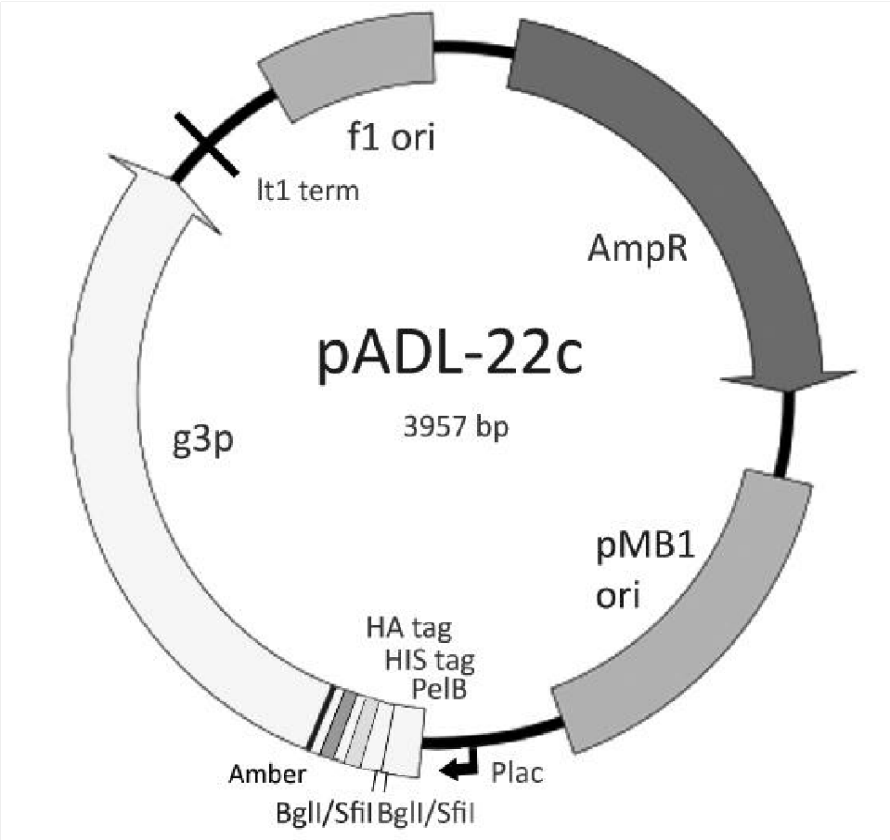


Figure 2.3: Vector Map of pADL™- 22c Phagemid Vector. Illustration of Antibody Design Laboratories pADL-22c Phagemid vector, including location of ampicillin resistance gene, HA and HIS tags. From Antibody Design Laboratories pADL-22c manual. *(*PADL-22c Phagemid*, n.d.)*

Restriction enzyme digestion of the phage display vector was carried out using BglI (NEB) according to the manufacturers' instructions. Digestion of the PCR product, containing the 400bp V_HH fragment, was achieved using SfiI (NEB). These were left on a heat block overnight at 37˚C, and the PCR product and vector were purified using a PCR Purification Kit (Invitrogen). These were then test ligated (1:3 molar ratio of Vector and insert) with T4 DNA Ligase (Thermofisher Scientific) for at 16^0C for overnight and purified using the QIAquick PCR purification kit (Qiagen).

2.9.3.4 Transformation and Screening

TG1 electrocompetent *E. coli* cells (Lucigen) were transformed with the purified, ligated vector. 25 µL of cells were incubated with 5 µL of the purified ligation on ice for one minute, before the mixture was electroporated (Gene Pulser Xcell). The cells were diluted with 1 mL of Recovery medium (Lucigen) and incubated in

a shaker at 37˚C for 1 hour at 200 rpm. Serial dilution of these bacteria (1:100; 1:1000; 1:10000, etc) were then plated on an LB agar plates with ampicillin (100µg/ml), 2% glucose and grown at 37˚C overnight.

The following day, 30 colonies were picked, and colony PCR was performed to check for the presence of nanobody gene. The primers used were Phis2 (forward) and PSIR2 (reverse) primers, which are vector-specific and code for the flanking regions of the V_HH insert.

2.9.3.5 Amplification of Transformation

Once at least 75% transformants were confirmed to have the insert, the experiment was amplified. The ligation was repeated as described above, with 15-25 times the amount of vector and insert, and the product purified. TG1 cells were incubated with the purified ligation mixture and electroporated. The cells were then grown in LB for 1 hour and plated onto LB agar plates with ampicillin as stated previously. The colonies were taken with a sterile scraper the next day and stored in LB with 20% glycerol at -80˚C for long term preservation.

2.9.3.6 Rescue and Amplification of Phage

10^9 cells of the immune library were grown in 60 mL of 2xTY medium, containing 100 µg/mL ampicillin and 2% glucose until they reached the mid logarithmic phase. This was determined by an OD (600nm) of 0.5-0.6. M13K07 Kanamycin resistant helper bacteriophage (Strategene) were added at 10-20 times the number of bacteria present. The mixture was kept at room temperature for 30 mins without any shaking for optimum phage adsorption. Infected cells were centrifuged, and the pellet was resuspended in 50 mL of 2xTY medium supplemented with 100 µg/mL ampicillin and 50 µg/mL kanamycin and incubated overnight at 37C, 200 rpm for amplification of nanobody-displaying phage. The next day, the bacteria were pelleted by centrifugation and the phage particles were precipitated using 20% Polyethylene Glycol (PEG6000; Sigma Aldrich), 2.5 M Sodium chloride, centrifuged (3200g, for 10 min), and resuspended in 500 µL of PBS.

2.9.3.7 Panning, Sequencing and Expression

Once the phagemid particles had been collected, the binders were selected for by panning. 96well microtiter plates (Nunc Maxisorp, Thermofisher) were coated with 5 µg/mL sIL-6R in a coating buffer and left overnight at 4℃. The following day, the plates were aspirated and washed 3x with PBS before being blocked with 1% skimmed milk (Marvel) in PBS for 2 hours. The blocking solution was decanted, and the wells washed PBS 0.1% Tween20. $5x10^{12}$ of phagemid virion in 200 µL of PBS with 2% milk were pipetted into each well. 2% skimmed milk in PBS was used as a negative control. The plates were incubated at room temperature for 2 hours before washing with PBST 0.05% Tween20 to remove unbound phage. Binders were eluted with 100ul trypsin per well. This mixture containing the eluted viruses was used to infect TG1 bacterial cells within their mid logarithmic phase (OD_{600} of 0.5-0.6). This was placed in in 2xTY medium, 100 µg/ml ampicillin, 2% glucose and incubated overnight at 37ºC on shaker. Glycerol stocks were made and stored at -80ºC.

Serial dilutions of infected TG1 cells were plated on LB agar plates with glucose overnight. Colonies formed on the plates, and each colony was inoculated into 1mL of 2xTY medium. The expression of the V_HH was induced with 1mM Isopropyl-β-D-thiogalactoside (IPTG; Sigma). The expressed fragments were collected from the periplasm by osmotic shock using Tris EDTA (Sigma) and 20% sucrose. Sequencing of V_HH clones and production was carried out by Ebbil LTD, in collaboration with ADLQ (anti-sIL-6R n=19, anti-CTGF n=1).

2.9.4 Characterisation of camelid antibodies

2.9.4.1 Optimization of secondary antibody

Secondary antibodies to be used for each antibody subclass were investigated by ELISA. 100µg/ml of anti-sIL-6R IgG1, IgG2/3 and V_HH, 1% BSA or non-immune serum were coated on a Nunc Immuno Maxisorp plate overnight at 4°C and then washed thrice with PBST. The plates were then blocked with 300µl 3% BSA in PBS for 1 hour and three types of secondary antibodies were incubated at a 1:2000 dilution. The secondary antibodies used were anti-camel IgG-HRP

(AD; Alpha Diagnostics), anti-camel IgG1 (AO; Antibodies Online) and anti-camel IgG2/3 (JI; Jackson Immuno Research).

2.9.4.2 Characterisation of antibodies in non-immune serum

Whole blood samples were taken via the jugular vein from 95 camels, and non-immune serum was separated by centrifugation. The total expression of the different IgG subclasses was determined by dot blot. In triplicate, 10μL of the serum from each camel was pipetted onto a nitrocellulose membrane. This was washed and blocked, and the different secondary antibodies were used to determine the expression of each subclass. AD was used as an overall anti-camel IgG secondary antibody, while AO and JI were used to estimate expression of IgG1 and IgG2/3 respectively. Results were analysed by densitometry using Image J.

2.9.4.3 Characterisation of antibodies in immune serum

Whole blood samples were taken via the jugular vein from an adult, male camel at baseline (pre-immune), and after four immunisations with CTGF over a 24-week period. The total expression of the different IgG subclasses was determined by dot blot. The secondary antibodies used were anti-camel IgG-HRP (AD; Alpha Diagnostics), anti-camel IgG1 (AO; Antibodies Online) and anti-camel IgG2/3 (JI; Jackson Immuno Research). Results were analyzed by densitometry using Image J (v 2.9.0)(Schneider et al., 2012).

2.9.5 Antibody Screening

Nunc maxisorp microtiter plates were coated with 100μl 5μg/ml of CTGF or sIL-6R in a carbonate-bicarbonate coating buffer or 1% BSA overnight at 4°C. The wells were decanted and washed with 300μl phosphate buffered saline (PBS) 0.05% w/v Tween20 (PBST) twice. This was then blocked with 300μl 3% BSA in PBS for 1 hour, and further washed with PBST as stated above. 100μl of anti-CTGF or anti-sIL-6R conventional (IgG1), HcAbs (IgG2/3) and V_HH fragments were incubated at concentrations of 100, 10, 1 and 0.1μg/ml for 1 hour before the plate was washed with PBST 5 times. Secondary antibodies used were anti-camel IgG-HRP (AD; Alpha Diagnostics), anti-camel IgG1 (AO; Antibodies

Online) and anti-camel IgG2/3 (JI; Jackson Immuno Research) at a dilution of 1:2000. This was incubated for 1 hour at room temperature and washed with PBST. 100µl of TMB substrate solution (5 mg TMB tablet (Sigma Aldrich), dissolved in 5ml DMSO (Sigma Aldrich) in 0.05M Phosphate-citrate buffer, pH 5.0) was added and observed until colour change developed (3-4 min). The reaction was quenched with 2M Sulfuric acid, and the absorbance was measured at 450nm using a Mithras LB 940 Multimode Microplate Reader (Berthold Technologies).

Chapter 3

Identification of cytokine targets for SSc using proteomics

3.1 Introduction

SSc is a complex autoimmune disorder, involving both genetic and environmental triggers, which lead to alterations in protein and metabolite expression. These alterations are associated with inflammation, fibrosis and vasculopathy (Solomon et al., 2013). The highest morbidity and mortality rates in SSc have been observed among patients with cardiopulmonary comorbidities. Leading pulmonary complications linked to mortality are SSc-PAH and SSc-PF (Elhai et al., 2017).

Various systemic cytokines and growth factors have been implicated in the pathogenesis of SSc, including TGFβ, IL-6 and CTGF (Brown & O'Reilly, 2019; Denton & Abraham, 2001). However, due to overlapping mechanisms, it is unclear, particularly in the early stages of disease, which pathways lead SSc patients to develop PAH, PF or no lung disease (NLD). While it has been suggested that SSc-PAH occurs due to metabolic dysfunction, and SSc-PF is the more inflammatory disease, further research is needed to identify specific targets which may guide future therapeutic development (Deidda et al., 2017; Paulin & Michelakis, 2014; Schoenfeld & Castelino, 2015).

Current treatment options for SSc-PAH and SSc-PF are limited, and few confer survival advantages to patients (Almaaitah et al., 2020). For SSc-PAH, drugs are aimed at improving vasodilation to reduce pulmonary arterial pressure. The prostaglandin epoprostenol, a potent vasodilator, and phosphodiesterase type 5 inhibitors (PDE-5Is) such as sildenafil and tadalafil, have been shown to improve exercise capacity, but not survival, in SSc-PAH (Badesch et al., 2000, 2007).

Combination therapies have shown some promise with regards to survival in PAH, however, it is unclear whether this approach may prove effective in SSc-PAH (Parikh et al., 2019). Therapeutic options for SSc-PF include immunosuppressants mycophenolate mofetil (MMF) and methotrexate (MTX). While these are effective for treatment of skin manifestations, their efficacy in SSc-PF requires further study, and their severe toxicities and side effects render long-term treatment plans problematic (Adler et al., 2018; Zhao et al., 2016). Biologics such as rituximab (RTX), which depletes $CD20^+$ B cells, and tocilizumab (TCZ), the IL-6R antagonist have indicated beneficial effects on lung function in SSc patients, however they are not specific for SSc-PF (Adler et al., 2018; Roofeh et al., 2021; Zhao et al., 2016). Recently, the anti-CTGF monoclonal antibody pamrevlumab was suggested to slow down disease progression in IPF, however in phase III clinical trials, this was not found to be effective (Richeldi et al., 2020). Discovery of robust biomarkers for SSc-PAH and SSc-PF could help to identify differences in their inflammatory pathways, and allow for detection of new, more specific, therapeutic targets. In recent years, omics-approaches, in particular proteomics, have made strides in detecting disease biomarkers.

Proteomics refers to the large-scale analysis and characterization of proteins. The concept of the 'proteome' was described as the separation and identification of the entire protein complement expressed by the genome of a cell, tissue or organism (Wasinger et al., 1995; Wilkins et al., 1996). Early techniques involved 2-dimensional gel electrophoresis, which separated proteins based on their molecular weights and isoelectric points, generating a unique protein signature, which could be compared against those of different disease states or experimental conditions (Wilkins et al., 1996). Technological advances in later years allowed for the use of mass spectrometry as the primary method of choice for proteomic analyses (Aebersold & Mann, 2003). Data generated through proteomics, both quantitative and qualitative, can aide in understanding the complexity and dynamics of protein expression, including structure, function and post-translational modifications. In disease applications, proteomics can help to elucidate protein functions, identify biomarkers, understand signalling pathways, and develop targeted therapies.

Within the context of SSc, proteomics studies have proven useful in understanding the molecular basis of the disease. The secretome of lung fibroblasts treated with TGF-β1, which has known implications in SSc, uncovered eleven proteomic biomarkers also identified in biological fluids of SSc patients (Kendall et al., 2021). Cultured dermal fibroblasts from patients with both SSc and nephrogenic systemic fibrosis, a non-autoimmune fibrotic condition, identified a 'fibrotic signature' of nine molecules, however these were not specific for SSc, nor did they differentiate between SSc-associated comorbidities (Del Galdo et al., 2010).

More recently, proteomics has been used to investigate protein expression in SSc-PAH and SSc-PF specifically. Serum from SSc-PAH patients has been reported to exhibit significantly increased concentrations of the chemoattractant chemerin, the growth factor midkine, and the glycoprotein follistatin-like 3 (FSTL3) compared to SSc patients with no PAH (Rice et al., 2018; Sanges et al., 2023). Another study proposed collagen IV, endostatin, insulin-like growth factor binding protein (IGFBP)-2, IGFBP-7, matrix metallopeptidase-2, neuropilin-1, N-terminal pro-brain natriuretic peptide and RAGE as discriminators between SSc-PAH and non-PAH (Bauer et al., 2021). In SSc-PF, proteomic analysis of bronchoalveolar lavage fluid (BALF) identified S100A6 and 14-3-3ζ, among other proteins known to be involved in inflammation (Landi et al., 2019; Rottoli et al., 2005). To date, studies comparing alterations in protein expression between SSc-PAH, SSc-PF and SSc patients with no lung disease have been scarce.

The aim of this chapter was to use proteomics to uncover systemic cytokines and growth factors that are specific to SSc-PAH and SSc-PF and identify pathways that differentiate these patients early in the disease.

3.2 Results

3.2.1 Clinical characteristics

Patient and healthy control sampling was carried out over a period of 15+ years (2000-2019) by the Department of Rheumatology, Royal Free Hospital, and stored at -80^{o}C. Participants (n=120) were categorized into four groups of equal number (**Figure 2.1**) under stringent clinical criteria: SSc-PAH, SSc-PF, SSc-NLD and HC. To limit population differences, all selected participants were European Caucasian. Participants were age-matched, to ensure protein concentrations were not due to variations in age, as circulating levels of pro-inflammatory cytokines have been shown to increase with age (López et al., 2018).

For SSc-PAH and SSc-PF, the first available sample from onset of pulmonary symptoms was used, to investigate biomarkers early in the disease. SSc-NLD patients were selected after minimum 5 years from SSc diagnosis, to ensure no lung complication development. Patients were also selected based on their autoantibody profiles (**2.2 patient recruitment and inclusion criteria**). Key cohort demographics and clinical characteristics are outlined in **Table 3.1.**

Table 3.1: Cohort demographics and clinical characteristics

Features	HC	SSc-NLD	SSc-PAH	SSc-PF
Participants (n)	30	30	30	30
Age (years)	46.1 ± 19.4	56.9 ± 8.6	59.9 ± 9.3	66.0 ± 11.6
Sex (Male: Female)	20:10	03:27	02:28	07:23
Disease duration (years)	NA	10 ± 4.7	8 ± 6.6	8 ± 4.7
Autoantibodies ACA n (%)	NA	16 (53.3)	30 (100)	0 (0)
Autoantibodies ATA n (%)	NA	14 (46.7)	0 (0)	30 (100)
Limited Subtype n (%)	NA	24 (80)	30 (100)	23 (76.6)
Diffuse Subtype n (%)	NA	6 (20)	0 (0)	7 (23.3)

Data are shown as mean ± SD, or n (%). ACA = Anti-centromere autoantibodies; ATA = Anti-topoisomerase autoantibodies; NA = not applicable. Differences in age were not significant (p = 0.36).

3.2.2 Cytokine identification using proteomics

Proteomics was carried out using the Olink platform cardiovascular II and immuno-oncology 96 plex immunoassay panels. Olink platform-based proteomics identified a total 167 plasma proteins using the cardiovascular II and immuno-oncology 96 plex immunoassay panels. Statistical analysis, utilizing a one-way ANOVA, identified 12 cytokines significantly altered between the four groups. These were IL-10, IL-12, IL-16, MCP-1, MCP-3, MCP-4, IL-33, IL1RL2, IL-6, IL-8, IL1RA and TNFRSF4. **Table 3.2** demonstrates the relationships between groups for each of the 12 significantly altered cytokines.

Table 3.2: Significantly altered cytokines by group

Cytokines	SSc-NLD	SSc-PAH	SSc-PF
IL-10	↑▲		
IL-12		↑▲◊	
IL-16		↑▲	
MCP-1			↑▲□●
MCP-3			↑▲□●
MCP-4			↑▲□●
IL-1RL2			↑▲
IL-33			↑▲
IL-6	↑▲	↑▲	↑▲
IL-8		↑▲	↑▲
IL-1RA		↑▲	↑□
TNFRSF4	↑▲	↑▲□◊	↑▲↓●

Significantly altered cytokines ($p<0.05$); ↑ up-regulated, ↓ down-regulated, ▲ comparison to HC, □ comparison to SSc-NLD, ● comparison to SSc-PAH, ◊ comparison to SSc-PF

The NPX scores of 8 cytokines were significantly increased in only one of the four groups. These were considered specific markers. For SSc-NLD, IL-10 was significantly increased compared to HC. In SSc-PAH, IL-12 was significantly higher compared to HC and SSc-PF, while IL-16 was significantly increased in SSc-PAH compared to HC only. MCP-1, MCP-3 and MCP-4 had significantly increased NPX scores in SSc-PF compared to all other groups. IL-33 and IL-1RL2 were both significantly higher in SSc-PF compared to HC. **Figure 3.1** illustrates the differences in NPX scores by group for these specific markers.

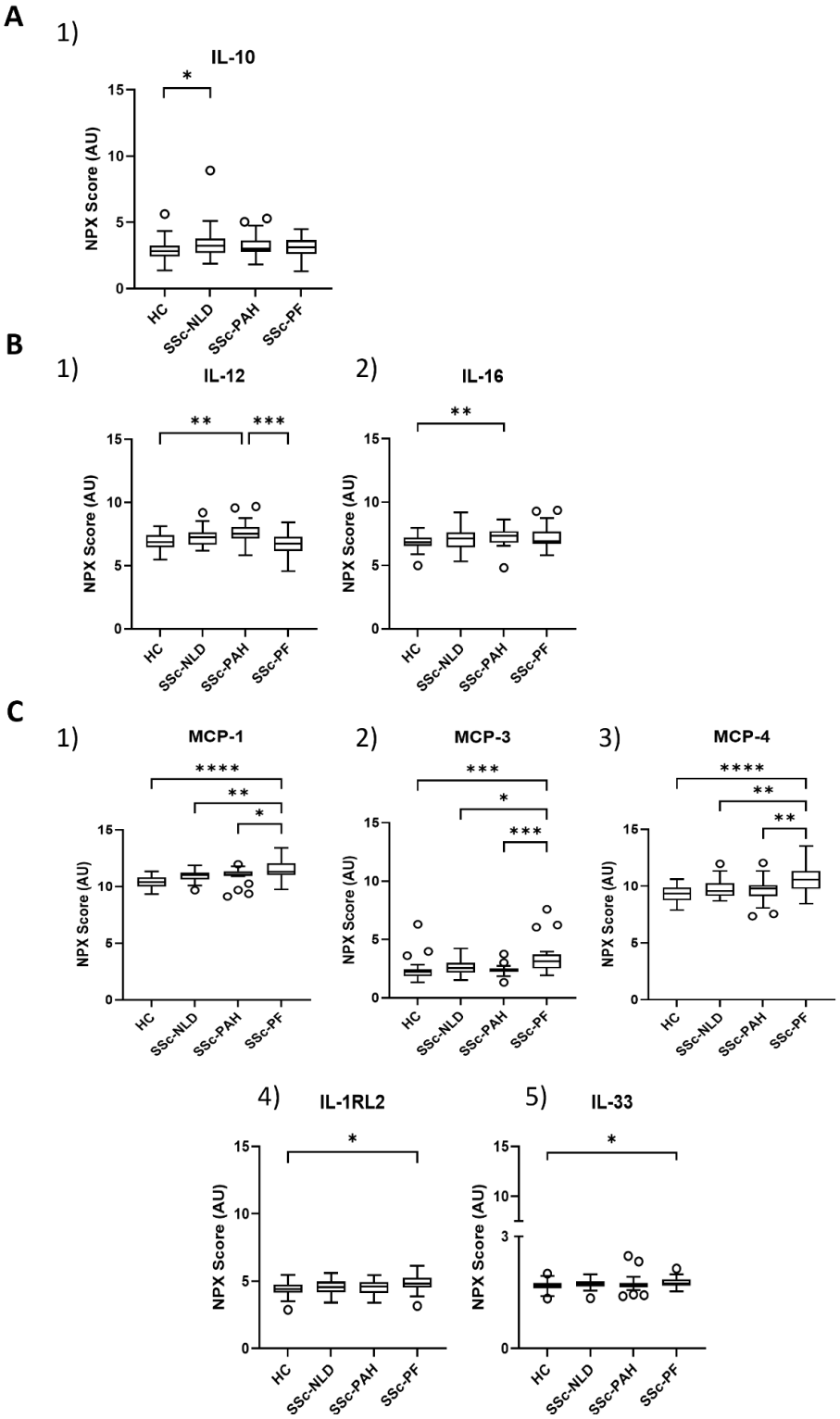


Figure 3.1: Proteomics uncovered 8 specific markers differentiating between SSc-NLD (n=30), SSc-PAH (n=30), SSc-PF (n=30) and HC (n=30). Boxplots detailing changes in NPX (AU) scores by group for 8 cytokines, with significant differences in **(A)** SSc-NLD; IL-10, **(B)** SSc-PAH; IL-12, IL-16 and TNFRSF4 and **(C)** SSc-PF; MCP-1, MCP-3. MCP-4, IL-1RL2 and IL-33. A one-way ANOVA was used for analysis. Sig. indicated by * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, **** = $p<0.0001$.

Four cytokines had NPX scores which were highest in more than one group and were not robust enough to differentiate between SSc-PAH and SSc-PF. These were considered non-specific markers. IL-6 was significantly increased in all SSc groups compared to HC. IL-8 was increased in SSc-PAH and SSc-PF compared to HC. IL1RA was increased in SSc-PAH compared to HC, and in SSc-PF compared to SSc-NLD. TNFRSF4 was significantly upregulated in SSc-NLD compared to HC and in SSc-PAH compared to HC, SSc-NLD and SSc-PF. In SSc-PF, TNFRSF4 was significantly higher compared to HC. **Figure 3.2** details boxplots for the non-specific markers.

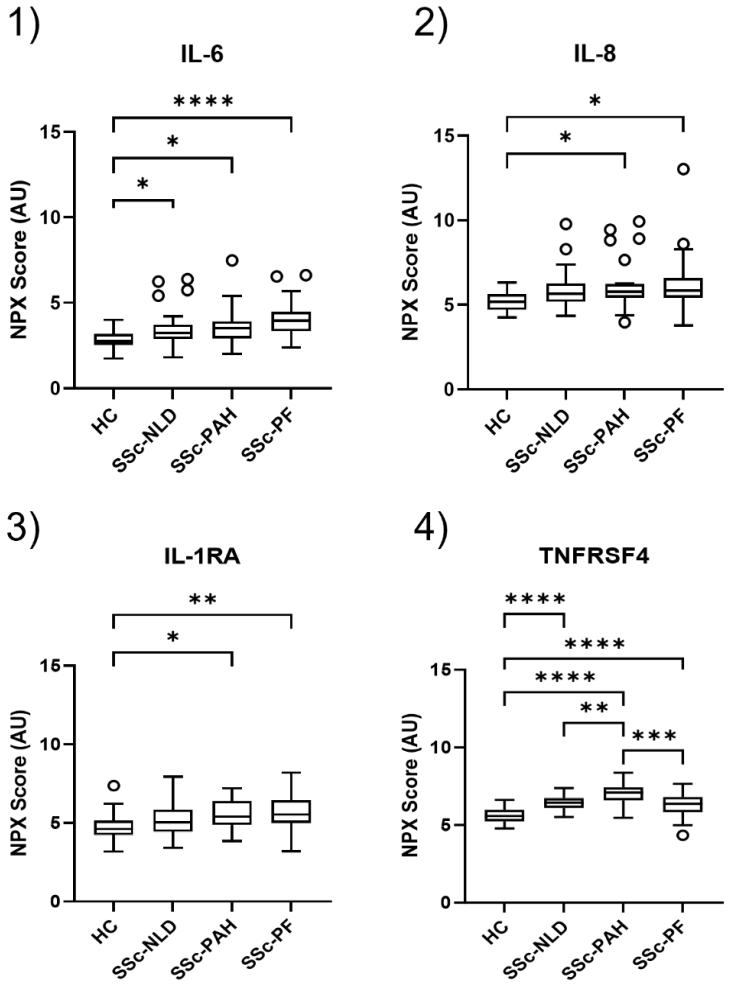


Figure 3.2: Proteomics identified four non-specific markers, elevated in more than one group of the four groups (SSc-NLD (n=30), SSc-PAH (n=30), SSc-PF (n=30) and HC (n=30)) . Boxplots detailing changes in NPX (AU) scores by group for non-specific cytokines; **(1)** IL-6, **(2)** IL-8, **(3)** IL-1RA and **(4)** TNFRSF4. A one-way ANOVA was used for analysis. Sig. indicated by * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, **** = $p<0.0001$.

3.2.3 Cytokine validation and investigation

Validation of 2 cytokines (17%) from the 12 shown to be significantly altered (**Table 3.2**) was completed. Serum levels of IL-6 and MCP-1 were measured by ELISA (**Figure 3.3A**). CTGF, as well as the soluble receptors of the IL-6 trans signalling pathway (sIL-6R and sgp130) were not part of the Cardiovascular II and Immuno-oncology 96 plex immunoassay panels, therefore these were also investigated by ELISA in the same cohort (**Figure 3.3B**).

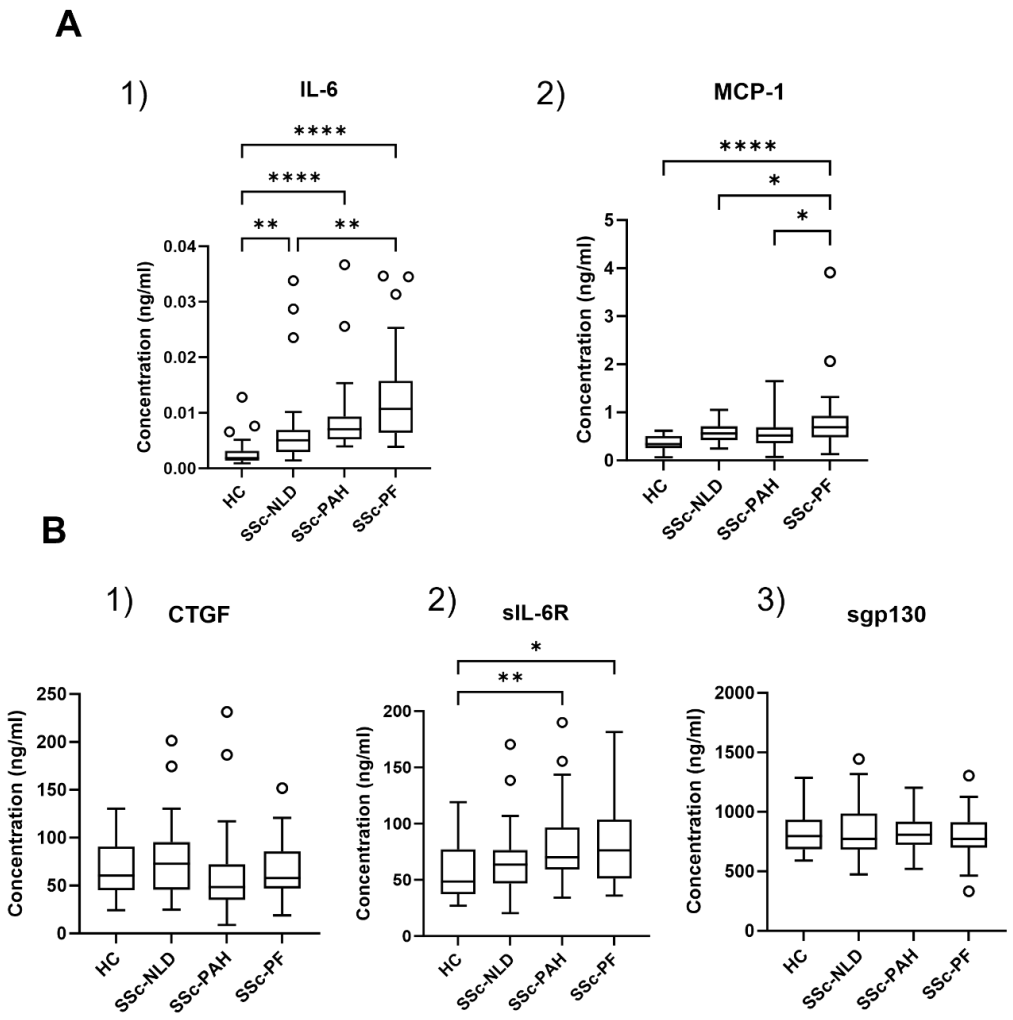


Figure 3.3: ELISA validation and investigation of systemic cytokines in SSc-NLD (n=32), SSc-PAH (n=32), SSc-PF (n=32) and HC (n=32). Serum concentrations of cytokines were measured in all groups **(A)** IL-6 and MCP-1 systemic concentrations were measured to validate proteomics data. Boxplots detailing serum concentrations (ng/ml) of IL-6 and MCP-1 were produced. **(B)** Serum concentrations (ng/ml) of CTGF, and trans signalling molecules sIL-6R and sgp130 were also investigated in the same cohort. A one-way ANOVA was used for analysis. Sig. indicated by * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, **** = $p<0.0001$.

IL-6 was significantly upregulated in all SSc groups compared to healthy controls, and significantly upregulated in SSc-PF compared to SSc-NLD. No significant difference was found in IL-6 expression in SSc-PF vs SSc-PAH as observed via proteomics. MCP-1 was significantly increased in SSc-PF compared to all other groups, as shown via proteomics (**Figure 3.3**). The soluble receptor for IL-6 (sIL-6R) has been suggested to be the receptor driving pro-inflammatory trans signalling. When measured by ELISA, sIL-6R was found to be upregulated in both SSc-PAH and SSc-PF compared to healthy controls, supporting the idea that sIL-6R is involved in inflammatory disease. There was no significant difference in the levels of the sgp130, or CTGF between the groups.

The interactions between these cytokines were analysed using Spearman's correlation analysis, and four significant associations were detected. When all participants were pooled together, there was a significant positive correlation between IL-6 and sIL-6R, and between IL-6 and MCP-1. In the SSc-NLD group, sgp130 was positively correlated with IL-6. This may be a protective mechanism against PAH and PF development in SSc patients. In SSc-PF, MCP-1 was significantly positively associated with CTGF (**Figure 3.4**).

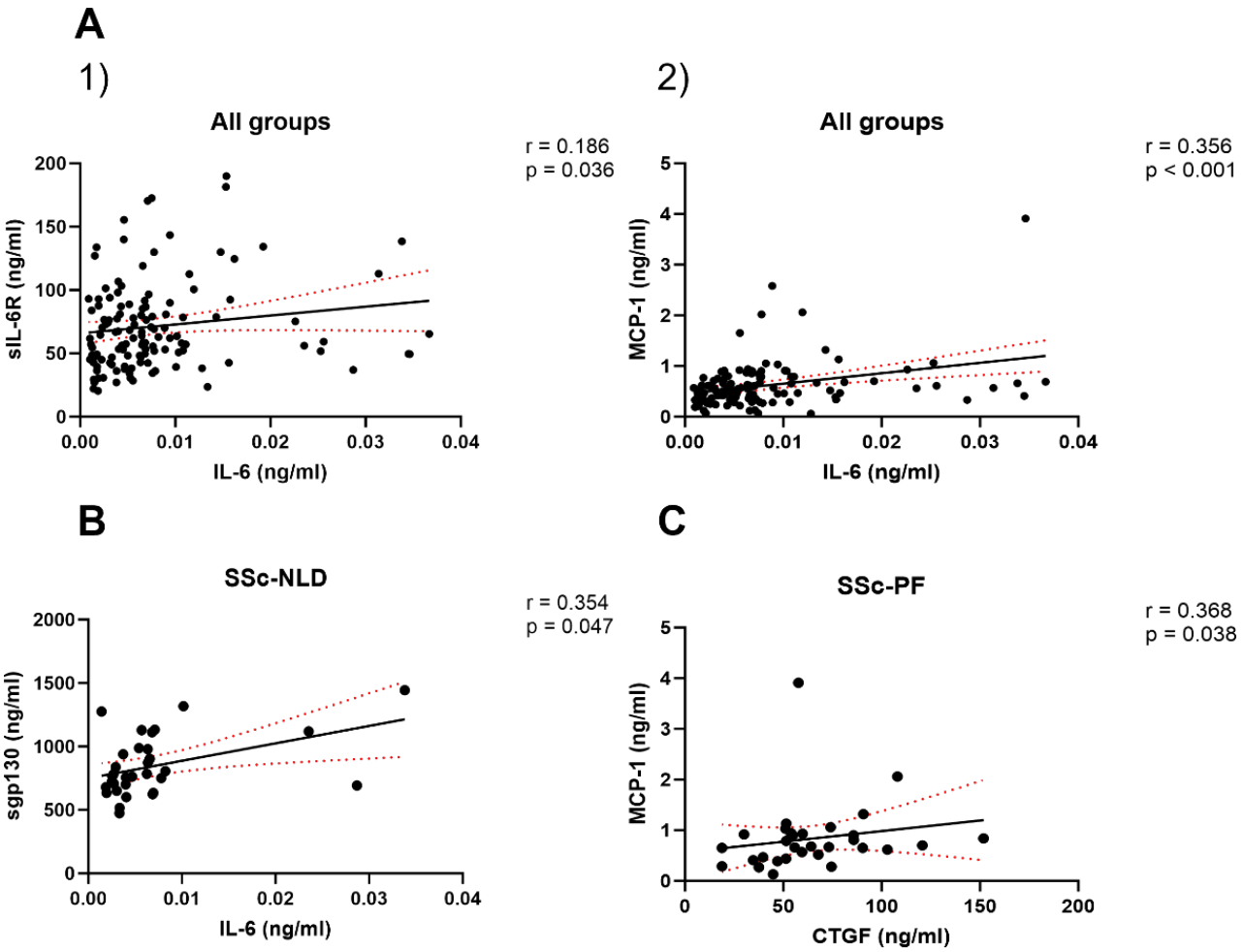


Figure 3.4: Correlation analysis of cytokines investigated by ELISA in SSc-NLD, SSc-PAH, SSc-PF and HC. Linear regression analysis demonstrating significant correlations between cytokines in **(A)** whole cohort (n=128); IL-6 and sIL-6R **(A1)** and IL-6 and MCP-1 **(A2), (B)** in the SSc-NLD group (n=32); IL-6 vs sgp130 and **(C)** SSc-PF (n=32); CTGF vs MCP-1. A p-value <0.05 was considered significant, r refers to correlation coefficient.

3.2.4 Protein-protein interactions

Significantly altered proteins identified by proteomics, and cytokines investigated by ELISA were used for investigation of protein-protein interactions and functional pathways in the disease groups (**Figure 3.5**). Network analysis was performed using the STRING database. The STRING platform assesses direct physical interactions and indirect functional associations from multiple sources, including primary curated databases, text mining, experimental interactions and co-expression interaction analyses (Szklarczyk et al., 2014). The network was assessed by node degree, a measure of the number of interactions that a protein has on average in the network, and clustering coefficients, a measure of how connected the nodes in the network are. Highly connected networks have high values. SSc-NLD had the lowest node degree (3.67) but the highest clustering coefficient (0.839). SSc-PAH had a node degree of 4.0 and the lowest clustering coefficient (0.713). SSc-PF had the highest node degree (6.67) and a clustering coefficient of 0.794. Biologics, either FDA-approved, or currently undergoing clinical trials targeting these cytokines were also included in the network. Of the identified biologics, only tocilizumab was found to be used in SSc.

In order to identify functional differences between groups, pathway analysis was conducted, using the Enrichr gene ontology (GO) database (**Figure 3.5**). The significantly altered proteins and cytokines investigated by ELISA were entered into the Enrichr tool for statistical overrepresentation testing and searched against biological process and molecular process GO terms. Statistical significance was determined using Fischer exact test with Benjamini-Hochberg corrections. The most significantly enriched biological pathway for SSc-NLD was T cell proliferation, and the most significantly enriched molecular pathway was TNFR activity. SSc-PAH and SSc-PF exhibited similar biological and molecular pathways, namely regulation of JAK activity and IL-6R binding.

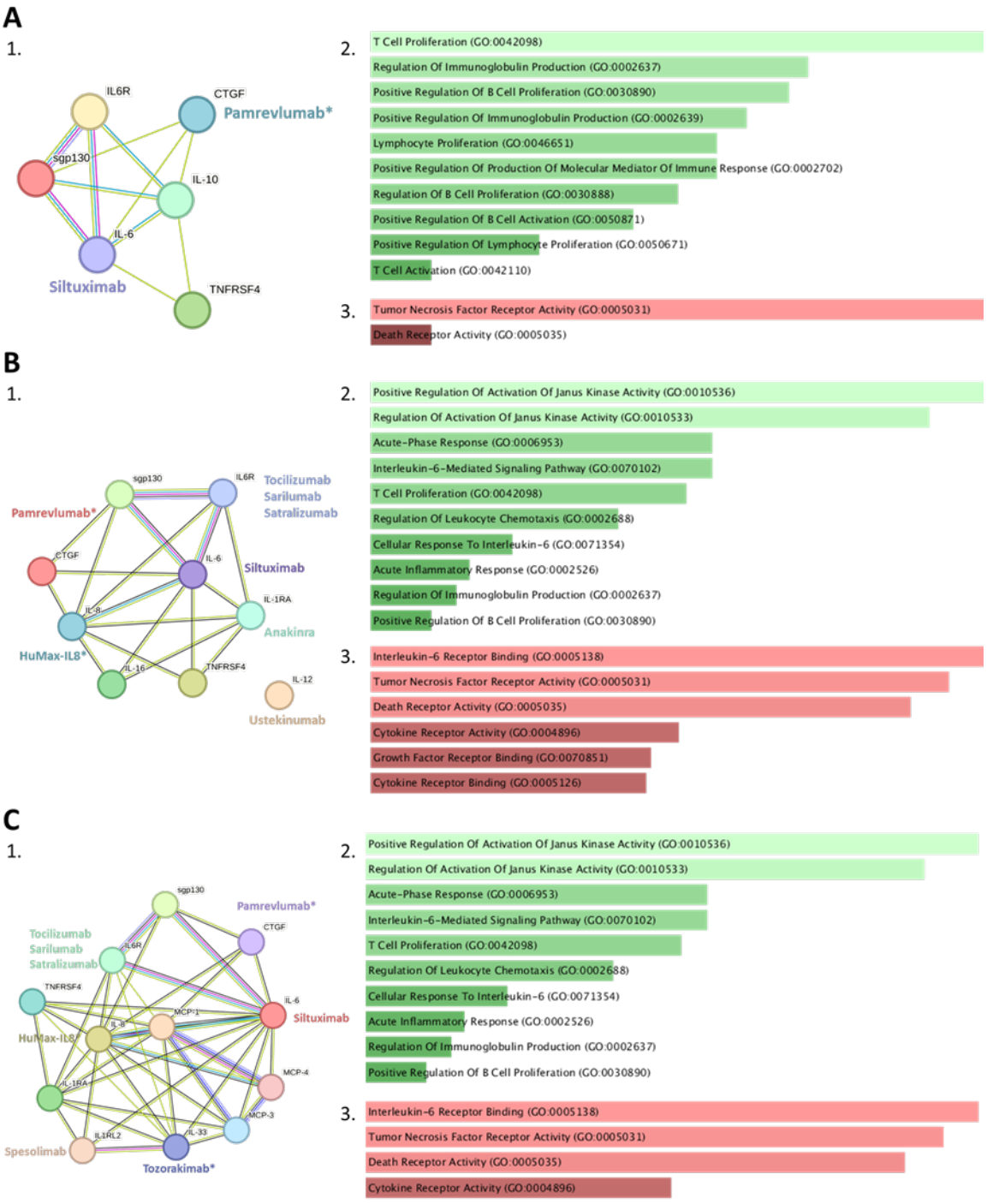


Figure 3.5: Protein-protein interactions and pathway analysis. Network analysis including FDA approved biologics and antibody therapeutics undergoing clinical trials for cytokines involved in SSc-NLD **(A)**, SSc-PAH **(B)**, and SSc-PF **(C).** Coloured nodes indicate different cytokines, edge colours represent type of evidence – experimental (purple), textmining (yellow), database (blue) and coexpression (black). Table of significantly ($p < 0.05$) enriched biological pathways **(2)** and molecular pathways **(3)** for each group.

3.2.5 Metabolite identification using Metabolomics

To investigate metabolic differences across systemic sclerosis (SSc) subtypes, a comprehensive metabolomics analysis was conducted that initially included 965 metabolites. This extensive profiling aimed to identify unique metabolic signatures associated with SSc-PAH (SSc with pulmonary arterial hypertension), SSc-PF (SSc with pulmonary fibrosis), and SSc-NLD (SSc with no lung disease). As part of the analysis, metabolites that showed significant elevations across multiple SSc subtypes were excluded, leaving a refined set of 27 metabolites for further study. This step ensured that only metabolites with specific associations to a single condition were retained, enhancing the ability to differentiate among the subtypes.

Among the 27 metabolites, a notable 22 were specifically altered in the SSc-PAH group, while only 5 were altered in SSc-PF (**Table 3.3**). Interestingly, no metabolites were found to be unique to the SSc-NLD group, suggesting a less distinctive metabolic profile for this subtype in the context of the studied panel.

Table 3.3: Significantly altered metabolites by group

Metabolites	SSc-NLD	SSc-PAH	SSc-PF
Dimethylarginine (SDMA + ADMA)		↑▲□◊	
Hydroxyasparagine		↑▲□◊	
Quinolinate (QUIN)		↑▲□◊	
N-acetylalanine (AYA)		↑▲□◊	
3-hydroxyhexanoate (3HH)		↑◊	
N6-carbamoylthreonyladenosine (T6a)		↑▲□◊	
Vanillactate (VLA)		↑▲□◊	
Deoxycarnitine		↑□◊	
3-hydroxyoctanoate (3HO)		↑◊	
4-acetamidobutanoate		↑▲□	
S-adenosylhomocysteine (SAH)			↓▲

Adenosine 5'-monophosphate (AMP)		↑▲	
N-acetylneuraminate (Neu5Ac)		↑▲□	
Pseudouridine (psi-Uridine)		↑□	
N-acetyl-isoputreanine		↑▲□◊	
N-acetylputrescine		↑▲	
C-glycosyltryptophan		↑▲□	
Gamma-glutamylhistidine (GGlu-His)			↓▲
Gamma-glutamylthreonine (GGlu-Thr)			↓▲
Sphinganine			↑▲
Succinate			↑▲
Vanillylmandelate (VMA)		↑▲◊	
3-methoxytyrosine		↑▲◊	
N2,N2-dimethylguanosine		↑▲	
N-palmitoylglycine (PalGly)		↑▲	
Gamma-glutamyl-alpha-lysine (GGlu-Lys)		↓▲	
Kynurenine (KYN)		↑▲	

Significantly altered metabolites ($p<0.0001$); ↑ up-regulated, ↓ down-regulated, ▲ comparison to HC, □ comparison to SSc-NLD, ● comparison to SSc-PAH, ◊ comparison to SSc-PF

Within the SSc-PAH group, 7 metabolites were significantly elevated when compared to all other SSc subtypes, highlighting them as potential biomarkers uniquely associated with SSc-PAH. These 7 metabolites were thus selected for inclusion in a candidate biomarker panel (**Figure 3.6**). This panel may offer diagnostic potential by distinguishing SSc-PAH from other SSc subtypes, providing a basis for further validation studies and potential clinical application.

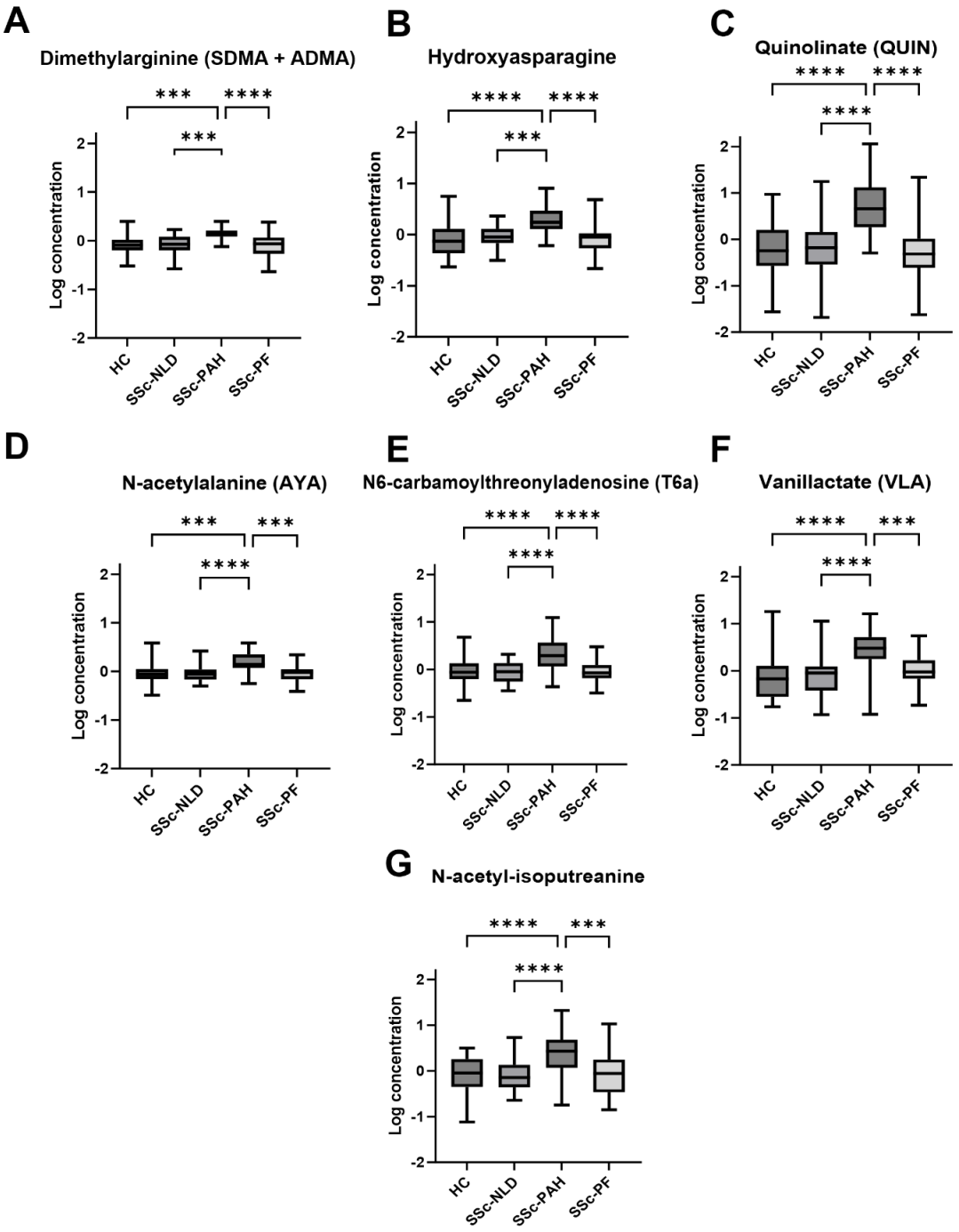


Figure 3.6: Relative levels of selected metabolites in SSc-NLD (n=30), SSc-PAH (n=30), SSc-PF (n=30) and HC (n=30). Boxplots (median, interquartile range) showing SSc-PAH compared to SSc-PF, SSc-NLD and HC A) Dimethylarginine (SDMA + ADMA) B) Hydroxyasparagine C) Quinolinate (QUIN) (D) N-acetylalanine (AYA) (E) N6-carbamoylthreonyladenosine (T6a) (F) Vanillactate (VLA) (G) N-acetyl-isoputreanine. A one-way ANOVA was used for analysis. Sig. indicated by * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, **** = $p<0.0001$.

3.2.6 Metabolite interactions and pathway analysis

Pathway enrichment analysis was conducted using the 27 significantly altered metabolites. Each pathway's enrichment was quantified by an enrichment ratio, defined as the ratio of observed hits to expected hits. Here, "hits" represented the number of metabolites identified in the pathway, while "expected" referred to the baseline number anticipated by chance. The analysis revealed substantial differences between the two conditions: 30 pathways were significantly enriched in SSc-PAH, compared to only 6 pathways in SSc-PF, indicating a higher level of metabolic disruption in SSc-PAH (**Figure 3.7**).

Network analysis mapped interactions between enriched pathways, uncovering 45 interactions among pathways enriched in SSc-PAH, suggesting functional connections among these pathways. In contrast, no interactions were observed among the pathways enriched in SSc-PF. The most significantly enriched pathways in SSc-PAH were nicotinate and nicotinamide metabolism, tryptophan metabolism, thiamine metabolism and phenylacetate metabolism. The metabolites involved in these pathways were AMP, QUIN and KYN. In SSc-PF, butanoate metabolism was the most significantly altered followed by citrate (TCA) cycle, sphingolipid metabolism and propanoate metabolism. Metabolites involved in SSc-PF related metabolic pathways were sphinganine and succinate.

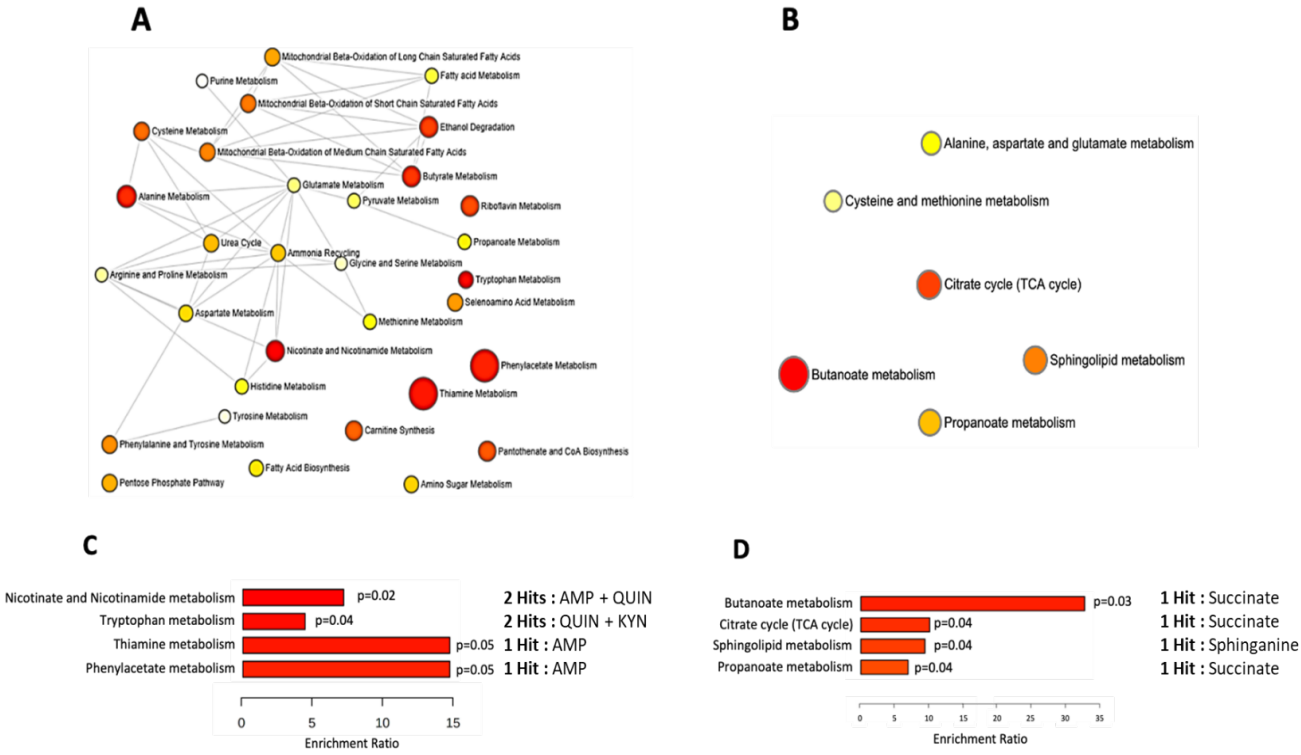


Figure 3.7: Enriched pathway interaction networks for selected metabolites. All enriched pathway network for SSc-PAH (A) and SSc-PF (B). Nodes indicate different metabolic pathways, size of the node refers to the extent of enrichment, while the colour indicates significance, with lowest p-values in red and highest in yellow. Significantly ($p<0.05$) enriched pathways with metabolite hits in SSc-PAH (C) and SSc-PF (D).

3.2.7 Cytokine-metabolite correlations

The cytokines and metabolites that showed significant differences between the groups were assessed using Pearson's correlation coefficient analysis. The correlation matrices showed distinct protein-metabolite profiles across the four groups (**Figure 3.8, 3.9, 3.10 & 3.11**).

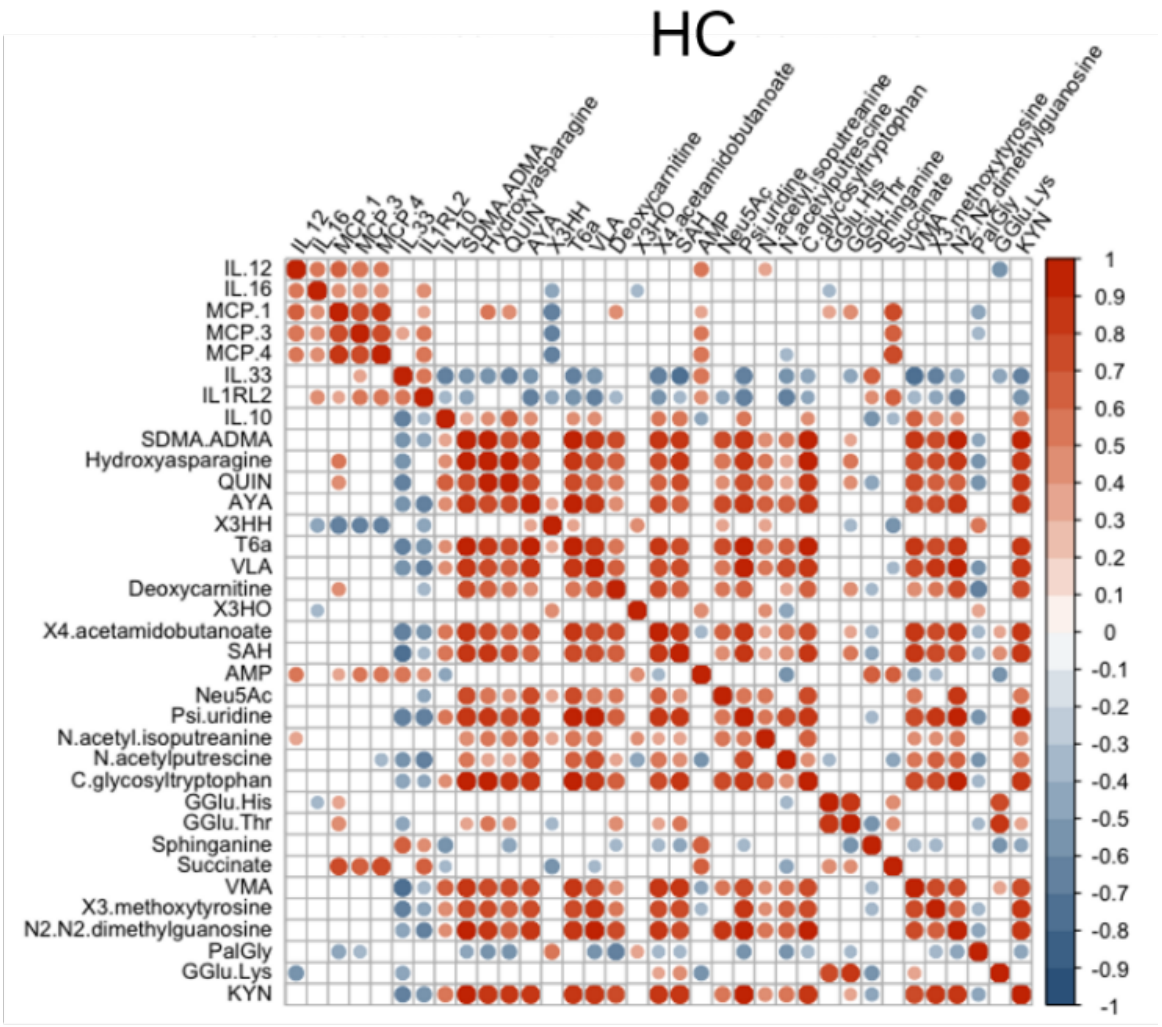


Figure 3.8: Correlation matrix of cytokines and metabolites in healthy controls (HC). The figure shows significant Pearson correlations. Significant ($p<0.05$) positive correlations are denoted in red, while negative correlations are shown in blue.

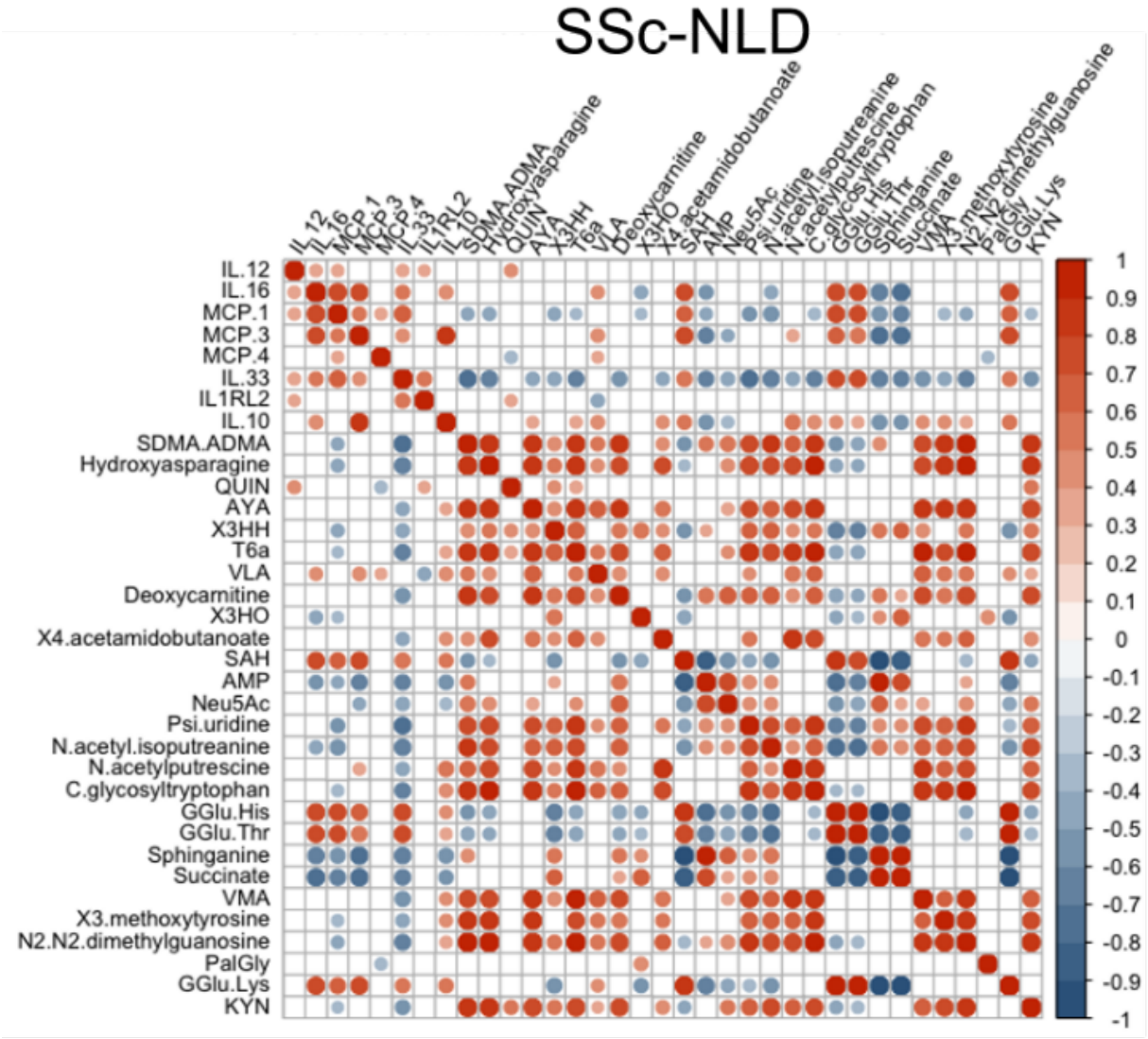


Figure 3.9: Correlation matrix of cytokines and metabolites in patients with SSc with no lung disease (SSc-NLD). The figure shows significant Pearson correlations. Significant ($p<0.05$) positive correlations are denoted in red, while negative correlations are shown in blue.

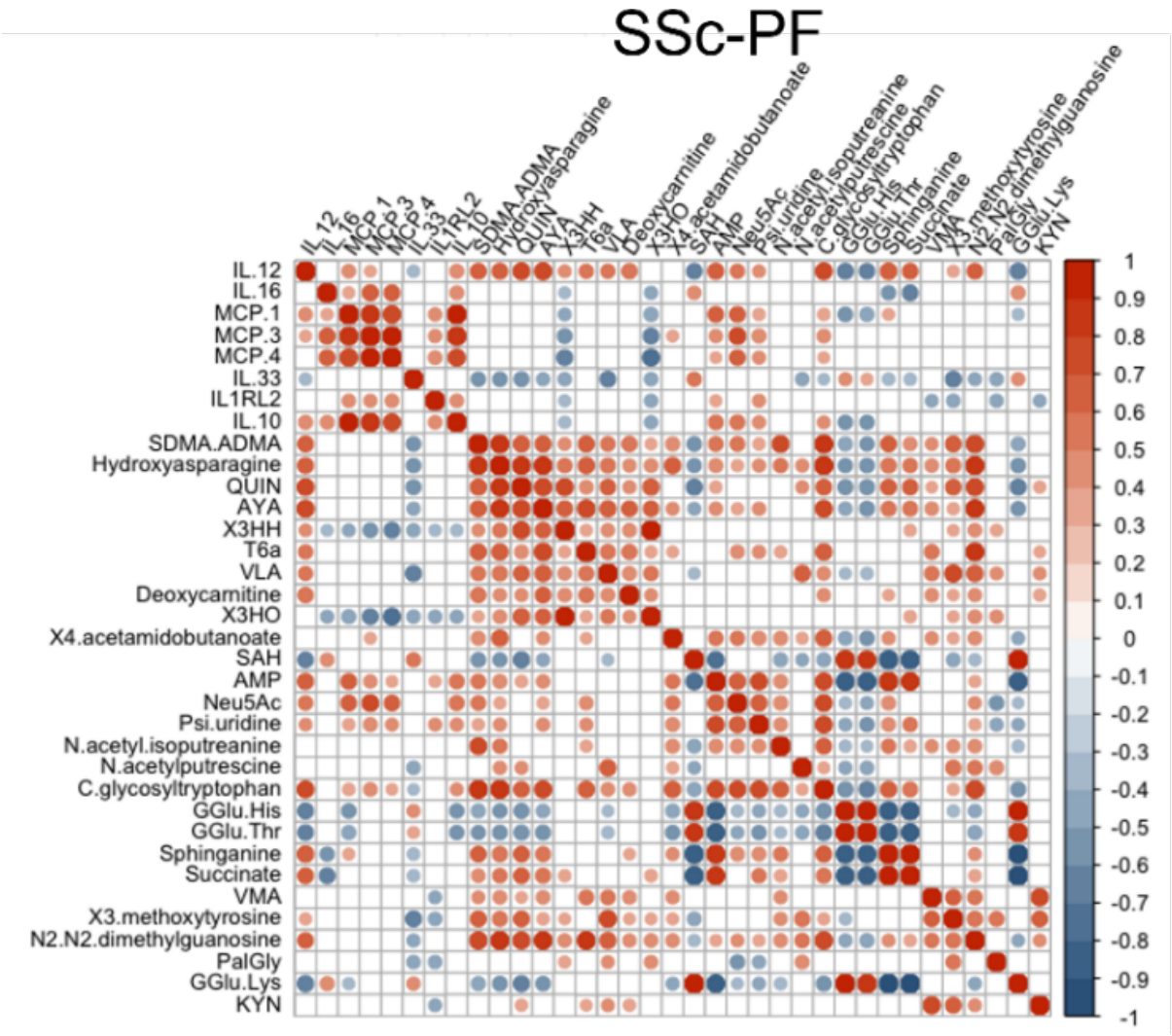


Figure 3.10: Correlation matrix of cytokines and metabolites in patients with SSc with pulmonary fibrosis (SSc-PF). The figure shows significant Pearson correlations. Significant ($p<0.05$) positive correlations are denoted in red, while negative correlations are shown in blue.

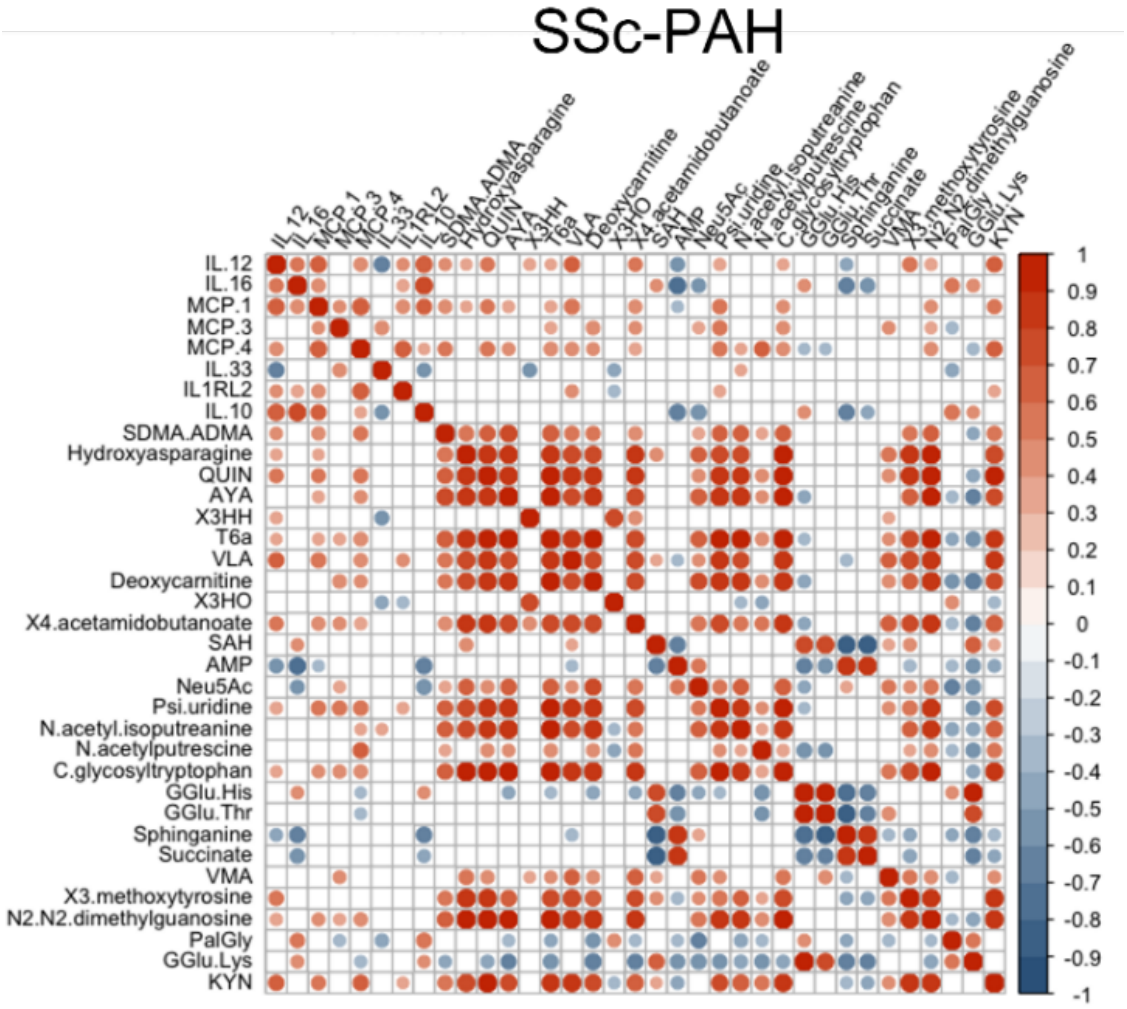


Figure 3.11: Correlation matrix of cytokines and metabolites in patients with SSc with pulmonary arterial hypertension (SSc-PAH). The figure shows significant Pearson correlations. Significant ($p<0.05$) positive correlations are denoted in red, while negative correlations are shown in blue.

3.3 Discussion

In SSc, pulmonary complications (SSc-PAH and SSc-PF) are the leading cause of mortality (Steen, 2003). The pathogenesis of both diseases is complex, with autoimmunity, vasculopathy and fibrosis acting as the main pathophysiologic drivers of disease. SSc-PF occurs due to alveolar epithelial cell damage, stimulating release of pro-inflammatory cytokines that encourage fibrosis, while in SSc-PAH, endothelial cell injury and release of vasoactive mediators leads to proliferation and vasoconstriction of the pulmonary arteries (Distler et al., 2020.; Guignabert et al., 2018).

Several inflammatory markers including IL-6 and CTGF have been implicated in the pathogenesis of SSc (M. Brown & O'Reilly, 2019; Denton & Abraham, 2001). Auto-antibodies targeting ATA and ACA, as well as a number of biomarkers have been associated with different pulmonary comorbidities, however, little is known about the underlying mechanisms that drive SSc patients to develop PAH, PF or no lung disease at all (K. T. Ho & Reveille, 2003b; Sanges et al., 2023). Research has suggested that SSc-PAH occurs due to metabolic dysfunction while SSc-PF is the more inflammatory disease, however, differences in the mechanisms of these diseases has not been thoroughly investigated, and robust, predictive biomarkers are scarce (Paulin & Michelakis, 2014; Schoenfeld & Castelino, 2015). Proteomics analysis performed in this chapter uncovered 3 inflammatory markers that differentiated SSc-PF from all other groups, and 7 metabolites specific for SSc-PAH. Other cytokines, including IL-6 and CTGF were not found to be discriminatory, however correlations between these cytokines uncovered indications of specific pathways for SSc-PAH, SSc-PF and SSc-NLD. Collectively, these results identify specific therapeutic targets, and provide greater insight into the pathology underlying these conditions.

Several plasma proteins were found to be significantly altered between the four groups. These were MCP-1, MCP-3, MCP-4, IL-6, IL-12, IL-8, IL1RA, IL-10, IL-33, IL1RL2, IL-16 and TNFRSF4. Of these, only MCP-1, MCP-3, and MCP-4 were considered discriminatory markers, as they were elevated in SSc-PF compared to all groups. Members of the monocyte chemoattractant protein family, MCP-1, MCP-3 and MCP-4 act as chemokines, recruiting immune cells to

sites of inflammation. Research has implicated MCP-1 in particular, to the initiation of inflammation in SSc, and high levels of its receptor, CCR2, have been found in $CD4^+$ T cells in the fibrotic lung of bleomycin mice (Distler et al., 2001; Osafo-Addo & Herzog, 2017). In SSc-PF patients, MCP-1 levels significantly correlated with development of pulmonary fibrosis (Hasegawa et al., 2001). Similarly, MCP-3 concentrations were positively associated with extent of skin fibrosis, and negatively correlated with FVC, indicating a profound role in SSc-related fibrosis and lung function (Yanaba et al., 2006). MCP-4, while not as extensively studied as other members of the MCP family in this disease, has been shown to be overexpressed in SSc, and aggravates lung fibrosis in patients with COVID-19 (Holton et al., 2024; Yanaba et al., 2010). Thus, MCP-4 may play a role within the fibrotic pathways leading to pulmonary complications such as SSc-PF. This study, along with others, has shown that MCP-1, MCP-3, and MCP-4 have implications in fibrosis, particularly in SSc and within the lung, and may be useful biomarkers for SSc-PF. Investigation of MCP-1 expression in the same cohort by ELISA, showed that MCP-1 was significantly increased in SSc-PF compared to all groups, validating proteomics results. However, further validation is needed for MCP-3 and MCP-4.

A number of cytokines identified were elevated in only one group but could not differentiate between all groups. These were IL-10, IL-12, IL-16, IL-33 and IL1RL2. While not discriminatory, these cytokines were of interest, to gain an understanding of mechanisms. IL-10 was elevated in SSc-NLD compared to HC. IL-10 is a known anti-inflammatory immunomodulator, involved in the resolution of inflammation through the inhibition of T-cells. Previous studies have suggested that IL-10 is elevated in SSc (De Vries, 1995; X.-L. Huang et al., 2020.). In this context, increased IL-10 in the SSc-NLD group may indicate a protective mechanism against pulmonary complication development.

In SSc-PAH, IL-12 and IL-16 were found to be elevated. IL-12 was increased in this group compared to HC and SSc-NLD. This cytokine is important for the differentiation and proliferation of several cytotoxic immune cells including macrophages, natural killer cells, and T cells (Vignali & Kuchroo, 2012). While traditionally considered a ‘pro-inflammatory’ cytokine, studies in angiotensin II-induced hypertensive mice have shown that IL-12 therapy markedly lowered

blood pressure (Balasubbramanian et al., 2019; Ye et al., 2019). In human studies of SSc-PAH, tadalafil treatment upregulated expression of IL-12 associated genes (Cheong et al., 2017). Moreover, serum IL-12 in SSc-PAH patients was reported to be increased post-treatment with bosentan (Heresi & Minai, 2008). Considering that tadalafil and bosentan are commonly prescribed therapies for SSc-PAH, combined with the absence of clinical treatment data within this study, it is possible that the observed increased IL-12 expression in SSc-PAH may be a consequence of treatment rather than a marker of disease. IL-16, also found to be elevated in this group compared to HC, is a known chemoattractant and activator for a variety of CD4+ immune cells and has associated with asthma and several autoimmune diseases (Cruikshank et al., 2000). While elevated levels of IL-16 in the skin and sera have been reported in SSc patients, its significance has not been well characterized in SSc-PAH. Nonetheless, the strong association between IL-16 and pulmonary disorders such as asthma suggests an inflammatory mechanism within the lung (S. Smith et al., 2018).

IL-33 and IL1RL2 were found to be marginally elevated in SSc-PF compared to HC, however previous research has indicated that these cytokines potentially play important role within SSc disease contexts. IL-33 is a cytokine from the IL-1 family released in response to damaged endothelial and epithelial cell barriers, due to allergens, infections, or inflammation (Cayrol & Girard, 2018). IL-33 binds to the suppression of tumorigenicity 2 (ST2) receptor, and through this axis activates signaling molecules such as MAPKs and NF-kB, which contribute to tissue inflammation and fibrosis (X. Wu et al., 2022). Both IL-33 and ST2 have been shown to be overexpressed in skin lesions of SSc patients, and IL-33 deficiency led to improved dermal fibrosis in bleomycin mice (Li et al., 2014.; X. Wu et al., 2022). As such it has been suggested as a potential target for SSc. Tozorakimab, an anti-IL-33 monoclonal antibody is currently undergoing clinical trials for asthma, however it is unclear whether this could be used in future for SSc-associated pulmonary conditions such as SSc-PF (England et al., 2023). IL1RL2 is the receptor for IL-36, a newly characterized member of the IL-1 superfamily which has pro-inflammatory, pro-fibrotic effects (Melton & Qiu, 2020). IL-36 signaling has been implicated in chronic inflammation and fibrosis, particularly in relation to inflammatory skin conditions (Melton & Qiu, 2020).

Spesolisumab, an IL1RL2 inhibitor was recently approved as a drug for pustular psoriasis (Blair, 2022; Kaplon et al., 2023). However, the effects of IL-36 signaling are wide ranging, with studies suggesting that inhibiting this pathway could reduce fibrosis in intestinal inflammation and improve cancer prognoses (Scheibe et al., 2019). Thus, targeting IL1RL2 may also improve lung fibrosis in patients with SSc-PF.

In order to validate proteomics results, quantification of serum concentrations for MCP-1 and IL-6 were carried out. MCP-1 was significantly elevated in SSc-PF compared to all groups, as observed via proteomics. IL-6 levels were significantly increased in all SSc groups compared to HC, validating proteomics findings. However, IL-6 in SSc-PF was also significantly elevated compared to SSc-NLD. While this trend is apparent in both experiments, this did not reach significance in the proteomics analysis. This may be due to differences in blood handling procedures, which have been shown to significantly alter IL-6 serum and plasma levels (Gong et al., 2019).

CTGF expression and the IL-6 trans signalling pathway molecules sIL-6R and sgp130 were of interest in this cohort, and thus were investigated by ELISA. CTGF has previously been reported to play a role in the pathogenesis of SSc (Abraham, 2008; Chujo et al., 2005; Leask, 2004). No differences in CTGF were observed within this group, which may be due to the limited sample size available. Significantly increased levels of IL-6, sIL-6R, but not sgp130 were observed in SSc-PAH and SSc-PF. This is consistent with previous research implicating the trans signalling pathway as a key contributor to the disease process, and in particular, development of pulmonary fibrosis in SSc (Hasegawa, Sato, Ihn1, et al., 1999). Tocilizumab, the IL-6R antagonist, which is prescribed in SSc, does not differentiate between membrane-bound and soluble forms of the receptor. More specific antibodies may be able to antagonize only the soluble, pro-inflammatory receptor without affecting the anti-inflammatory, membrane bound form, and this may improve the significant side effects of the drug. Linear regression analysis of the entire cohort showed significant positive correlations between IL-6 and sIL-6R, and between IL-6 and MCP-1. Thus, increased levels of IL-6 may be driving disease by increasing MCP-1 and sIL-6R levels. Interestingly, in the SSc-NLD group, there was a positive correlation between IL-

6 and sgp130. As sgp130 is the antagonist to the trans signalling pathway, this may be a protective mechanism against pulmonary complication development within this group. In SSc-PF, a significant positive association was observed between CTGF and MCP-1. Since MCP-1 was shown to be elevated in this group, this suggests that CTGF may play a role in the inflammatory pathway within this group, although it was not seen to be elevated in this instance.

Network analysis of cytokines involved in the three disease groups was carried out using the STRING database. SSc-PF had the highest node degree, a measure of the number of interactions that a protein has on average in the network. Cytokines upregulated in SSc-PF having more interactions with one another is characteristic of the inflammatory cascade that drives the disease. SSc-PAH was found to have the lowest clustering coefficient, a measure of how connected the nodes in the network are. This is indicative of the metabolic nature of the disease, where inflammation plays a part, but may not be the preliminary cause. Several biologics which target cytokines within the disease network were identified, however, only tocilizumab was found to be routinely used in SSc. The majority of drugs within the network were treatment for other rheumatic, autoimmune diseases such as rheumatoid arthritis, systemic juvenile idiopathic arthritis and psoriatic arthritis. One biologic, tozorakimab, was identified as undergoing clinical trial for symptomatic Chronic Obstructive Pulmonary Disease (COPD). The targets identified in this study may similarly play a role in lung function, and it may be of interest to repurpose these drugs for SSc, and in particular, SSc-associated pulmonary complications.

Significantly enriched biological and molecular pathways were identified for cytokines involved in each of the disease groups. A clear difference was apparent between the biological and molecular pathways in SSc-NLD and those of SSc-PAH and SSc-PF. SSc-NLD was associated with biological pathways involved in T and B lymphocyte proliferation and activation, as well as IgG production. CD4+ T cells have been shown to contribute to SSc pathogenesis, and abnormal T cell responses precede inflammation and fibrosis in SSc patients (W. Jin et al., 2022). Studies have suggested that increased cytotoxic CD4+ T lymphocyte activation may induce apoptotic death of endothelial cells, and this is followed by excessive tissue repair processes that lead to fibrosis (Maehara et al., 2020). Dysregulation

of B cell function has also been suggested to play a role in the pathogenesis and development of SSc, and B cell depletion therapy with rituximab has shown promise in treating the disease (Beesley et al., 2023; Zamanian et al., 2021). While overall B cell numbers are increased in SSc, the number of IL-10 producing regulatory B cells (Bregs) are reduced, as is their capacity to produce anti-inflammatory IL-10(Matsushita et al., 2016; Mavropoulos et al., 2016, 2017). The differences in B cell regulation between SSc-NLD and SSc-associated pulmonary complications has not been fully explored. However, in this study, anti-inflammatory IL-10 was found to be increased in SSc-NLD, but not the pulmonary groups. In this instance, Bregs anti-inflammatory mechanisms may be play a protective role in SSc-NLD, shielding against development of pulmonary disorders. SSc-PAH and SSc-PF were found to undergo the same biological and molecular pathways, highlighting the overlapping mechanisms that drive both diseases forward.

Untargeted metabolomics analysis identified 27 metabolites as significantly different between groups. Of these, 22 metabolites were altered in SSc-PAH, and 5 were altered in SSc-PF. The difference in the number of dysregulated metabolites is reflective of the idea that metabolic abnormalities are present to a lesser extent in SSc-PF compared to SSc-PAH.

A panel of 7 metabolites were significantly upregulated in SSc-PAH compared to all groups. These metabolites, SDMA + ADMA, Hydroxyasparagine, QUIN, AYA, T6A, VLA and N-acetyl isoputreanine are involved in energy metabolism, mitochondrial oxidation, and amino acid catabolism, and have been shown to play inflammatory roles within the vasculature (S. Zhang et al., 2015; Markiewski & Lambris, 2007; Smallridge et al., 2003). Notably, QUIN, a metabolite formed through the kynurenine pathway (KP) of tryptophan metabolism, was highly expressed in SSc-PAH patients. Research in human lung primary cells has suggested that treatment with IL-6/sIL-6R may lead to similar KP metabolic profiles as in PAH, suggesting that the metabolic dysfunction within the KP is driven by IL-6 trans signaling (Cai et al., 2022).

In order to investigate interactions between the cytokines and metabolites, correlation analyses were carried out on all cytokines and metabolites with

significant differences between groups. In the pulmonary disease groups, clear differences relating to IL-33 associations was observed. In SSc-PAH, IL-33 exhibited strong negative correlations with MCP-1, MCP-4, IL-12, IL-8, IL1RL2, IL-16 and TNFRSF4. In HC and SSc-NLD these correlations were positive, indicating that IL-33 may play a central role in inflammatory pathways driving SSc-PAH. Similarly, in the SSc-PF group, IL-33 seemed to have lost its associations with most cytokines, suggesting disruption within this signalling pathway. These associations provide insight into the signalling mechanisms of SSc-associated pulmonary complications. The IL-33 pathway in particular may be of interest, and Tozorakimab, the anti-IL-33 monoclonal antibody could be of therapeutic benefit in these diseases.

Cytokine-metabolite correlations within the SSc-PAH group showed that 6 of the 7 metabolites in the biomarker panel, with the exception of N-acetyl-isoputreanine, were significantly positively correlated with MCP-1 and to a lesser extent, MCP-4, indicating a link between the inflammatory and metabolic pathways in these patients. In the SSc-PF group, differences in correlations between metabolites were less obvious, confirming previous suggestions that the disease is pro-inflammatory cytokine-driven, rather than metabolic (Paulin & Michelakis, 2014; Schoenfeld & Castelino, 2015).

Taken together, these results provide specific biomarkers and highlight potential mechanisms which drive SSc-NLD, SSc-PAH and SSc-PF. This chapter confirmed previous indications that SSc-PAH pathogenesis is more a result of metabolic dysfunction while SSc-PF is more inflammatory, and also suggested protective mechanisms which may slow down disease progression. In this chapter, 17% of the cytokines uncovered by proteomics were validated by ELISA. Experiments to validate the remaining proteins are necessary in order to confirm their involvement in the disease. Additionally, an increase in the number of participants would increase power for statistical analysis, and perhaps draw attention to other cytokines which were not found to be significantly altered at this time. Biomarkers, and in particular those discovered using omics methods, are highly specific and indicative of disease pathology, however, combining these with traditional clinical criteria is key for optimal diagnostic and prognostic benefit.

While many of the differences noted have been previously reported, it is important to note within the limitations of this study, that the participants were not age-matched, nor matched for gender. Many cytokines and metabolites have been shown to be altered between males and females, and many of these also change with the aging process. Thus, some of the differences between the disease groups and healthy controls could be attributed to gender or age differences. However, the SSc groups were closer in age, as well as having similar male: female ratios, meaning they could be more accurately compared. In order to make full use of these biomarkers, further research is required to correlate the cytokines identified in this study to clinical parameters and symptoms experienced by patients.

Chapter 4

Investigation of SSc-related fibrotic pathways *in vitro*

4.1 Introduction

It is still unclear whether the immune dysregulation observed in SSc is a primary cause, or a consequence of the disease. Nonetheless, the characteristic fibrosis of the skin and internal organs in SSc is generally considered a downstream effect, occurring as a result of a chronic inflammatory environment.

Fibrosis ensues when inflammation is unresolved, and the balance between ECM protein production, and degradation is disturbed. Cytokines, chemokines and growth factors, many of which have been established in SSc, are key drivers of inflammation and fibrosis. Released by local cells within the affected tissue, or by recruited immune cells, these mediators activate fibroblasts to transdifferentiate into myofibroblasts, migrate to the site of damage, and deposit ECM. Normally, these myofibroblasts would undergo apoptosis, and the excessive ECM would be degraded by matrix metalloproteinases (MMPs). However, in SSc, fibroblasts are continuously stimulated to transdifferentiate into myofibroblasts, producing more ECM which does not get degraded (Peng et al., 2012). Additionally, studies have suggested that the presence of a stiff, fibrotic matrix results in continual pro-fibrotic stimulation, leading to more inflammatory cytokine release and sustained persistence of fibrosis (Leask, 2015). Myofibroblasts can also be generated from other sources including resident mesenchymal cells, epithelial and endothelial cells through epithelial/endothelial-mesenchymal transition (EMT/EndoMT) (Cipriani et al., 2014). This process is a crucial link between epithelial/endothelial injury observed in SSc, to the progression and development of dermal and internal organ fibrosis.

The presence of inflammatory cytokines, chemokines and growth factors has been well-established in SSc. Immunomodulatory drugs such as MMF, while still used as first-line treatment for SSc, have exhibited limited efficacy with regards to pulmonary complications (Daoussis & Liossis Mediterr, 2019; Jerjen et al., 2022). Understanding the role of cytokines and discerning how they interact with their environment, and with each other, is critical for developing targeted therapies. Several drugs that target cytokines or their receptors have been investigated as potential treatments for SSc to modulate the immune response, reduce inflammation, and limit fibrosis (Daoussis & Liossis Mediterr, 2019; Jerjen et al., 2022), 2019; Jerjen et al., 2022). However, the treatment of SSc is complex and may require a combination approach.

Interleukin-6 (IL-6), Connective Tissue Growth Factor (CTGF), and Monocyte Chemoattractant Protein-1 (MCP-1) are three distinct molecules that play important roles in the immune system and tissue physiology in health and disease and have previously been reported to play a role in the pathogenesis of SSc. IL-6 has been suggested to contribute to increased collagen deposition in SSc skin fibroblasts (O'Reilly et al., 2012). Elevated levels of IL-6, and sIL-6R were identified in SSc compared to healthy controls, and sIL-6R levels correlated significantly with the severity of pulmonary fibrosis in SSc patients (Hasegawa et al., 1998; Hasegawa, Sato, Ihn1, et al., 1999). Additionally, IL-6 levels were positively correlated with total skin score and skin thickness (Kitaba et al., 2012; Muangchan & Pope, 2012). Representational difference analysis (RDA) showed increased CTGF gene transcription in fibroblasts isolated from SSc patients (Shi-wen et al., 2000). Selective deletion of the CTGF gene in mouse models of PAH and PF significantly reduced pulmonary interstitial scarring and vessel remodelling (Tam et al., 2021). Chemokines MCP-1 and MCP-3 have been shown to be overexpressed by SSc fibroblasts compared to healthy controls, and polymorphisms in the MCP-1 gene have been reported to contribute to the development of SSc (Distler et al., 2001; W Distler et al., 2009). While all three molecules have the capacity to lead to fibrosis via their signalling pathways, it is unclear whether they have a synergistic effect, and whether those integrated pathways are involved in SSc pathogenesis.

Severity of skin fibrosis in SSc can provide insight into systemic disease progression. Studies have suggested a correlation between skin fibrosis and decline in lung function (W. Wu et al., 2019). Thus, to investigate overall fibrotic pathways, skin fibroblasts were utilised. The aim of this chapter was to investigate pathways involving IL-6, CTGF and MCP-1 *in vitro*.

4.2 Results

4.2.1 Cohort characteristics

Healthy control (n=3) skin fibroblasts were taken by Dr Xu Shiwen using punch biopsy from consenting adults at the Department of Rheumatology, Royal Free Hospital, and stored at in liquid nitrogen. To limit population differences, and to control for natural variations in cytokine levels, all selected participants were age-matched, female, European Caucasians (López et al., 2018; Peoples et al., 2016). Cohort demographics and clinical characteristics are outlined in **Table 4.1.**

Table 4.1: Cohort demographics

Features	Healthy Controls
Participants (n)	3
Age (years)	49.7±7.6

Data are shown as mean ± SD. Differences in age were not significant (p=0.95)

4.2.2 IL-6 and sIL-6R signaling in healthy skin fibroblasts

Constitutive release of IL-6 by number of different cell types was measured by ELISA (**Figure 4.1A**). Investigation of the IL-6 trans signalling pathway was initially conducted using Jurkat cells (JC), a T lymphocyte cell line, as they did not release IL-6 without stimulation. JC have also been reported to lack expression of the membrane-bound IL-6R (Igaz et al., 2000). To investigate if IL-6 trans signalling occurs through the JAK/STAT pathway, cells were treated either with IL-6, sIL-6R or both at either low (physiological) or high (pharmacological) levels (**Figure 4.1B**). The physiological (phys) levels were obtained from the normal concentrations of IL-6 and sIL-6R in circulation (Baran et al., 2018; Montero-Julian, 2001). Pharmacological (pharm) were determined from levels measured in disease (Almuraikhy et al., 2016.; Hasegawa et al., 1998; Mohamed-Ali et al., 2001a). Protein was extracted and the expression of STAT3 and pSTAT3 were analysed by western blotting (**Figure 4.1B, 4.1C**). There was no difference in the level of STAT3 across the treatment groups, however phosphorylation was detected only when IL-6 and sIL-6R were incubated together at high concentrations.

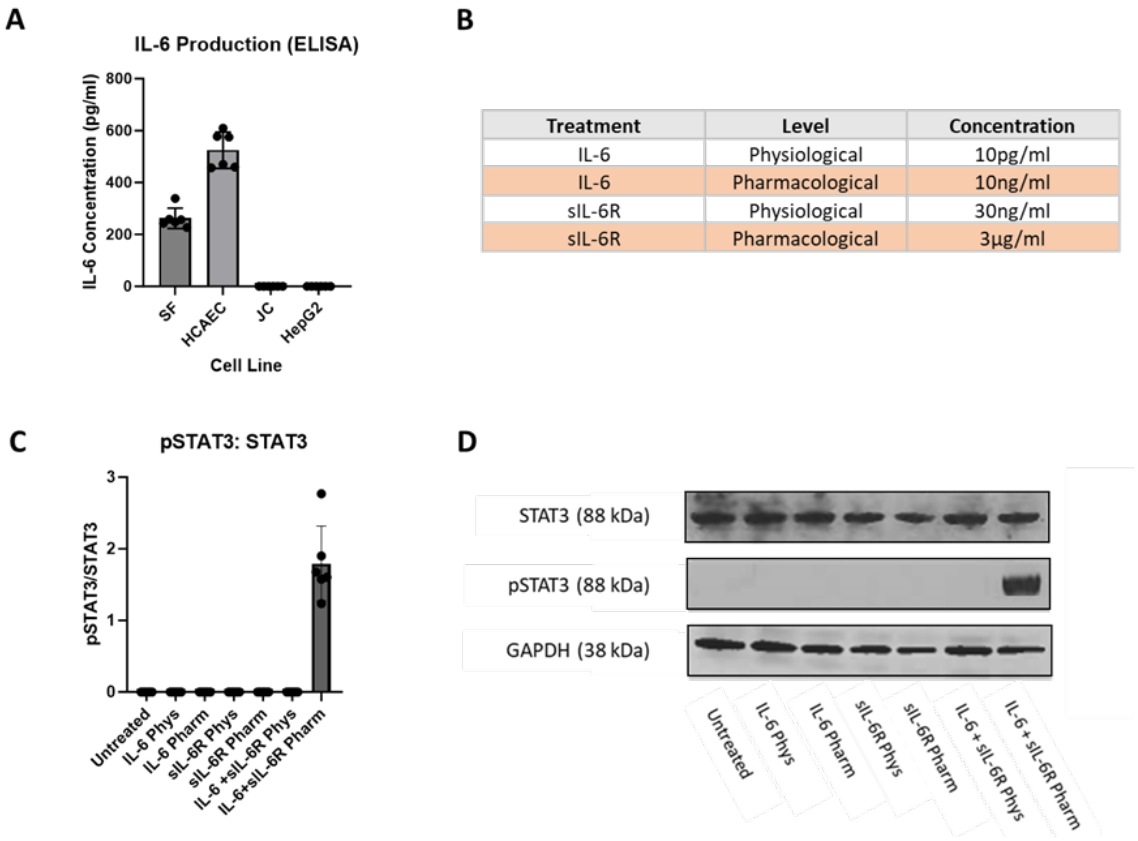


Figure 4.1: Investigation of IL-6 trans signalling in JC. (A) Graph of constitutively released of IL-6 concentrations in culture media for skin fibroblasts (SF), human coronary arterial endothelial cells (HCAEC), Jurkat cells (JC) and HepG2 cell lines (n=6). **(B)** Table detailing physiological and pharmacological concentrations of IL-6 and sIL-6R (n=6). **(C)** Chart of pSTAT3:STAT3 ratio for untreated, IL-6, sIL-6R and IL-6 + sIL-6R treated cells (n=6). **(D)** Blots of STAT3, pSTAT3 and GAPDH for all treatments.

To determine the optimal concentration of IL-6 and sIL-6R in healthy control skin fibroblasts for STAT3 phosphorylation, and to identify if this relates to collagen expression, a dose response of IL-6 and sIL-6R was used. Type I Collagen, STAT3 and pSTAT3 were measured by western blot (**Figure 4.2A, 4.2B**). A range of concentrations, up to pharmacological concentrations, were used for IL-6 and sIL-6R (Figure 4.2C). Protein was extracted and the expression of Collagen I, STAT3 and pSTAT3 were analysed by western blotting. Collagen I and pSTAT3 expression increased with increasing concentrations of IL-6 and sIL-6R There was no difference in the level of STAT3 with increasing concentrations of IL-6 and sIL-6R. To further ascertain the optimal concentrations of IL-6 and sIL-6R (3ng/ml IL-6, 1µg/ml sIL-6R), and to increase power for statistical analysis two technical repeats with each healthy control were used (**Figure 4.2D**).

A

Treatment	IL-6 concentration	sIL-6R concentration
0: Untreated	0ng/ml	0ng/ml
1: IL-6 + sIL-6R	0.0123ng/ml	0.037ng/ml
2: IL-6 + sIL-6R	0.37ng/ml	0.111ng/ml
3: IL-6 + sIL-6R	1.11ng/ml	0.333ng/ml
4: IL-6 + sIL-6R	3.33ng/ml	1µg/ml
5: IL-6 + sIL-6R	10ng/ml	3µg/ml

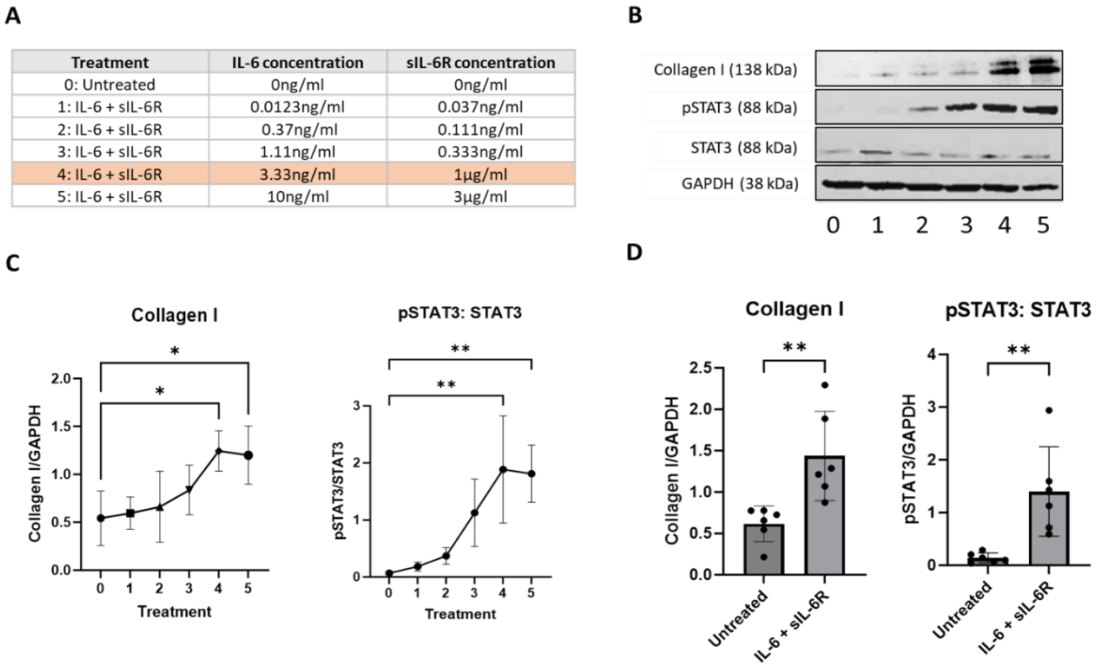


Figure 4.2: Investigation of IL-6 trans signalling in healthy control SF. **(A)** Table detailing dose response concentrations of IL-6 + sIL-6R **(B)** Blots of Collagen I, pSTAT3, STAT3 and GAPDH for all treatments **(C)** Dose response showing quantification of relative Collagen I and pSTAT3:STAT3 protein levels, obtained via ImageJ for all treatments (n=3). A one-way ANOVA was used for analysis. **(D)** Bar chart of relative Collagen I and pSTAT3:STAT3 protein levels, obtained via ImageJ for cells treated with optimal doses of IL-6 + sIL-6R (n=3; two technical repeats). A T-test was used for analysis. Significance is indicated by * = p<0.05, ** = p<0.01 throughout.

4.2.3 CTGF signaling in healthy skin fibroblasts

To investigate if truncated CTGF induces collagen in healthy skin fibroblasts, cells were incubated with increasing doses of CTGF. Previous studies have suggested that pg/ml concentrations were sufficient to produce collagen I expression, and that CTGF signals through phosphorylation of ERK1/2 (Z.-M. Song et al., 2018). Expression of Collagen I, ERK1/2, pERK1/2, STAT3, pSTAT3 were analysed by western blotting. Collagen I and pERK1/2 concentrations increased with higher doses of CTGF, peaking at 12.5pg/ml (**Figure 4.3A, 4.3B**). Doses higher than 12.5pg/ml produced a refractory effect. No significant difference was in STAT3 expression was observed, and no pSTAT3 was detected. The experiment was repeated with fewer concentrations and increased sample size to confirm previous results (**Figure 4.3C, 4.3D**).

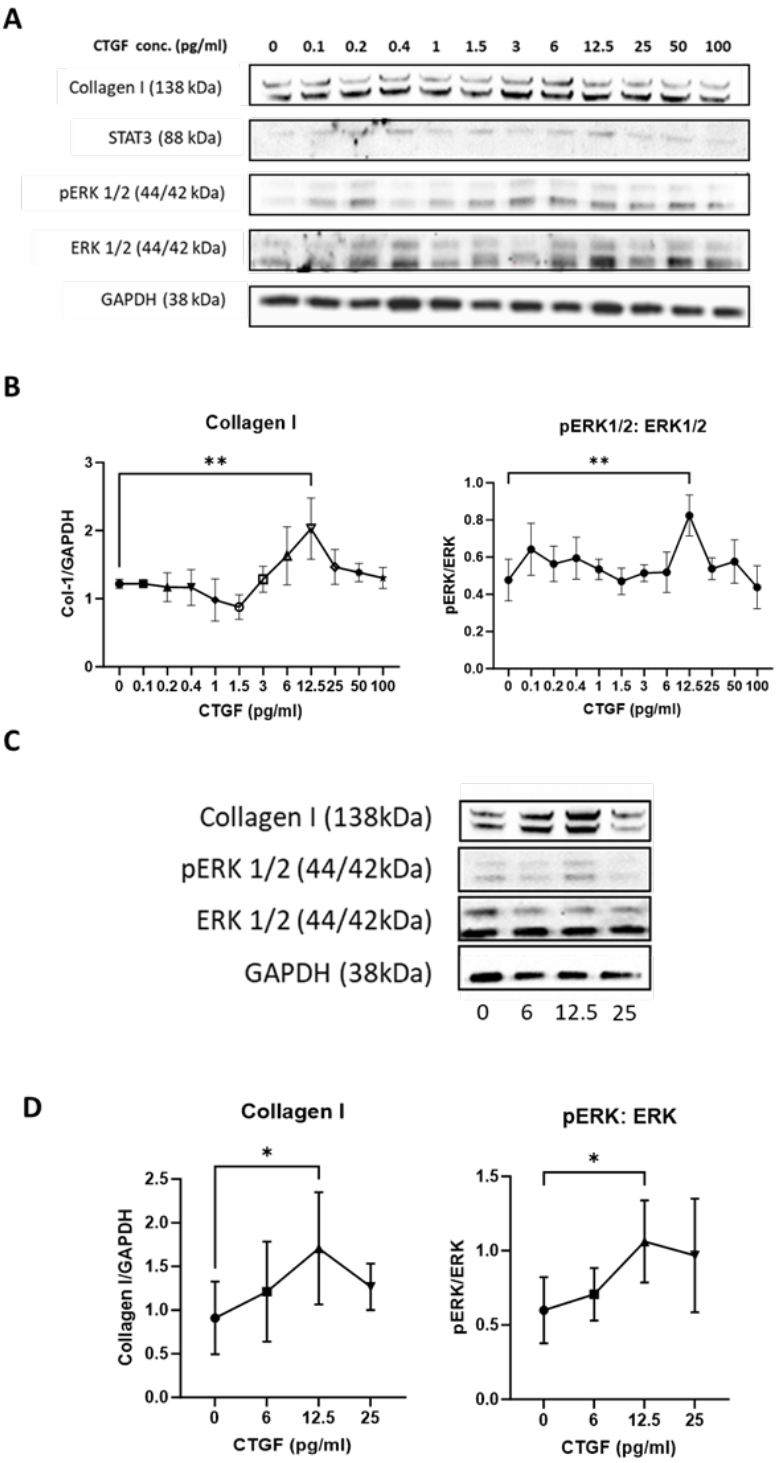


Figure 4.3: Effect of increasing CTGF on markers of fibrosis in healthy SF. (A) Blots of Collagen I, STAT3, pERK1/2, ERK1/2 and GAPDH for all doses **(B)** Dose response showing quantification of relative Collagen I, pERK1/2:ERK1/2 for all doses (n=3) **(C)** Blots of Collagen I, pERK1/2, ERK1/2 and GAPDH for optimal doses **(D)** Dose response showing quantification of relative Collagen I and pERK1/2:ERK1/2 ratio for optimal doses (n=3; two technical repeats). A one-way ANOVA was used for analysis. Significance is indicated by * = $p<0.05$, ** = $p<0.01$ throughout.

Similarly, secretion of IL-6 and MCP-1 in response to treatment with increasing concentrations CTGF was measured in healthy control skin fibroblasts by ELISA. A small sample size (n=3) was used to optimise CTGF dose initially (**Figure 4.4A, 4.4B**). To further ascertain the optimal concentrations of CTGF (12.5 pg/ml), and to increase power for statistical analysis two technical repeats with each healthy control were used (**Figure 4.4C, 4.4D**).

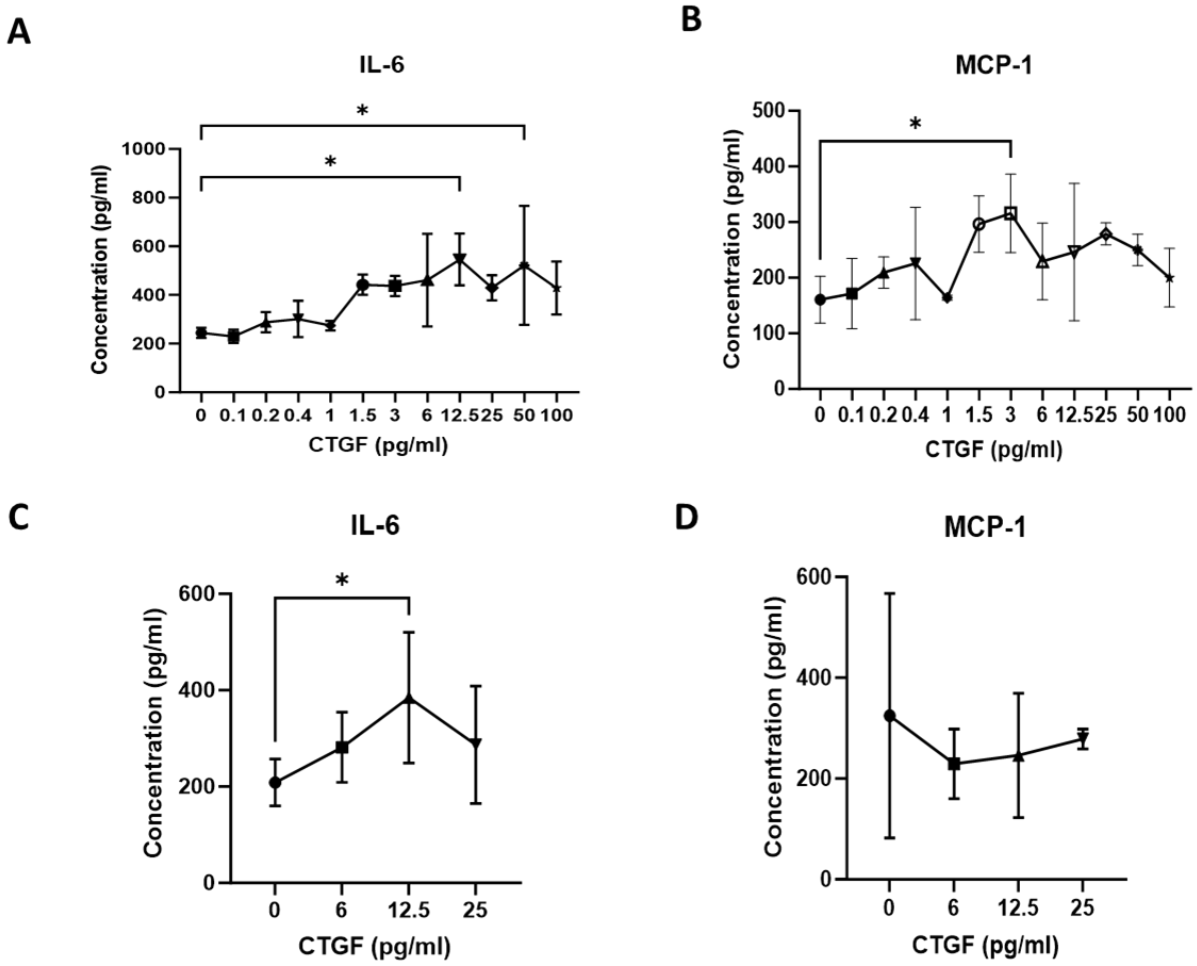


Figure 4.4: Effect of increasing CTGF on IL-6 and MCP-1 release in healthy SF. (A) IL-6 concentration in culture media for all doses of CTGF (n=3) **(B)** MCP-1 concentration in culture media for all doses of CTGF (n=3) **(C)** IL-6 concentration in culture media for optimal doses of CTGF (n=6) **(D)** MCP-1 concentration in culture media for optimal doses of CTGF (n=3, two technical repeats). A one-way ANOVA was used for analysis. Significance is indicated by * = $p<0.05$, ** = $p<0.01$ throughout.

CTGF consists of four conserved structural domains: insulin-like growth factor binding protein (IGFBP), von Willebrand factor type C (VWC), thrombospondin type 1 repeat (TSP-1), and C-terminal cystine knot (CT). Full-length CTGF refers to the complete, unaltered form, containing of all the functional domains. Truncated CTGF refers to a form of the CTGF in which a part of the protein has been removed. This can occur through various biological mechanisms, such as alternative splicing of the CTGF gene or post-translational modifications. Truncated CTGF may have altered or reduced activity compared to full-length CTGF. The presence, or absence, of specific domains can have different effects on cellular processes and signalling pathways. Truncated forms of CTGF can be produced by different cells under certain conditions, and truncated variants may have regulatory roles in in specific diseases or conditions. To date, research highlighting differences between truncated and full length forms of CTGF, with regards to mediation of fibrosis, has been scarce. To investigate different forms of CTGF within the previously identified pathways, healthy skin fibroblasts were treated with increasing concentrations of truncated CTGF (CTGF-T) (**Figure 4.5A, 4.5B**), and full length CTGF (CTGF-FL) (**Figure 4.5C, 4.5D**). Expression of Collagen I and pERK1/2 were analysed by western blot. Truncated CTGF was found to significantly increase Collagen I and pERK1/2 expression at 12.5pg/ml, however no differences were observed with CTGF-FL.

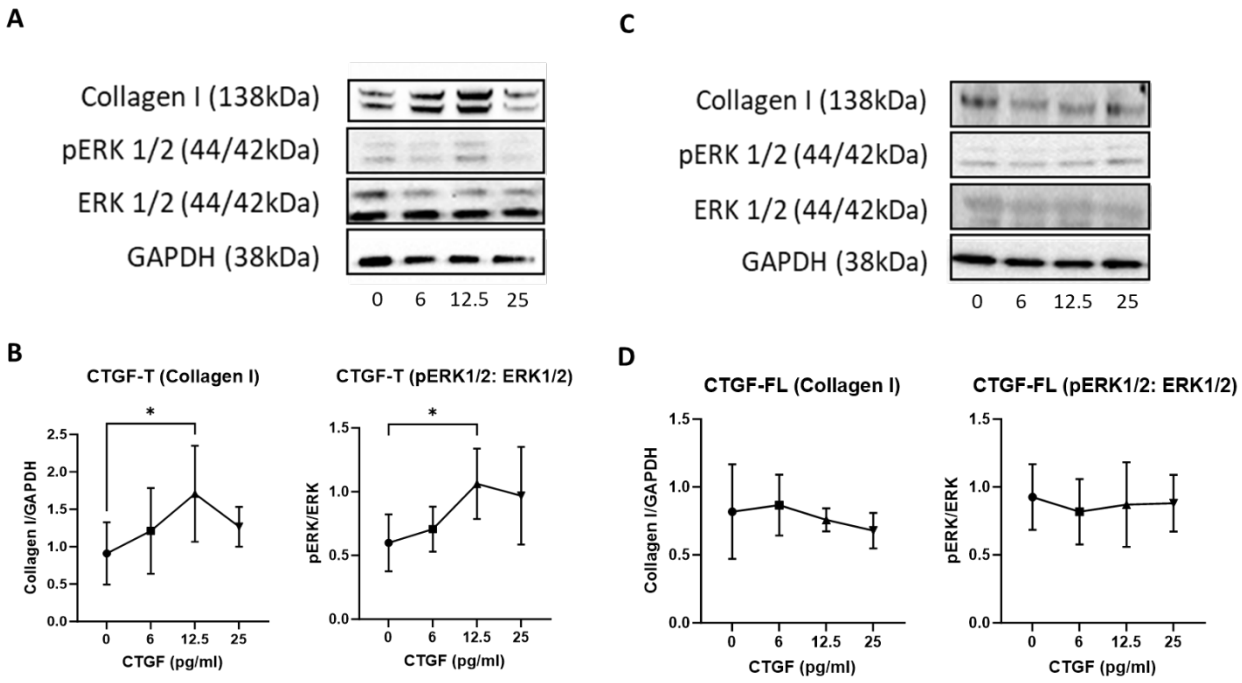


Figure 4.5: Difference in Collagen I and pERK1/2 expression with increasing concentrations of CTGF-T and CTGF-FL. (A) Blots of Collagen I, pERK1/2, ERK1/2 and GAPDH after treatment with 0, 6, 12.5 and 25pg/ml CTGF-T. **(B)** Dose response of Collagen I, pERK1/2: ERK1/2 ratio after treatment with 0, 6, 12.5 and 25pg/ml CTGF-T (n=6). **(C)** Blots of Collagen I, pERK1/2, ERK1/2 and GAPDH after treatment with 0, 6, 12.5 and 25pg/ml CTGF-FL. **(D)** Dose response of Collagen I, pERK1/2: ERK1/2 ratio after treatment with 0, 6, 12.5 and 25 pg/ml CTGF-FL (n=3, two technical repeats). A one-way ANOVA was used for analysis. Significance is indicated by * = $p<0.05$, ** = $p<0.01$ throughout.

4.2.4 sIL-6R with CTGF is the most fibrotic in healthy skin fibroblasts

Previous studies have suggested key roles for both sIL-6R and CTGF individually within the inflammatory/fibrotic process (Brigstock, 2010; Rose-John, 2012a). In this study, treatment of healthy skin fibroblasts with either sIL-6R or CTGF, led to an increase in Type I Collagen expression, albeit through different pathway mediators (**Figure 4.2, 4.3**). The sIL-6R was shown to act through pSTAT3 (**Figure 4.2D**), while CTGF mediates its effects through pERK1/2 (**Figure 4.3D**). Additionally, treatment with CTGF in this study resulted in increased production, and release of IL-6. More readily available IL-6 molecules in circulation could result in more IL-6/sIL-6R complexes formed, and a more inflammatory environment. Furthermore, a more inflammatory environment could stimulate

fibroblasts to release a ‘cytokine storm’, within which more CTGF and more sIL-6R could be produced. Thus, it is probable that sIL-6R and CTGF could work in synergy to drive a pro-inflammatory, pro-fibrotic environment which is characteristic of many diseases.

To investigate this, healthy skin fibroblasts were treated with IL-6, CTGF, sIL-6R alone, at concentrations determined previously, or with a combination of IL-6 + sIL-6R, and CTGF + sIL-6R. Expression of Collagen I, STAT3, pSTAT3, pERK1/2 and ERK1/2 were determined by western blotting (**Figure 4.6A, B**). Concentrations of IL-6, MCP-1, MCP-3 and MCP-4 were measured by ELISA (**Figure 4.6C**).

Treatment with CTGF, sIL-6R, IL-6 + sIL-6R and CTGF + sIL-6R resulted in a significant increase in Collagen I expression. With sIL-6R, IL-6 + sIL-6R and CTGF + sIL-6R, pSTAT3 was apparent. When the cells were treated with either CTGF, or CTGF + sIL-6R, there was a significant increase in pERK1/2. Similarly, treatment with CTGF, sIL-6R, IL-6 + sIL-6R and CTGF + sIL-6R all resulted in a significant increase IL-6, MCP-1, MCP-3 and MCP-4 concentration. Interestingly, the highest expression of all investigated fibrotic mediators was observed when fibroblasts were treated with a combination of CTGF and sIL-6R, suggesting that the two work in synergy to create a pro-fibrotic, pro-inflammatory environment.

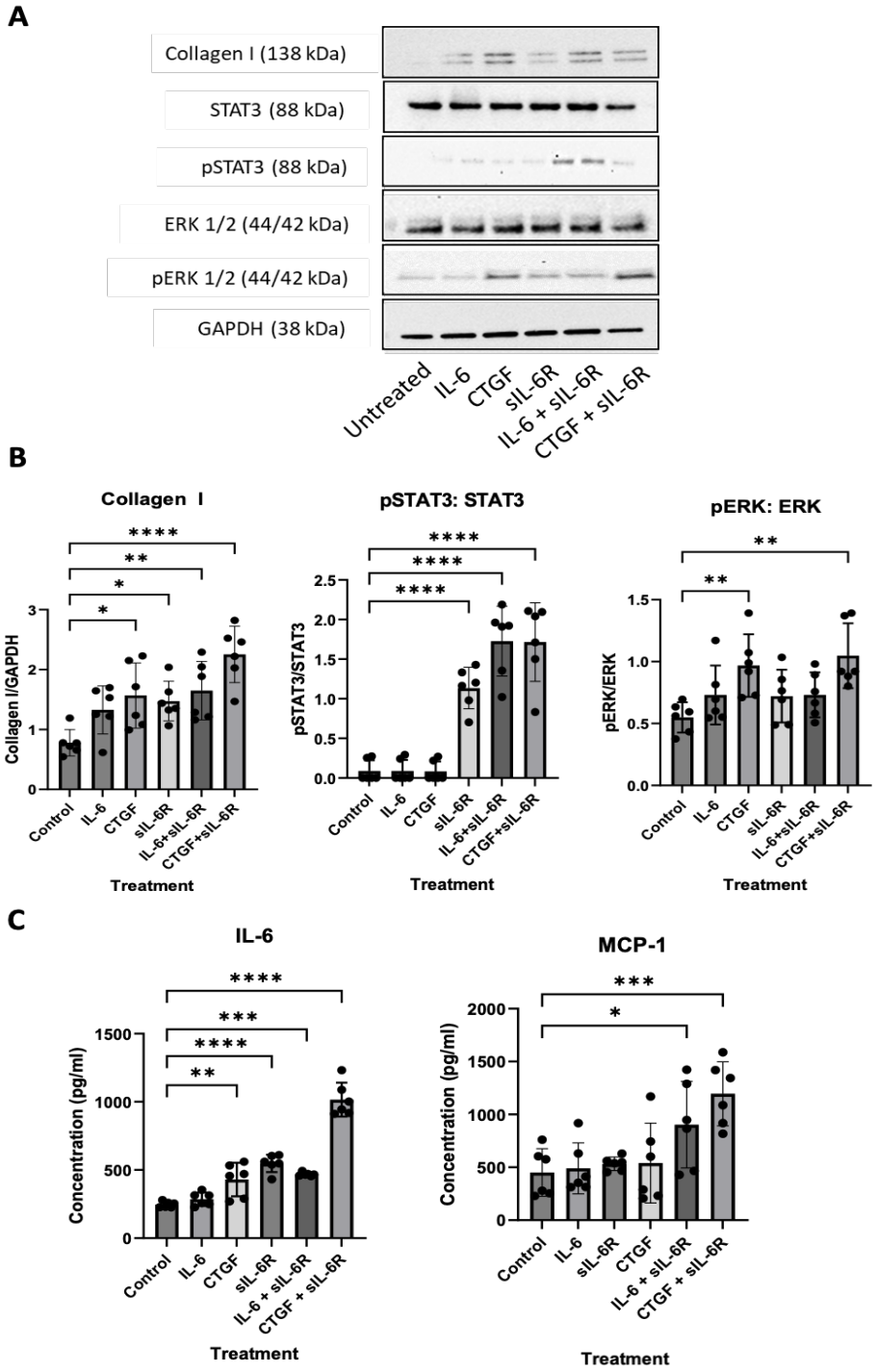


Figure 4.6: Comparison of IL-6, CTGF and sIL-6R used alone, or in combination on fibrotic markers in healthy skin fibroblasts. (A) Blots of Collagen I, STAT3, pSTAT3, ERK1/2, pERK1/2 and GAPDH after treatment with IL-6, CTGF, sIL-6R alone, IL-6+sIL-6R, or CTGF + sIL-6R **(B)** Graphs of Collagen I pSTAT3:STAT3 ratio, and pERK1/2:ERK1/2 ratio for all treatments (n=3, two technical repeats). **(C)** Concentration of IL-6, MCP-1, MCP-3 and MCP-4 after treatment with IL-6, CTGF, sIL-6R alone, IL-6 + sIL-6R, or CTGF + sIL-6R (n=3; two technical repeats). A one-way ANOVA was used for analysis. Significance is indicated by * = $p<0.05$, ** = $p<0.01$ throughout.

4.3 Discussion

Fibrosis represents a common manifestation associated with various chronic diseases, such as coronary heart disease, obesity and SSc (Buechler et al., 2015; Y. Y. Ho et al., 2014; Lei et al., 2013). The immunoregulatory actions of certain cytokines drives the fibrotic process, either directly, or through recruitment of chemoattractants and/or acute phase proteins. Of these cytokines, IL-6 and CTGF are of particular interest, and are hallmarks of chronic inflammation in these diseases.

IL-6 is a uniquely endocrine cytokine that has been shown to exhibit both pro and anti-inflammatory effects. IL-6 plays a key role in the resolution of inflammation during the acute phase response, decreasing circulating levels of pro-inflammatory cytokines including TNFα and IFNγ (Xing et al., 1998) and inducing the release of anti-inflammatory IL-1Ra (Gabay et al., 1997). However, IL-6 has been shown to mediate the switch from acute to chronic inflammation, leading to secretion of the chemokines IL-8 and MCP-1, and localisation of leukocytes to the inflammatory site (Romano et al., 1997). Many interleukins, including IL-6, have been shown to progress the pathogenesis of idiopathic pulmonary fibrosis (She et al., 2021). In CHD, sustained elevation of IL-6 releases fibrinogen, leading to scarring and further exacerbation of the fibrotic process, and constitutive IL-6 release from adipocytes has been shown to advance the progression of obesity (Yudkin et al., 2000a), (Mohamed-Ali et al., 2001b).

Thus, IL-6 exhibits two contradictory inflammatory profiles. There is evidence to suggest that IL-6's two signalling pathways, classical signalling and trans signalling, account for this difference. While it is unclear how these pathways lead to such varied outcomes, current research indicates that classical signalling may be the more anti-inflammatory pathway, while trans signalling leads to more activation of pro-inflammatory genes (Reeh et al., 2019), (Rose-John, 2012b). This arises from IL-6 binding to two variations of its receptor: the transmembrane form (IL-6Rα) and the soluble form (sIL-6Rα). The sIL-6Rα, produced by either proteolytic cleavage of IL-6Rα (Mülberg et al., 1993) or alternative mRNA splicing (Lust et al., 1992), has been shown to exist in molar excess in the circulation (Mohamed-ali et al., 1999). Classical signalling occurs when IL-6 binds to IL-6Rα.

This complex binds to the signal transducer gp130, and leads to the transcription of anti-inflammatory genes (Schaper & Rose-John, 2015). In trans signalling, the sIL-6Rα/IL-6 complex associates with the gp130 subunit, causing pro-inflammatory effects (Taga et al., 1989). In both pathways, the primary signalling pathway has been suggested to be via activation of JAK/STAT. Interestingly, the gp130 subunit, exists in a soluble form (sgp130) which may antagonise the IL-6/IL-6Rα and IL-6/sIL-6Rα complexes.

CTGF is a matricellular protein that is crucial for normal functionality, playing a considerable role in cell proliferation, angiogenesis and fibrogenesis (Holbourn et al., 2008b), (Ponticos, 2013). CTGF is induced primarily by TGFβ, via activation of the SMAD pathway (Ponticos, 2013), (Holmes et al., 2001), however, VEGF and integrins have also been implicated (Chen et al., 2020). Pathologically, CTGF has been suggested to modulate cardiac, renal and hepatic fibrosis (Dorn et al., 2018), (Toda et al., 2018) (Kodama et al., 2011). In SSc patients, isolated fibrotic fibroblasts showed overexpression of CTGF (Shi-Wen et al., 2007), and in mouse models of SSc, monoclonal antibodies blocking CTGF signalling attenuated fibrosis (Makino et al., 2017). While CTGF signalling remains vastly uncategorised, studies have increasingly shown the involvement of the extracellular signal-regulated kinase (ERK) pathway mediated CTGF in the progression of various inflammatory diseases (Z.-M. Song et al., 2018). A bleomycin-induced mouse model of pulmonary fibrosis highlighted the importance of CTGF and ERK phosphorylation in development of lung fibrosis, with the effects partly ameliorated by Atorvastatin (Zhu et al., 2013). Still, there is much to be uncovered about CTGF signalling, and it's relationship with IL-6 remains mostly uncategorized, although there is some evidence to suggest that CTGF increases IL-6 expression in fibroblasts (S. C. Liu et al., 2012). As both CTGF and IL-6 are paramount to the fibrotic process, they may play a synergistic role in the pathology of fibrotic diseases. Studying the cross-talk between these two cytokines in fibrotic models such as SSc would provide a greater insight into their molecular mechanisms.

MCP-1 is a chemokine, which plays a crucial role in the recruitment and activation of monocytes to sites of inflammation or injury within the body. Once activated, monocytes can differentiate into macrophages, which are important for the

clearance of pathogens, dead cells, and debris, as well as for tissue repair and remodelling (Deshmane et al., 2009). MCP-1 dysregulation has also been implicated in various fibrotic conditions, and mice with MCP-1 deficiency have been reported to have diminished skin fibrosis (Ferreira et al., 2006). The relationship between IL-6 and MCP-1 has been well documented. IL-6 can induce MCP-1 expression in certain cell types, thereby promoting the recruitment of monocytes to inflammatory sites (Marino et al., 2008). The trans signalling pathway may contribute to MCP-1 dysregulation in chronic inflammation, as the IL-6/sIL-6R complex has been shown to induce endothelial cells to shift from neutrophil-recruiting IL-8 to a monocyte-recruiting MCP-1 cytokine profile (Kaplanski et al., 2003). While this serves to reduce neutrophil infiltration, and is an important step in the resolution of inflammation, disruption within this system could lead to sustained mononuclear cell permeation to damaged tissues, which can contribute to fibrosis and disease pathogenesis.

With regards to CTGF, the interactions with MCP-1 are lesser understood. MCP-1 and CTGF may interact indirectly through their involvement in common pathways related to inflammation and tissue repair. MCP-1-induced monocyte infiltration at sites of tissue injury or inflammation can lead to the production of various cytokines and growth factors, including CTGF, which in turn promotes fibrosis and tissue remodelling. There is some evidence that CTGF can directly induce MCP-1 expression through multiple signalling pathways, including phosphorylation of ERK in synovial fibroblasts (S.-C. Liu et al., 2013).

In order to determine an appropriate cell model to investigate IL-6 signalling, 4 different cell types (skin fibroblasts, HCAECs, Jurkat cells and HepG2 cells) were grown and starved for 24hours, and IL-6 levels in the culture media were measured by ELISA. HCAECs released the most amount of IL-6, followed by skin fibroblasts. Both Jurkat cells and HepG2 cells showed no constitutive release of IL-6. No sIL-6R was measured in any of the cell culture mediums. HepG2 cells have been shown to express the membrane-bound IL-6R, while studies have suggested that Jurkat cells lack the cell-surface receptor, and therefore cannot signal through classical signalling (Igaz et al., 2000). As Jurkat cells neither constitutively produced IL-6, nor expressed the membrane-bound IL-6R, these were taken forward to investigate IL-6 trans signalling.

Jurkat cells were treated with IL-6 and/or sIL-6R at physiological and disease/pharmacological levels (Baran et al., 2018b; Jones et al., 2018), and protein expression of STAT3 and pSTAT3 were determined by western blot. STAT3 phosphorylation only occurred in instances where IL-6 and sIL-6R were incubated together at high, pharmacological doses. This result is in line with current research indicating that IL-6 trans signalling occurs through phosphorylation of STAT3. The concentrations taken forward were 10ng/ml IL-6 and 3ug/ml sIL-6R.

Skin fibroblasts were used to determine if trans signalling lead to fibroblast activation and the formation of a pro-fibrotic environment. Cells were treated with increasing doses of IL-6 and sIL-6R up to the pharmacological dose determined previously. Western blotting was used to estimate STAT3, pSTAT3 and Type I Collagen expression. Expression of pSTAT3 and Collagen I increased in a dose-dependent manner, which plateaued at 3.33ng/ml IL-6, 1ug/ml sIL-6R. No changed were observed in STAT3 expression. This indicated that trans signalling is associated with increased ECM protein deposition in healthy skin fibroblasts and may trigger an inflammatory fibrotic environment in these cells.

To investigate if CTGF also produces a similar pro-fibrotic effect in these cells, healthy control skin fibroblasts were treated with increasing concentrations of CTGF. Expression of STAT3, ERK1/2 and Collagen Type I were measured by western blot. Expression of pERK1/2 and Collagen I were significantly increased when treated with 12.5pg/ml CTGF. This is consistent with previous studies indicating that picogram per millilitre levels of CTGF are sufficient to induce fibrosis (Fan et al., 2000). To determine if CTGF treatment can directly contribute to IL-6 and MCP-1 production, the cell culture media was analysed by ELISA. Levels of IL-6 and MCP-1 were also significantly increased with increasing CTGF, and for IL-6 this was also at the 12.5pg/ml concentration. No difference was found with pSTAT3, likely because no sIL-6R was present in the culture media. Research has shown, however, that the sIL-6R exists in molar excess in the circulation. As these cytokines do not exist in isolation and tend to interact with each other, it may be that CTGF and sIL-6R have a synergistic effect in inducing fibrosis.

Interestingly, when truncated and full length CTGF were compared, the truncated form appeared to induce fibrosis to a larger extent. While reasons for this are unclear, truncated CTGF variants may have altered binding affinities, resulting in enhanced or aberrant signalling activities that promote fibrotic processes. Additionally, CTGF has been shown to have both pro-fibrotic and anti-fibrotic effects depending on the cellular context and signalling environment. Truncation of CTGF may result in the loss of regions that normally mediate its anti-fibrotic effects or its interaction with negative regulators, leading to loss of negative regulation.

Considering skin fibroblasts were shown previously to release IL-6 constitutively (**Figure 4.1A**), and to further understand the complex interactions between these molecules, the experiments were repeated to include treatments of IL-6, CTGF and sIL-6R alone, as well as IL-6 with sIL-6R and CTGF with sIL-6R. It has been well documented that IL-6 induces CTGF, however these results suggest that CTGF also induces IL-6 and MCP-1 which in turn, lead to an increase in Collagen type I through pSTAT3. CTGF may also directly contribute to this Collagen deposition through pERK1/2.

When cells were treated with 12.5pg/ml CTGF, 1μg/ml sIL-6R or a combination of both, pSTAT3 was present only when sIL-6R was available in the culture media. As skin fibroblasts produce IL-6 constitutively, activation of the trans signalling pathway can still occur without further addition of IL-6 into the culture media. In cases where CTGF was present, pERK1/2 was significantly increased, however this was not activated through IL-6/sIL-6R signalling. Levels of collagen were significantly increased in all treatment conditions, except with IL-6 alone. This may be due to the lack of sIL-6R in the cell culture media, thus the trans signalling pathway was not induced. The highest expression of Collagen was apparent when the cells were treated with a combination of CTGF and sIL-6R.

Concentrations of IL-6 and MCP-1 were also measured by ELISAs in the cell culture media. IL-6 levels were significantly increased when treated with CTGF, sIL-6R, IL-6+sIL-6R and CTGF+sIL-6R. As with Collagen expression, the highest concentration of IL-6 was apparent when CTGF was combined with sIL-6R. Similarly, MCP-1 was also significantly increased in both IL-6+sIL-6R and

CTGF+sIL-6R treatments, with the combination of CTGF and sIL-6R having the largest effect.

Skin fibroblasts taken from 3 patients with SSc were also treated with IL-6, CTGF, sIL-6R alone or combinations of IL-6 with sIL-6R and CTGF with sIL-6R. Similar to the healthy fibroblasts, pSTAT3 was only present when cells were treated with sIL-6R, alone or in combination. However there was no significant difference in pERK1/2 or Collagen I expression when analysed by western blot, and no significant difference in IL-6 and MCP-1 concentration when measured by ELISA. There was high variability among the SSc group, which may have contributed to the lack of differences resulting from treatment. When healthy control and SSc fibroblasts were compared for IL-6 and Collagen Type I expression, there were no significant differences. It is well documented in the literature, and proteomics results from Chapter 3 (**Figure 3.4**) indicate that SSc patients have increased levels of IL-6 and MCP-1, as well as high collagen levels which contribute to skin thickness. In this instance, lack of significant difference may be due to patient treatments regimens taken at the time of sampling. Routine therapies for SSc include immunosuppressants such as cyclophosphamide (CyP), methotrexate (MTX), and mycophenolate mofetil (MMF), which have clear effects on circulating cytokine levels as well as ECM protein production (Katsiari et al., 2018). Additionally, the anti-IL-6R monoclonal antibody tocilizumab is also used clinically to treat SSc, which, if taken by the patient, would have a direct dampening effect on both the trans signalling and CTGF signalling pathways discussed. Thus, the lack of documentation of the patient treatment plans is a clear limiting factor in this experiment. Ultimately, healthy control fibroblasts were used for further experiments as they produced more consistent results and did not carry the confounding variables related to treatment.

Taken together, these results suggest that CTGF and the trans signalling pathway act in synergy to initiate and contribute to a pro-fibrotic environment. This occurs through activation of genes coding for ECM proteins such as collagen, via phosphorylation of signalling molecules STAT3 and ERK1/2. Novel therapeutic antibodies that can selectively target CTGF and sIL-6R may help to ameliorate fibrosis.

Emerging evidence suggests intricate crosstalk between the IL-6 and CTGF signalling pathways. IL-6 has been shown to modulate CTGF expression, and reciprocally, CTGF can influence IL-6 signalling. Understanding the molecular mechanisms governing this interplay is crucial for unravelling the complexities of fibrogenesis (**Figure 4.7**). Given the central roles of IL-6 and CTGF in fibrosis, targeting these pathways presents promising therapeutic strategies. Inhibitors of IL-6 signalling, such as monoclonal antibodies or small molecule inhibitors, are under investigation for their efficacy in ameliorating fibrotic conditions. Similarly, interventions targeting CTGF, including antibodies and gene therapies, hold potential for disrupting the fibrotic cascade.

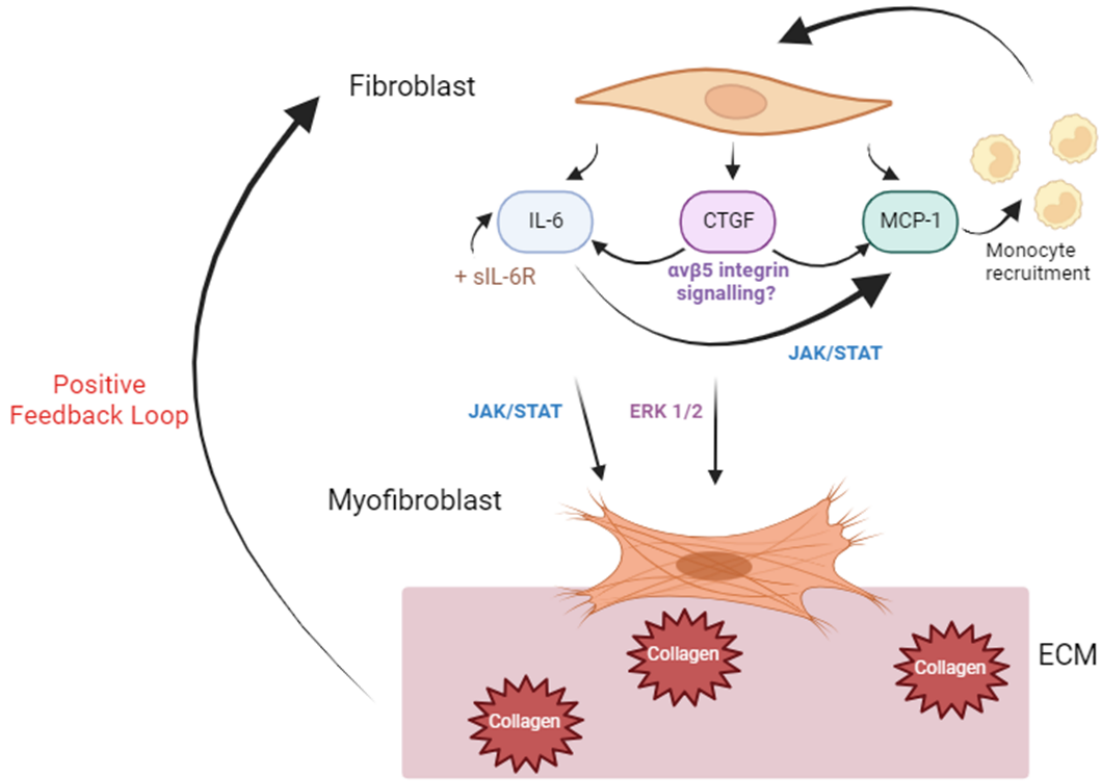


Figure 4.7: Schematic detailing fibroblast activation to a myofibroblast. The fibrotic process involving the effects of and interactions between IL-6, sIL-6R, CTGF, and MCP-1, and their downstream signalling pathways.

This chapter explored the intricate relationship between IL-6 and CTGF signalling pathways in the context of fibrosis. Elucidating the molecular mechanisms governing this interplay enhances our understanding of fibrogenesis, providing valuable insights for the development of targeted therapeutic interventions. The

potential success of targeting IL-6 and CTGF opens new avenues for the treatment of fibrotic disorders, bringing us closer to more effective and specific therapeutic strategies for patients suffering from these debilitating conditions.

Biologics have increasingly shown promise in the treatment of SSc, and SSc-related pulmonary complications, however their high toxicities and variable effects remain challenging. Novel therapeutic agents targeting pathways in a more specific manner could prove beneficial. Two inflammatory mediators identified in this chapter, CTGF and sIL-6R, were chosen as targets to produce new, more specific, camel-derived biologics.

Chapter 5

Production, characterisation and use of camel-derived antibodies targeting CTGF and sIL-6R

5.1 Introduction

Unlike conventional antibodies that consist of two heavy and two light chains, HcAbs are smaller in size, consisting of a single monomeric variable domain (Hamers-Casterman et al., 1993b). Both conventional and HcAbs are naturally occurring antibodies found in camelids. V_HH fragments, also known as nanobodies, are bioengineered antibody fragments consisting of the antigen binding (Fab) portion of HcAbs (Muyldermans, 2013b). HcAbs and V_HH fragments offer several advantages over conventional antibodies. Their smaller sizes (50 KDa and 15 KDa respectively), make them more compact and better suited for penetrating tissues and reaching targets that might be challenging for larger antibodies. Due to their structure, they are more stable and less susceptible to unfolding or denaturation under various conditions (Vincke & Muyldermans, 2012). Moreover, they exhibit unusual binding profiles, binding convexly into the grooves of the antigen rather than attaching concavely to its surface. This allows them to access hidden epitopes, otherwise undetected by conventional antibodies (Wesolowski et al., 2009).

V_HH fragments confer further advantages, as they can be efficiently produced in microbial systems such as bacteria or yeast. Using phage display technology further can simplify and accelerate this production process. They can also be easily engineered and modified for various applications, such as fusing them to other proteins or creating bispecific antibodies. Finally, their stability and small

size of could make them suitable candidates for oral delivery, although challenges related to the gastrointestinal environment must be overcome (Arbabi-Ghahroudi et al., 2005; Dumoulin et al., 2002; Pothin et al., 2020). The use of V_HH fragments in therapeutic applications has increased in recent years, with three being approved for clinical use, and many others undergoing various phases of clinical trials (Steeland et al., 2016).

Previous chapters of this thesis identified the roles of IL-6, sIL-6R and CTGF in signalling pathways leading to fibrosis *in vitro.* This chapter aimed to investigate the therapeutic potential of novel camelid antibodies targeting sIL-6R and CTGF. The central hypothesis for this chapter is targeting sIL-6R and CTGF with camel-derived conventional, HcAbs and V_HH fragments may effectively reduce activation of the STAT3 and ERK1/2 pathways, and thus fibrosis, *in vitro*.

5.2 Results

All animal experiments conducted were carried out at Anti-Doping Lab Qatar (ADLQ) in compliance with the Animals Scientific Procedures Act (1986) and University College London Research guidelines. Immunisations and blood sampling was carried out by Dr Babiker. Production of HcAbs and V_HH fragments was conducted both at UCL, Royal Free Hospital and at ADLQ, with support of Dr Konduru Seetharama Sastry. Recombinant protein following periplasmic expression in *E. Coli* of one anti-CTGF clone and several anti-sIL-6R clones of V_HH fragment antibodies were generated. The clones were selected based on binding to their respective antigens.

5.2.1 Camelid antibody production

5.2.1.1 Production of polyclonal antibodies from immune serum

Camel whole blood samples were taken at baseline, and after each immunisation with CTGF or sIL-6R. Serum was extracted and antigen binding of the anti-sera was measured by ELISA. The collected serum with the best titres were taken forward for antibody purification. For CTGF, this was the 2nd immunisation, and for sIL-6R this was the 5th immunisation (**Figure 5.1**). Serum from these was used to purify the conventional and HcAbs.

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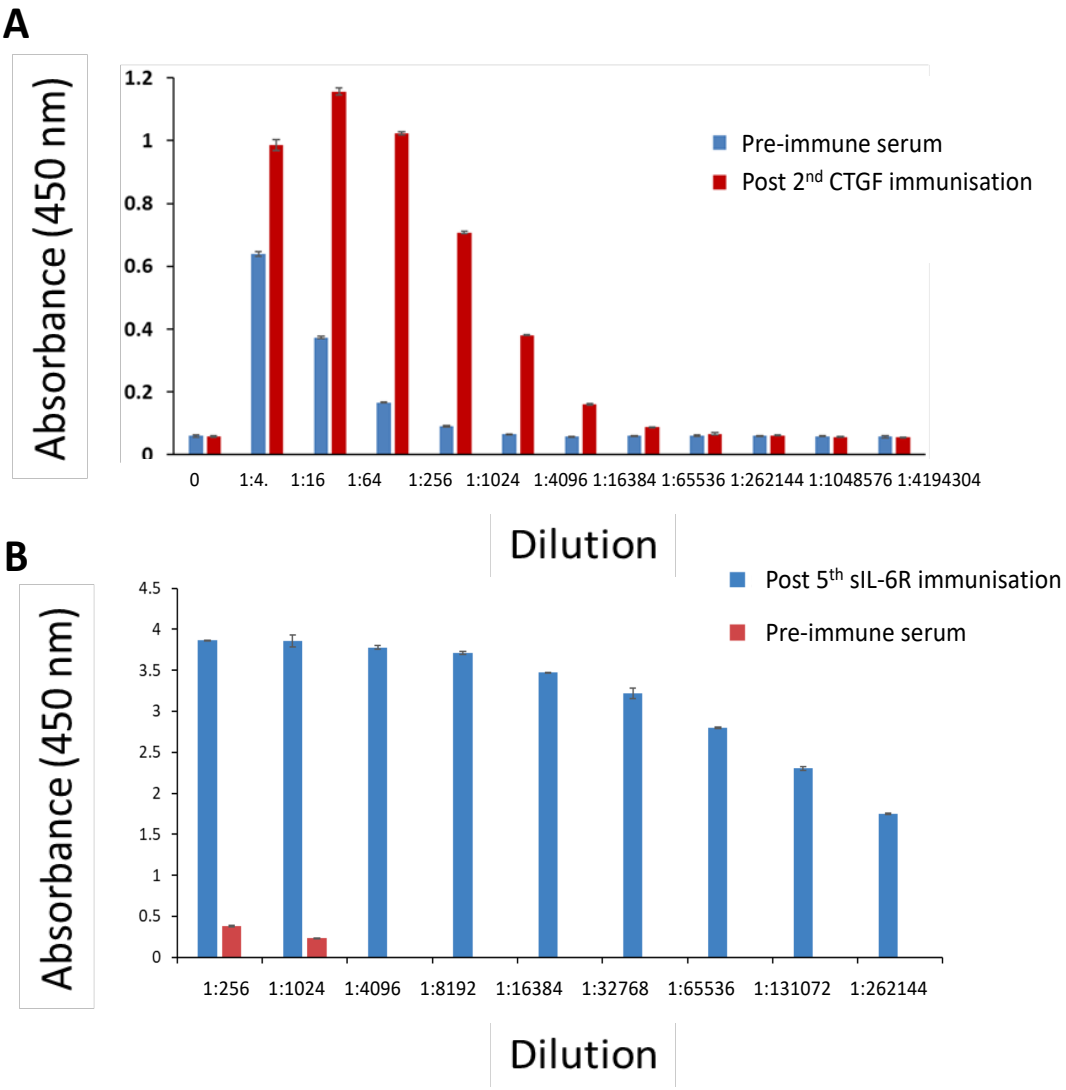

Figure 5.1: CTGF and sIL-6R immune serum titrations. Pre-and post-immune serum titers after (A) 2 CTGF immunisations, and (B) after 5 sIL-6R immunisations.

Anti-CTGF and anti-sIL-6R polyclonal antibodies were purified from the immune sera using Protein A and Protein G chromatography. Both Proteins A and G have varying affinities for different species and subclasses of IgG, thus were purified separately. IgG1 and IgG3 both show adsorption onto Protein G columns, likely because of their similar amino acid hinge region sequence (De Genst et al., 2005). They were differentially eluted with buffers of different pH (**Figure 5.2**)

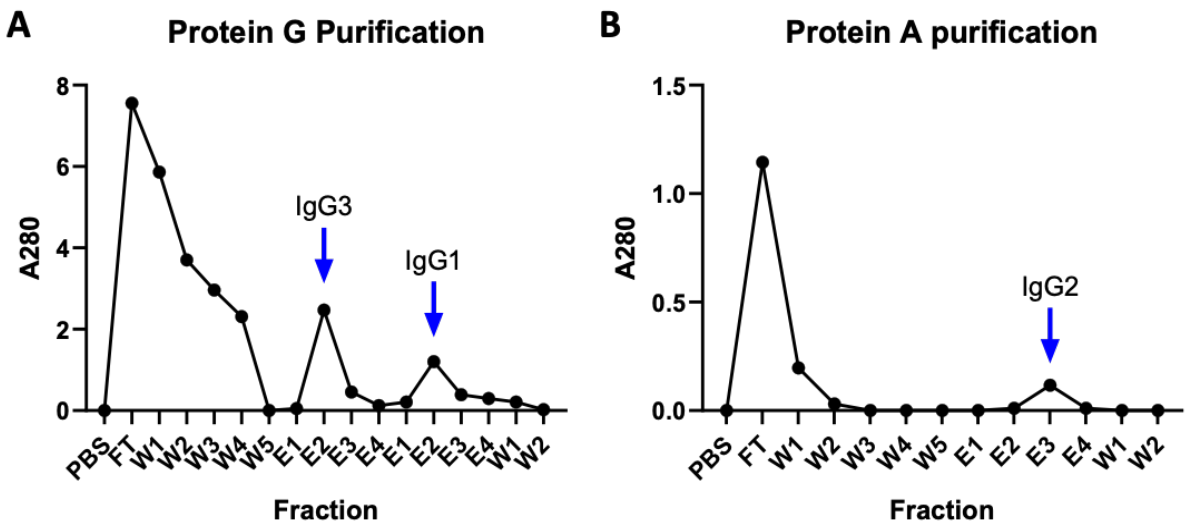

Figure 5.2: Chromatogram of IgG3 and IgG1 purification. Purification of anti-CTGF conventional (IgG1) and HcAbs (IgG3) by protein G column chromatography, using buffer A (NaCl + acetic acid buffer-pH 3.5) to elute IgG3, and buffer B (Glycine buffer-pH 2.7) was used to elute IgG1.

After collection of all fractions, the purity of the elutes was assessed by SDS PAGE and Western blot (**Figure 5.3**). The unpurified sera, flow through (FT) of both columns and pre-elution fractions were also run on the same gel. The conventional (IgG1) fraction showed two clear bands at 55 KDa and 30 KDa, corresponding to the heavy and light chains respectively. HcAbs (IgG2 and IgG3) both showed one band around 45-50 KDa indicating a lack of light chain.

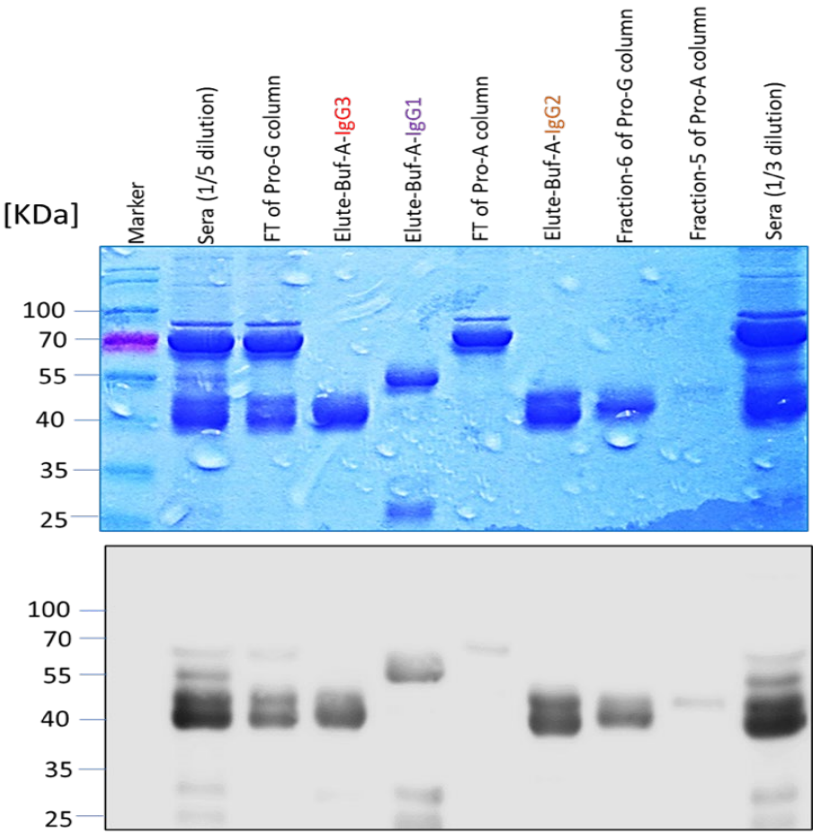

Figure 5.3: **SDS PAGE and western blot of eluted fractions.** Gel and western blot showing (left to right) protein marker, all proteins in diluted (1/5) serum, FT of protein G column, eluted fractions of protein G column (IgG3 and IgG1), FT of protein A column, eluted fraction of protein A column, fraction 6 (pre-elution fraction of protein G column), fraction 5 (pre-elution fraction of protein A column) and diluted (1/3) serum.

5.2.1.2 Production of V_HH fragments

Anti-sIL-6R and anti-CTGF V_HH fragment antibodies were largely generated by Dr Sastry at ADLQ using phage display technology methods outlined previously (Pardon et al., 2014). Screening experiments were carried out at UCL, Royal Free Hospital.

One anti-CTGF V_HH fragment was produced and was used for screening experiments. Several (n=18) anti-sIL-6R V_HH fragment clones were produced. For screening and skin fibroblast *in vitro* experiments, clone 1A4 was utilized. Experiments in HepG2 cell, utilized all 10 anti-sIL-6R V_HH fragment clones.

5.2.2 Characterisation of camelid antibodies

5.2.2.1 Optimization of anti-camel secondary antibody

The purified anti-CTGF and anti-sIL-6R antibodies were assessed for their antigen binding capacity. Firstly the secondary antibodies to be used in subsequent experiments were assessed by ELISA. The secondary antibodies used were anti-camel IgG-HRP (AD; Alpha Diagnostics) which was not specific for different subclasses, anti-camel IgG1 (AO; Antibodies Online), which was specific for conventional antibodies, and anti-camel IgG2/3 (JI; Jackson Immuno Research), which was specific for HcAb.

Overall, the AD anti-camel IgG HRP antibody recognised both conventional and heavy chain IgG classes, and did not recognise the V_HH fragment antibody. However, the antibody did show a significantly higher binding efficiency for conventional over HcAbs. The AO anti-camel IgG1 antibody showed specificity for IgG1, equal to that of AD, but no binding efficiency for HcAb (IgG2/3) or V_HH fragment antibodies. The JI anti-camel IgG2/3 antibody had a high affinity for HcAbs and did not significantly recognise the IgG1 subclass. It did, however, have a slight binding capacity for V_HH, albeit not equal to that of the HcAbs (**Figure 5.4**). Based on these results subsequent assays utilized AO for assessing the conventional antibodies and JI for assessing heavy-chain only antibodies and V_HH fragments antibodies.

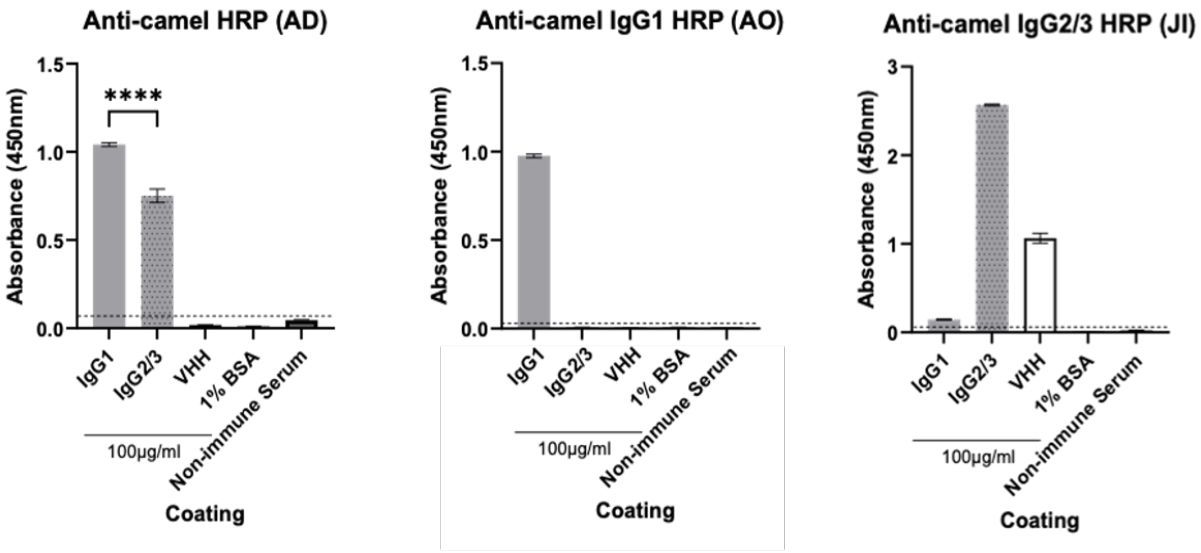

Figure 5.4: Specificity of anti-camel secondary antibodies to the different IgG subclasses to sIL-6R. Boxplots detailing the antigen binding capacity of (**A**) anti-camel IgG (AD), (**B**) anti-camel IgG1 (AO) and (C) anti-camel IgG2/3 (JI) to purified anti-siL-6R IgG1, IgG2/3 and V_HH. 1% BSA and camel non-immune serum were used as negative controls. A one-way ANOVA was used for analysis. Significance is indicated by * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, **** = $p<0.0001$.

5.2.2.2 Effect of immunization on the production of heavy chain only antibodies

The secondary antibodies identified were used to estimate the difference in secretion of conventional and heavy-chain antibodies in non-immune and immune serum (**Figures 5.5 & 5.6**). Overall, there was approximately 4-fold greater levels of IgG2/3 than IgG1 in the systemic circulation, consistent with previous studies that suggest up to 80% higher expression of HcAbs naturally (Vincke & Muyldermans, 2012). However, immunisation did not have an impact of the IgG subclass during the timeframe studied.

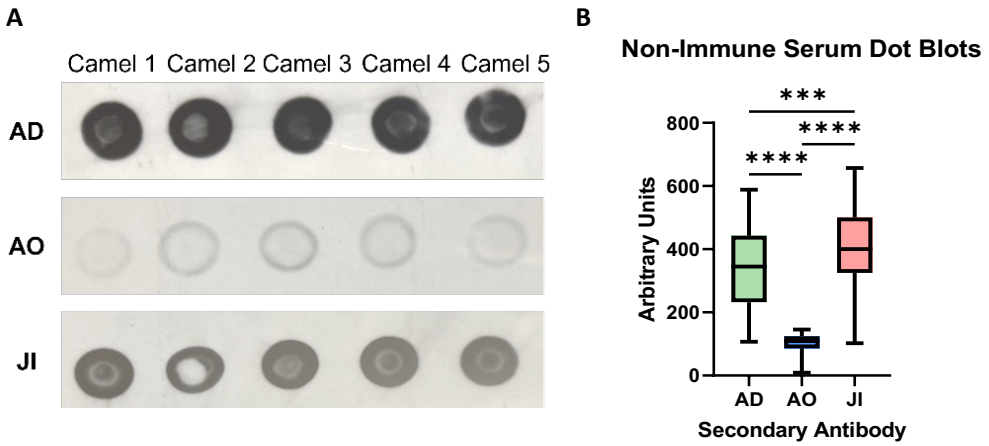

Figure 5.5: Expression of the different IgG subclasses in non-immune serum. (A) Dot blots showing expression of overall IgG, IgG1 and IgG2/3 expression in non-immunised camels (n=5) **(B)** Bar charts detailing differences in expression of overall IgG, IgG1 and IgG2/3 in non-immunised camels (n=95). Optical density was calculated and divided by exposure time in seconds to normalise (AU). 90% of samples were taken at the same exposure time, however 10% required differing exposure time points. A one-way ANOVA was used for analysis. Significance is indicated by * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001.

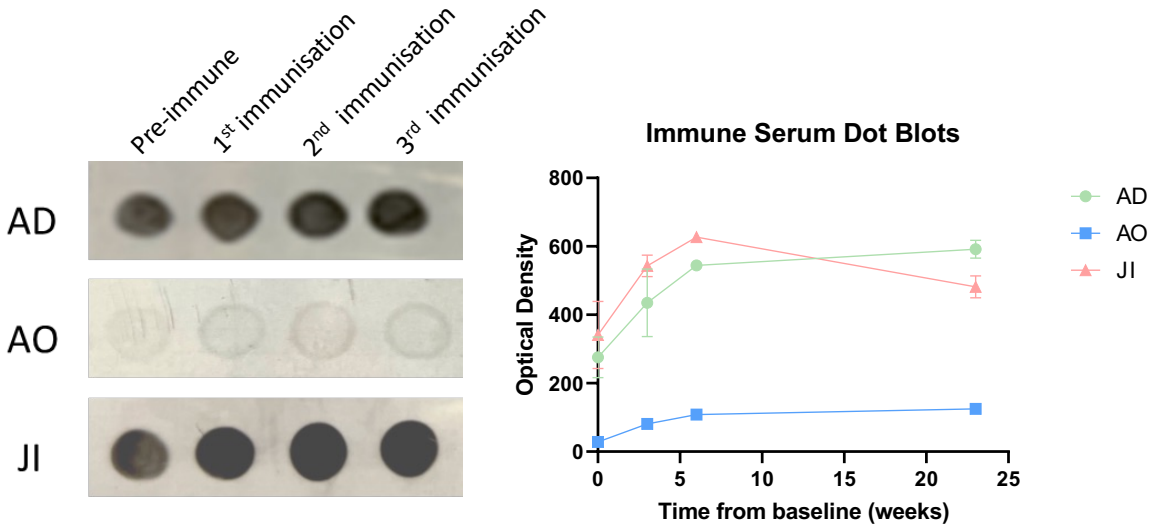

Figure 5.6: Expression of the different IgG subclasses in non-immune serum. (A) Dot blots showing expression of overall IgG, IgG1 and IgG2/3 expression in non-immunized camels (n=5) **(B)** Line graph detailing differences in expression of overall IgG, IgG1 and IgG2/3 in one camel after multiple immunisations with CTGF. Optical density was calculated and divided by exposure time in seconds to normalise (AU). 90% of samples were taken at the same exposure time, however 10% required differing exposure time points.

5.2.3 Antibody screening

Purified antibodies were assessed for their antigen binding capacity by ELISA. The anti-CTGF V_HH fragment was unable to recognize the antigen, despite the fact that the clone was derived from a HcAb library showing better binding to CTGF, compared to the conventional antibody (**Figure 5.7A**). Because of this, no subsequent experiments were conducted with the anti-CTGF V_HH fragment clone. In contrast the anti-sIL-6R V_HH fragment antibodies showed relatively higher binding to the ligand, compared to either the conventional or HcAbs (**Figure 5.7B**).

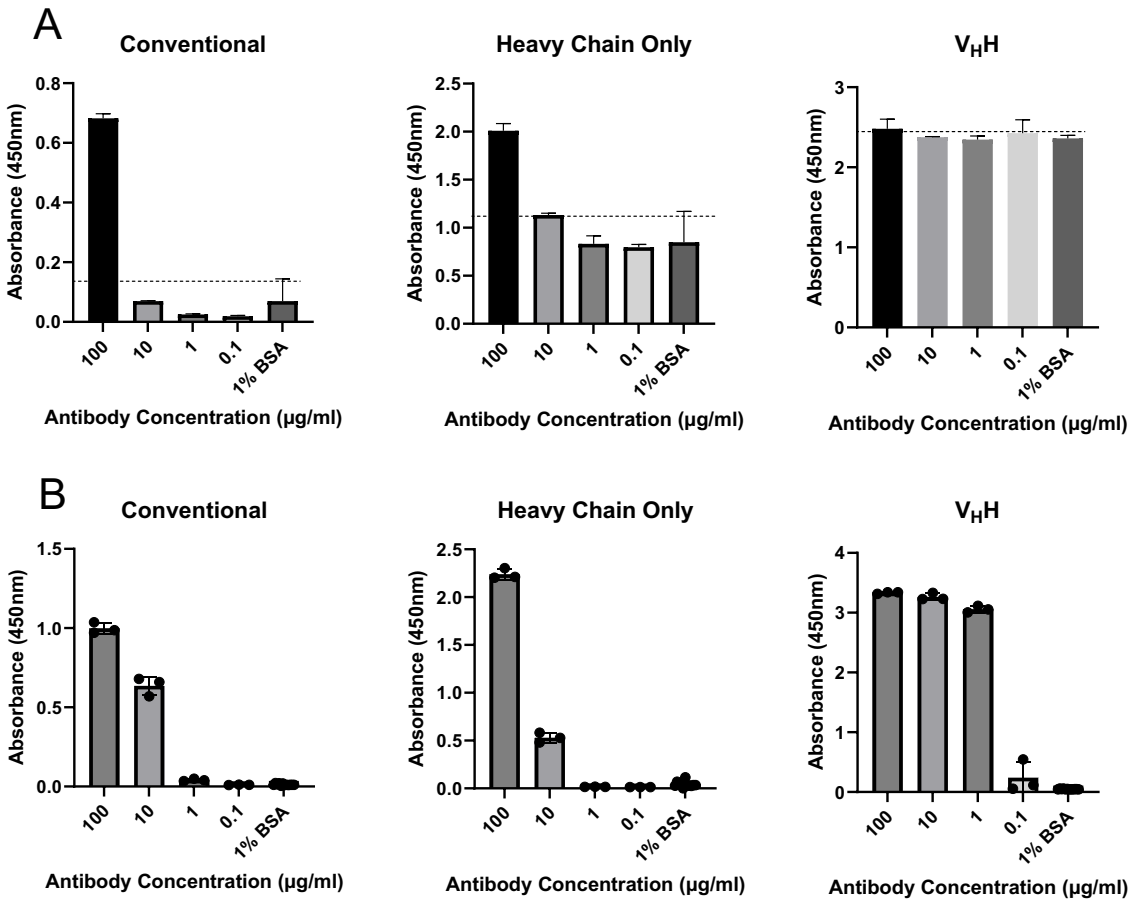

Figure 5.7: Screening of conventional, HcAbs and V_HH fragments. Antibody binding capacity of **(A)** anti-CTGF conventional, HcAbs and V_HH fragments at 100, 10, 1 and 0.1μg/ml concentrations **(B)** and anti-sIL-6R conventional, HcAbs and V_HH fragments at 100, 10, 1 and 0.1μg/ml concentrations**.**

5.2.4 Antibody functionality *in vitro*

5.2.4.1 Skin Fibroblasts

In healthy skin fibroblasts treated with sIL-6R and CTGF, anti-sIL-6R IgG1 and IgG2/3 antibodies significantly reduced the expression of pSTAT3, to a similar level as Tocilizumab. However, anti-sIL-6R V_HH fragment did not have an ameliorating effect at this concentration. In contrast, expression of pERK1/2 was significantly reduced by the anti-sIL-6R V_HH fragment clone, as well as all CTGF polyclonal antibodies and Tocilizumab (**Figure 5.8**).

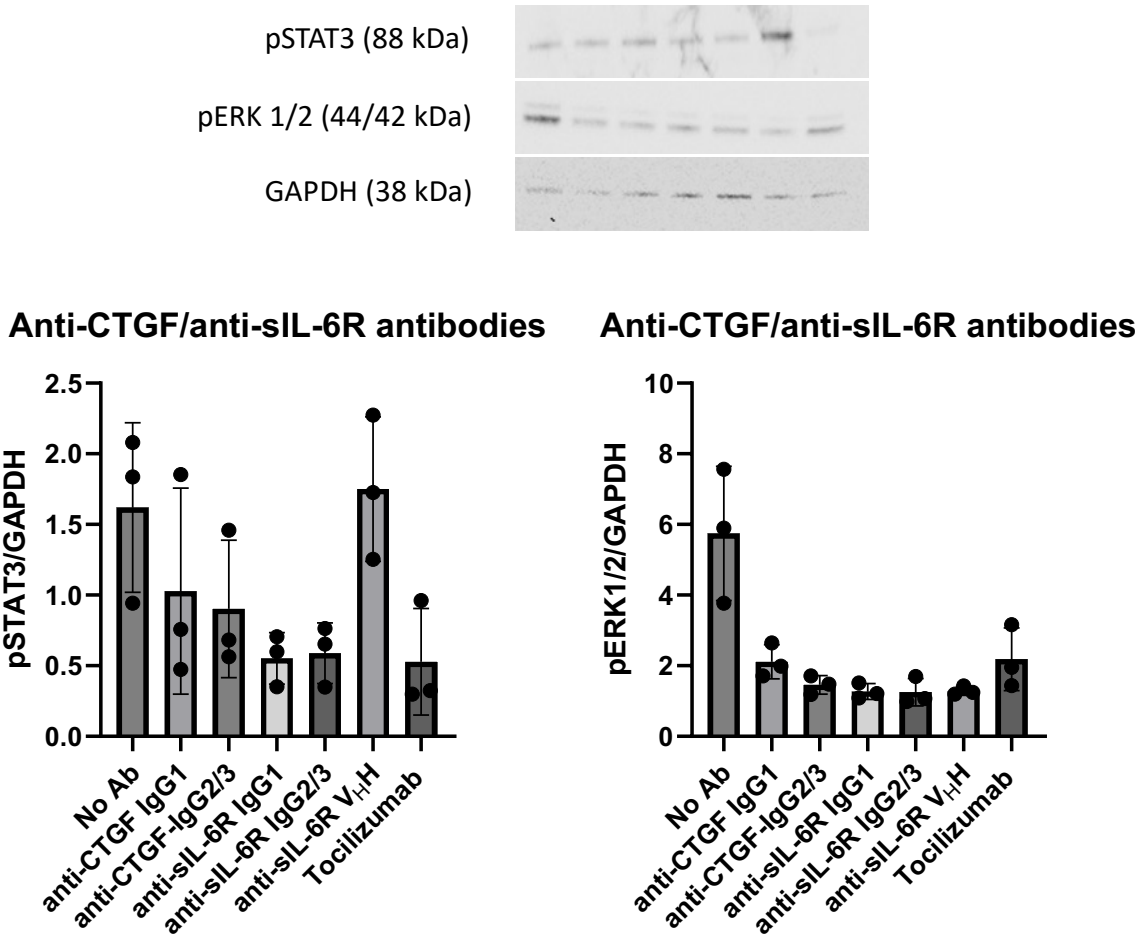

Figure 5.8: Effect of purified camelid conventional, HcAbs and V_HH fragments on the phosphorylation of STAT3 and ERK1/2 in treated skin fibroblasts. Western blots of camelid antibodies (anti-CTGF IgG1, IgG2/3 and anti-sIL-6R IgG1, IgG2/3 and V_HH) compared to Tocilizumab on pSTAT3, ERK1/2 and GAPDH. Graphs of pSTAT3/GAPDH and pERK1/2/GAPDH for all treatments (n=3).

Combinations of anti-sIL-6R and anti-CTGF antibodies were used in healthy skin fibroblasts, and expression of pSTAT3 and pERK1/2 were analyzed by western blotting. Anti-CTGF with anti-sIL-6R conventional and HcAbs dampened down expression of pSTAT3, however the presence of anti-sIL-6R V_HH appeared have little effect on pSTAT3. All antibody combinations had a diminishing effect on pERK1/2 expression (**Figure 5.9**).

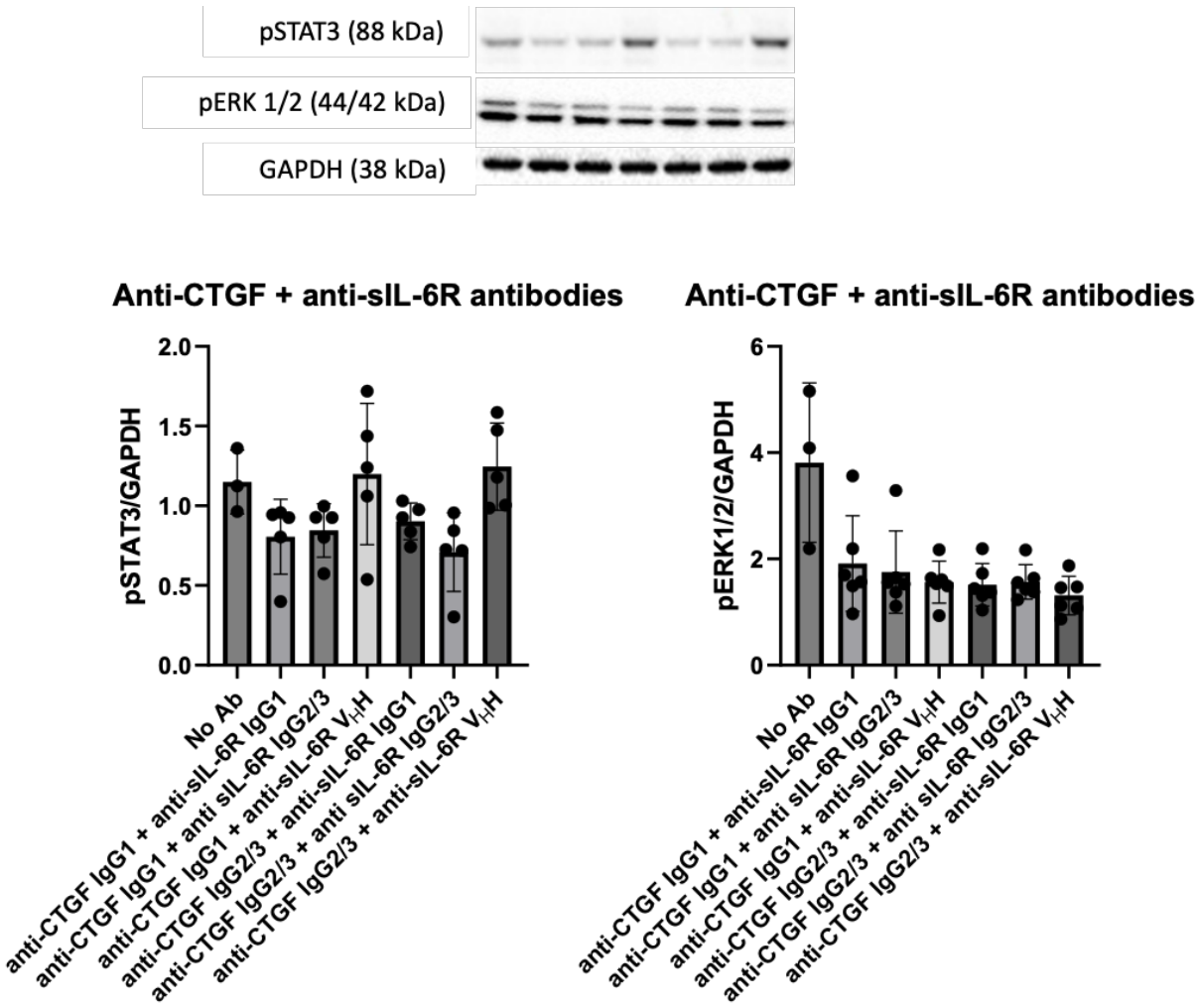

Figure 5.9: Effect of different combinations of camelid conventional, HcAbs and V_HH fragments on the phosphorylation of STAT3 and ERK1/2 in treated skin fibroblasts. Western blots of camelid antibody combinations compared to Tocilizumab on pSTAT3, ERK1/2 and GAPDH (A). Graphs of pSTAT3/GAPDH and pERK1/2/GAPDH for all treatments (n=6).

Concentration of IL-6 and MCP-1 in the cell culture media of skin fibroblasts treated with combinations of anti-CTGF and anti-sIL-6R antibodies were

measured by ELISA. A combination of anti-CTGF and anti-sIL-6R polyclonal antibodies reduced the concentration of IL-6 and diminished MCP-1 levels to a greater extent than Tocilizumab. However, treatment with the anti-sIL-6R V_HH fragment had no effect on either IL-6 or MCP-1 concentration (**Figure 5.10**).

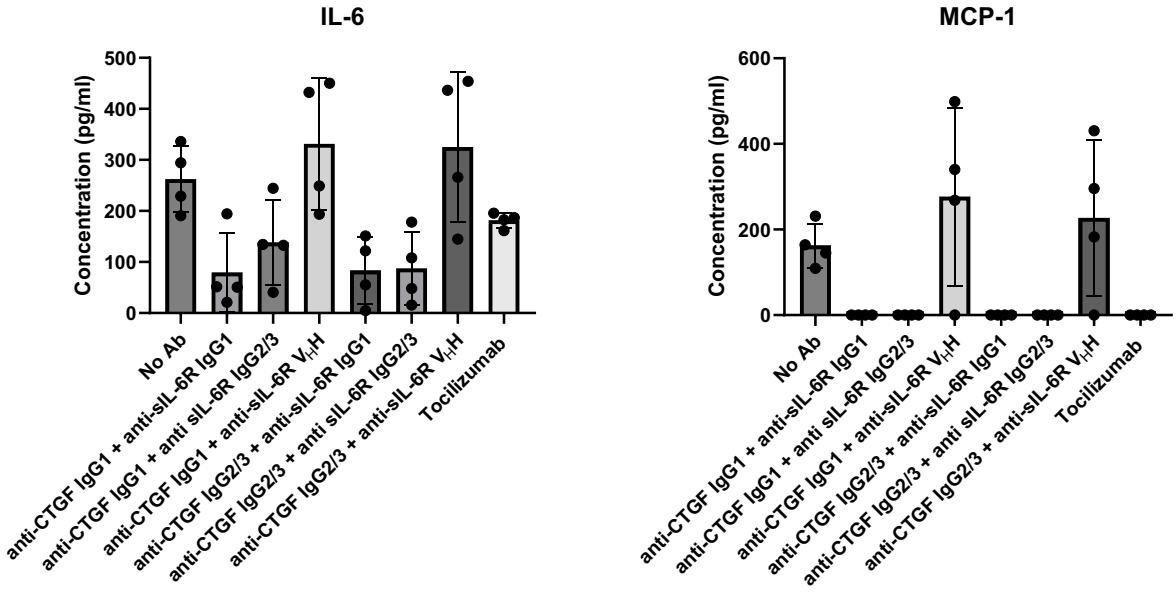

Figure 5.10: Effect of different combinations of camelid conventional, HcAbs and V_HH fragments on IL-6 and MCP-1 in treated skin fibroblasts. Graphs of IL-6 and MCP-1 concentration for all treatments (n=4).

5.2.4.2 HepG2 cells

In HepG2 cells treated with sIL-6R and CTGF, anti-sIL-6R polyclonal sera, and multiple (n=18) V_HH fragment clones were assessed for their effect on the STAT3 and ERK1/2 signaling pathways. The anti-sIL-6R polyclonal sera was not purified, as both conventional and HcAbs were found to behave in a similar manner.

Anti-sIL-6R polyclonal sera inhibited pSTAT3 to a similar degree to Tocilizumab. However, the polyclonal sera was not shown to inhibit pERK1/2. Due to the constitutive expression of ERK1/2 and pERK1/2 in untreated cells, it's likely that the pathway could not be effectively explored in HepG2 cells under these conditions. It may be that longer periods of serum starvation are required to accurately assess this pathway in future. Similar to the skin fibroblasts, in HepG2 cells, the anti-sIL-6R V_HH clone 1A4 did not inhibit pSTAT3. However, when other

clones were analysed, 4 were found to inhibit STAT3 phosphorylation to levels comparable to Tocilizumab (**Figure 5.11**).

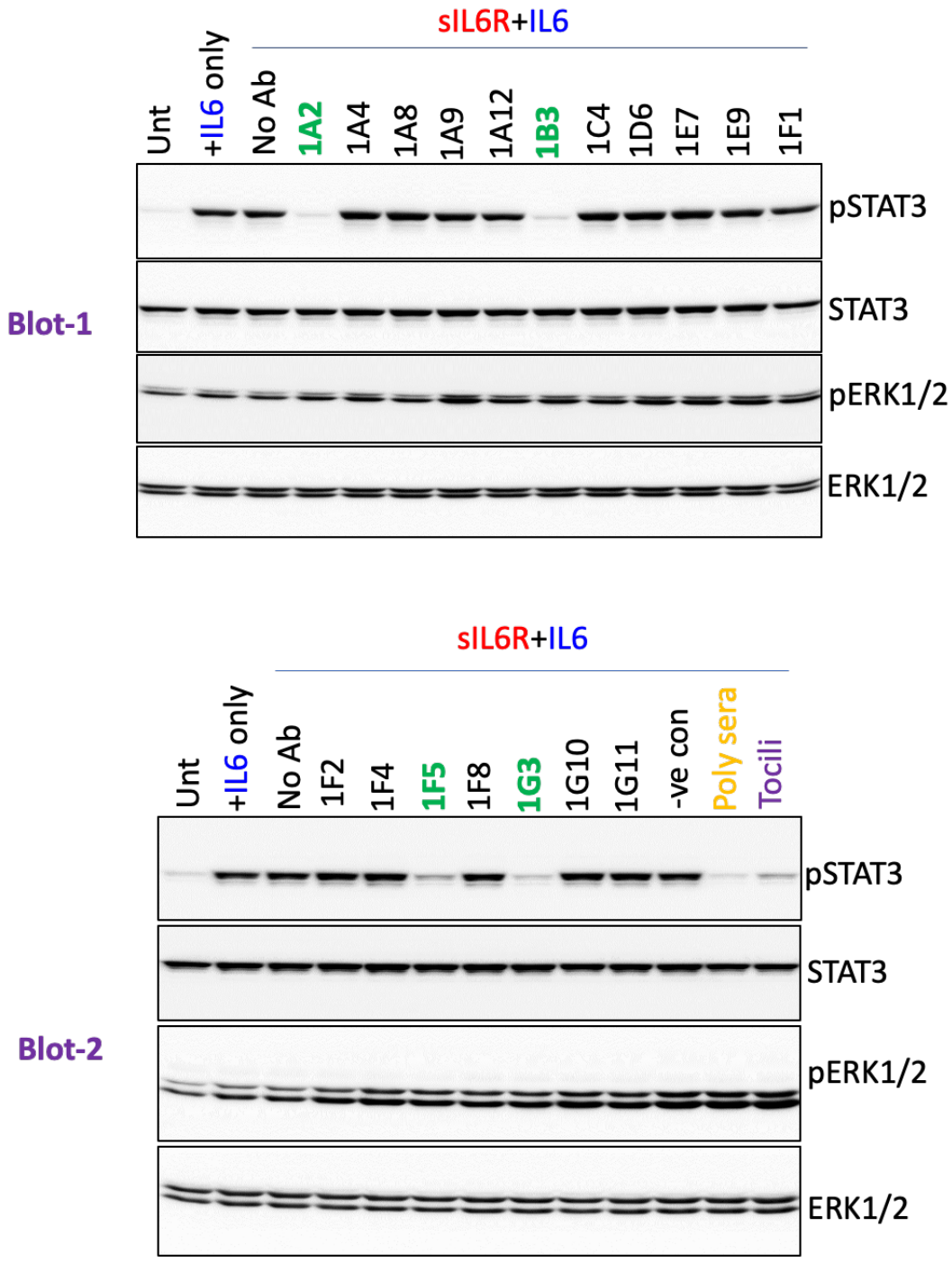

Figure 5.11: Effect of anti-sIL-6R V_HH clones, polyclonal sera and Tocilizumab on HepG2 cells treated with IL-6 and sIL-6R. Western blots showing the effect of 18 sIL-6R V_HH fragment clones on the expression of pSTAT3, STAT3, pERK1/2 and ERK1/2.

5.2 Discussion

Polyclonal anti-CTGF and anti-sIL-6R antibodies were produced from camels immunized with CTGF and sIL-6R. The serum titres were analyzed to determine the maximum antibody production. For the camel immunized with CTGF, this was determined to be the 2nd immunisation. For the camel immunized with sIL-6R, the 5th immunisation was the point at which antibody production plateaued. These serum samples were successfully used to extract polyclonal anti-CTGF and anti-sIL-6R antibodies using Protein A and Protein G purification.

Previous research regarding the distribution of antibody types in camels has been inconsistent, suggesting that HcAbs make up anywhere between 50-80% of their natural repertoire (Vincke & Muyldermans, 2012). In this study, the distribution of antibody subtypes was examined, using serum samples from 95 camels. The serum was analysed by dot blot, and expression of the antibodies per dot was estimated using 3 different types of secondary antibody. Alpha Diagnostics (AD) anti-camel IgG was used to estimate overall IgG expression. Antibodies Online (AP) anti-IgG1 was used to analyse the expression of conventional antibodies, and anti-camel IgG2/3 was utilized to estimate the expression of HcAbs. All antibodies were HRP-linked for chemiluminescence imaging.

In previous experiments analysing the antigenicity of extracted conventional and HcAbs, the AD anti-camel antibody alone was used as a secondary antibody. Results from these experiments suggested that the conventional antibodies were up to 9-fold more antigenic. It was considered unlikely that HcAbs are truly less antigenic to that extent, considering they are the major subclass in camels naturally (Vincke & Muyldermans, 2012). Thus, it was hypothesised that the difference in these results came from the different antibodies affinity to the secondary anti-camel antibody, rather than an innate difference antigenicity of the subclasses. For this reason, more specific AD and AO antibodies were used in subsequent experiments.

Immunization had no effect on the subtype of antibody expressed. In both non-immune and immune sera, HcAbs were estimated to make up 4-fold more of the naturally-produced antibodies than conventional antibodies. This is similar to the estimates in prior studies, suggesting that the majority of naturally occurring

antibodies in camels are HcAbs (Vincke & Muyldermans, 2012). This suggests that they are a key component of the camels immune system, and therefore may be useful as therapeutics to target disease pathways in humans. It is important to note, however, the western blotting technique used cannot adequately quantify this the concentration of each antibody subclass. Further studies using different strategies such as radial immunodiffusion may be more effective in determining concentration of HcAbs vs Conventional Abs (Van Der Giessen et al., 1975). Furthermore, in this experiment, the expression of each antibody subtype was estimated using optical density, which is highly dependent on exposure time. While measures were taken within the experiment to ensure the same exposure was given to all samples, there are operational differences which may skew the results. This was mitigated, however by normalising the optical density to the exposure time in arbitrary units (AU).

The change in expression of the different IgG subclasses were compared in one camels pre-immune sera, and after multiple antigen immunisations with CTGF. As with the previous experiment, 3 HRP-linked antibodies were used; AD (anti-camel IgG), AO (anti-camel IgG1) and JI (anti-camel IgG2/3). The trends in the expression of both conventional and HcAbs showed a sustained increase between the increased until the 3rd immunisation, with HcAbs sustaining its huge lead in expression. After the 3rd immunisation, however, the expression of HcAbs began to decrease, while conventional antibodies continued to rise. These results suggest that HcAbs are a large part of the camels innate response, while conventional antibodies may play a more adaptive role, with the camel slowly transitioning to conventional antibodies with repeated exposure. While this is an interesting finding which may help to explain the evolutionary mechanisms of camelid antibody production, this experiment was conducted with only one antigen. The results from immunisation with CTGF, may not mirror those of other antigens.

To date, no studies have compared the efficacy of conventional, HcAbs, and V_HH fragments targeting the same antigen. In this experiment, the purified anti-CTGF and anti-sIL-6R Conventional antibodies, HcAbs and V_HH fragments were assessed for their antigen-binding capacity by ELISA. For the anti-CTGF antibodies, conventional and HcAbs both showed affinity for the antigen,

particularly at the highest (100μg/ml) concentration. At the highest concentration, the HcAbs exhibited an absorbance at 450nm which was 2-fold higher than that of the conventional antibody. This is an interesting finding as it may be an indication that the HcAb are more antigenic. The absorbance of the plates incubated with the anti-CTGF V_HH fragment was not found to be increased from that of the BSA control, suggesting that the fragment had lost its antigen binding capability during the production process. For this reason, this antibody was not taken forward for further experimentation. This is a limitation with producing V_HH fragments using phage display. In this instance, it may be due to lack of stability in the clone identified, and utilization of a different clone may produce a more desirable effect. Alternatively, as the biological activity of V_HH fragments can be influenced by the expression system, it may be useful to evaluate a mammalian system for this particular antibody to help ensure that the produced V_HH retains its antigen-binding capability and overall functionality.

All anti-sIL-6R antibody subclasses showed affinity to the antigen compared to the BSA control, particularly at the 100μg/ml concentration. Both the conventional and HcAb also showed affinity to the antigen at the 10μg/ml concentration. Similar to CTGF, the sIL-6R HcAbs were more antigenic than the conventional, exhibiting a 2-fold increase in absorbance (450nm) than that of the conventional antibodies. Interestingly, the V_HH fragment had the highest absorbance, 3-fold higher than that of the conventional antibodies suggesting that it has a substantially increased affinity to sIL-6R than its conventional and HcAb counterparts. The V_HH fragment also exhibited a similar absorbance at the 10μg/ml and 1μg/ml concentrations. If indeed the V_HH fragment does bind the antigen and may produce a similar effect at the lower concentrations, this adds value to its use as a therapeutic. It is plausible that a lower concentration of antibody will be cleared from the system more rapidly, and produce fewer side effects, which a key concern with monoclonal antibody therapeutics as a whole (Arbabi-Ghahroudi, 2022).

In order to assess the antigenicity of the antibodies *in vitro*, primary healthy skin fibroblasts were treated with the profibrotic cocktail consisting of 12.5pg/ml CTGF, 1μg/ml sIL-6R as described in Chapter 4. This was then incubated with either anti-CTGF conventional and HcAbs or anti-sIL-6R conventional, HcAbs and V_HH fragment antibodies, or the anti-IL-6R antagonist Tocilizumab.

A trend was identified in dampening the expression of pSTAT3 in cells incubated with either anti-CTGF or anti-sIL-6R conventional, HcAbs. Particularly, with the anti-sIL-6R polyclonal antibodies, this effect was comparable to that of Tocilizumab. Similarly, both anti-CTGF and anti-sIL-6R polyclonal antibodies appeared to reduce expression of pERK1/2 in a manner comparable to Tocilizumab.

Interestingly, the anti-sIL-6R V_HH fragment antibody did not produce a decline in pSTAT3 expression, however it did have an inhibitory effect on pERK1/2. This indicates that the 1A4 anti-sIL-6R V_HH fragment clone is highly specific. It is possible that other V_HH fragment clones could be equally as selective to other pathways, within the same cell, and a cocktail of carefully selected V_HH fragment clones could be beneficial to induce precise, highly targeted effects. Moreover, as indicated by its antigen binding capabilities, the antibody may also be used at a lower concentration which may reduce its side effects. The production of a therapeutic antibody that is specific to the ERK/1/2 pathway is of great consequence, as there is considerable interest in the development of inhibitors for the MAPK/ERK pathway members, which are estimated to be mutated in 30% of all cancers. Currently no FDA approved ERK inhibitors are available on the market though several are under clinical investigation (LY3214996; Eli Lilly, LTT462; Novartis, BVD-523; BioMed Discoveries).

The effect of using a combination of anti-CTGF and anti-sIL-6R antibodies was assessed in primary skin fibroblasts to identify if the antibodies had a synergistic effect. The resulting reduction in pSTAT3 and pERK was similar to that of the antibodies on their own. The antibodies may be competitively inhibiting similar epitopes within the antigen and therefore do not produce compounding effects. It is plausible that incubation with different V_HH clones that activate different downstream pathways may aid in getting a compounding effect.

Culture media of skin fibroblasts was collected after incubation with anti-CTGF and anti-sIL-6R antibodies, as well as tocilizumab, and the concentration of IL-6 and MCP-1 was measured by ELISA. The combination of anti-CTGF and anti-sIL-6R conventional and HcAbs depleted expression of IL-6 and MCP-1 in a similar manner to Tocilizumab. In fibroblasts, these results suggest that camel-

derived anti-CTGF and anti-sIL-6R antibodies have the potential to be used therapeutically, however further research is required for more robust analysis of these compounds.

Experiments in HepG2 cells using several (n=18) anti-sIL-6R V_HH fragment antibodies further confirmed that clone 1A4 had no effect on phosphorylation on STAT3. However, 4 clones (1A2, 1B3, 1F5 and 1G3) significantly inhibited pSTAT3 levels comparable to polyclonal sera and Tocilizumab. Constitutive release of both ERK1/2 and pERK1/2 meant that any additional effects were not apparent. Therefore the effect of those clones on ERK1/2 signalling pathway could not be evaluated in HepG2 cells under those conditions. Future experiments should aim to serum starve cells for 48hrs to reduce expression of pERK1/2 in untreated cells.

Overall, these results provide much needed insight into the properties of camelid antibodies. The results in this chapter show that camelid polyclonal antibodies can be successfully extracted and maintain their antigen binding capacity and effectiveness when used *in vitro*. Anti-CTGF and anti-sIL-6R camelid conventional and HcAbs may be equally effective as currently available drugs such as Tocilizumab in reducing pSTAT3 and pERK1/2 expression, and thus may be interesting alternatives to similar drugs on the market, albeit in different cell types. However, further research would need to be conducted on their effects *in vivo* and toxicology studies would need to be implemented. Furthermore, their high production cost would need to be taken into account when comparing with readily available marketed therapeutics. Additionally, while the antibodies exhibited effectiveness in depleting IL-6 and MCP-1 production in these cells, this research has not identified a link between key fibrotic effects such as Type I Collagen production that is characteristic of diseases such as SSc. Further experiments will be required to measure collagen I production with anti-CTGF and anti-sIL-6R antibody use.

Chapter 6

General discussion and future perspectives

Systemic Sclerosis (SSc) poses a significant challenge in the realm of autoimmune diseases due to its complex pathogenesis and diverse clinical manifestations. SSc-associated cardiopulmonary complications SSc-PF and SSc-PAH are associated with higher morbidity and mortality compared to SSc patients with no lung disease (SSc-NLD). Current therapies for SSc-PF and SSc-PAH are target towards symptom relief and have not shown significant improvement in mortality rates for these patients. There is also currently no method to distinguish between patients who may go on to develop SSc-PF and SSc-PAH from those who will not show symptoms of lung disease. Uncovering robust biomarkers for each of these conditions is a crucial step towards stratifying patients, predicting their disease manifestation and allowing for early intervention. Additionally, specific discriminatory markers could allow for the identification of new therapeutics targeting the underlying causes. In recent years, omics-approaches have made large strides in detecting new biomarkers and therapeutic targets. However, to date, there have been no studies using a multi-omics approach to identify markers of SSc-PAH and SSc-PF within the same cohort. Therefore, the aim of this chapter was to use proteomics and metabolomics, to uncover systemic cytokines/growth factors and metabolites with the potential to differentiate between SSc-PAH and SSc-PF patients early in the disease.

Proteomics analysis uncovered significant differences in the fibrotic signalling mechanisms of SSc-related pulmonary arterial hypertension (SSc-PAH) and SSc-related pulmonary fibrosis (SSc-PF). Proteomics identified 3 cytokines significantly elevated in SSc-PF compared to all other groups (MCP-1, MCP-3 and MCP-4), while metabolomics identified 7 metabolites significantly overexpressed in SSc-PAH compared to all other groups (SDMA + ADMA,

Hydroxyasparagine, QUIN, AYA, T6a, VLA and N-acetyl putrescine). The SSc-PF panel was entirely cytokine based, while the biomarkers for SSc-PAH consisted entirely of metabolites. Results from this thesis suggest that SSc-PF patients exhibit the most inflammatory disruption, while SSc-PAH appears to be more related to metabolic dysregulation. These insights highlight the necessity for different targeted therapeutic approaches for these distinct conditions.

In order to validate proteomics results, quantification of serum concentrations for MCP-1 and IL-6 were carried out. MCP-1 was significantly elevated in SSc-PF compared to all groups, as observed via proteomics. IL-6 levels were significantly increased in all SSc groups compared to HC, validating proteomics findings. CTGF expression and the IL-6 trans signaling pathway molecules sIL-6R and sgp130 were of interest in this cohort, and thus were investigated by ELISA. The sIL-6R was also found to be significantly increased in SSc patients with lung disease. While CTGF levels were not found to be significantly increased, CTGF did exhibit a positive correlation with MCP-1 in the SSc-PF cohort, suggesting that it may play a role in disease pathogenesis. Additionally, in the SSc-NLD cohort, there was a significant positive correlation between IL-6 and sgp130, suggesting a protective mechanism. The results from this chapter identified the IL-6 trans signaling pathway and CTGF as key players in SSc-associated lung disease. Therefore, these molecules were taken forward for subsequent *in vitro* experiments to further elucidate their pathways.

Chapter 4 of this thesis explored the optimisation of IL-6, sIL-6R and CTGF, in order to trigger pathological fibrosis in healthy primary cells. Healthy skin fibroblasts were chosen due to their ability to produce collagen, a marker of fibrosis, and constitutive secretion of IL-6. It was found that treatment of skin fibroblasts with 3ng/ml IL-6 and 1μg/ml sIL-6R induced a significant increase in Type 1 collagen expression and phosphorylation of STAT3. When the cells were treated with CTGF, it was found that levels as low as 12.5pg/ml CTGF triggered phosphorylation of ERK 1/2 and production of Type 1 collagen. This concentration of CTGF also triggered significantly increased secretion of IL-6 into the cell culture media. In order to evaluate the synergistic effects of sIL-6R and CTGF, cells were treated with a cocktail of 1μg/ml sIL-6R and 12.5pg/ml CTGF, without any addition of IL-6. This produced the most fibrotic result, with levels of

Type 1 collagen significantly exceeding that of either of the molecules alone. This chapter elucidated the molecular mechanisms governing this interplay and provided valuable insights for the development of targeted therapeutic interventions. CTGF and sIL-6R, were thus chosen as targets to produce novel, specific, camel-derived biologics.

Chapter 5 explored novel therapeutic avenues by investigating anti-sIL-6R and anti-CTGF camel-derived antibodies and V_HH fragments. The findings presented in this thesis underscore the exceptional properties of camel-derived antibodies and V_HH fragments, including their small size, high specificity, and robust binding affinity. These characteristics make them attractive candidates for precision targeting of key molecular players implicated in SSc pathogenesis. By interrogating fibrotic disease pathways at the molecular level, this study has demonstrated the ability of camel-derived antibodies and V_HH fragments to disrupt aberrant signaling cascades and attenuate fibrotic tissue remodeling.

One of the notable contributions of this research is the specificity of the 1A4 anti-sIL-6R V_HH fragment, which modulated the ERK1/2 pathway without affecting STAT3 signaling. This provides compelling evidence of the efficacy of these novel biologics in blocking or neutralizing pro-fibrotic factors in a highly precise and targeted manner, thus offering a potential therapeutic strategy for halting disease progression in fibrotic conditions such as SSc. Furthermore, 4 clones (1A2, 1B3, 1F5 and 1G3) were identified. These clones depleted pSTAT3 to a similar extent to currently available market drug Tocilizumab, suggesting that these clones may have the potential to ameliorate pSTAT3-induced fibrosis.

Several avenues for future research and development emerge from the findings presented in this thesis. Further research into the mechanisms of action of anti-CTGF and anti-sIL-6R polyclonal antibodies and V_HH fragment antibody clones is necessary. Additionally, comprehensive pre-clinical studies in models of SSc, such as bleomycin-induced fibrosis in mice, are warranted to further elucidate the safety profile, pharmacokinetics, and optimal dosing regimens of camel-derived antibodies and V_HH fragments. Understanding their *in vivo* behaviour and potential immunogenicity is crucial for their successful translation into clinical settings.

In conclusion, the investigation of camel-derived antibodies and V_HH fragments represents a promising frontier in the quest for effective treatments for SSc. Through continued research endeavours and collaborative efforts, these novel biologics hold the potential to revolutionize SSc therapy, offering new avenues for improving patient outcomes and quality of life. The findings of this thesis lay the groundwork for future studies aimed at harnessing the unique properties of anti-CTGF and anti-sIL-6R camel-derived antibodies and V_HH fragments to combat the complex and multifaceted challenges posed by fibrotic diseases, including but not limited to SSc.

References

Abeywardena, M. Y., Leifert, W. R., Warnes, K. E., Varghese, J. N., & Head, R. J. (2009). Cardiovascular Biology of Interleukin-6. *Current Pharmaceutical Design*, *15*(15), 1809–1821. https://doi.org/10.2174/138161209788186290

Abraham, D. (2008). Connective tissue growth factor: growth factor, matricellular organizer, fibrotic biomarker or molecular target for anti-fibrotic therapy in SSc? *Rheumatology (Oxford, England)*, *47 Suppl 5*(SUPPL. 5). https://doi.org/10.1093/RHEUMATOLOGY/KEN278

Adler, S., Huscher, D., Siegert, E., Allanore, Y., Czirják, L., DelGaldo, F., Denton, C. P., Distler, O., Frerix, M., Matucci-Cerinic, M., Mueller-Ladner, U., Tarner, I. H., Valentini, G., Walker, U. A., Villiger, P. M., & Riemekasten, G. (2018). Systemic sclerosis associated interstitial lung disease - individualized immunosuppressive therapy and course of lung function: Results of the EUSTAR group. *Arthritis Research and Therapy*, *20*(1). https://doi.org/10.1186/s13075-018-1517-z

Aebersold, R., & Mann, M. (2003). Mass spectrometry-based proteomics. *Nature 2003 422:6928*, *422*(6928), 198–207. https://doi.org/10.1038/nature01511

Akhurst, R. J. (2017). *Targeting TGF-b Signaling for Therapeutic Gain*. https://doi.org/10.1101/cshperspect.a022301

Akira, S., & Kishimoto, T. (1992). IL-6 and NF-IL6 in acute-phase response and viral infection. *Immunological Reviews*, *127*(1), 25–50. https://doi.org/10.1111/J.1600-065X.1992.TB01407.X

Almaaitah, S., Highland, K. B., & Tonelli, A. R. (2020). *Management of Pulmonary Arterial Hypertension in Patients with Systemic Sclerosis*. https://doi.org/10.2147/IBPC.S232038

Almuraikhy, S., Kafienah, W., Bashah, M., Diboun, I., Jaganjac, M., Al-Khelaifi, F., Abdesselem, H., Mazloum, N. A., Alsayrafi, M., Mohamed-Ali, V., & Elrayess, M. A. (2016). Interleukin-6 induces impairment in human subcutaneous adipogenesis in obesity-associated insulin resistance. *Diabetologia*. https://doi.org/10.1007/s00125-016-4031-3

Anstee, Q. M., Targher, G., & Day, C. P. (2013). Progression of NAFLD to diabetes mellitus, cardiovascular disease or cirrhosis. *Nature Reviews Gastroenterology & Hepatology 2013 10:6*, *10*(6), 330–344. https://doi.org/10.1038/nrgastro.2013.41

Arbabi-Ghahroudi, M. (2022). Camelid Single-Domain Antibodies: Promises and Challenges as Lifesaving Treatments. *International Journal of Molecular Sciences*, *23*(9). https://doi.org/10.3390/IJMS23095009

Arbabi-Ghahroudi, M., Tanha, J., & MacKenzie, R. (2005). Prokaryotic expression of antibodies. *Cancer Metastasis Reviews*, *24*(4), 501–519. https://doi.org/10.1007/S10555-005-6193-1

Ayers, N. B., Sun, C., & Chen, S.-Y. (2017). *Transforming growth factor-β signaling in systemic sclerosis*. https://doi.org/10.7555/JBR.31.20170034

Badesch, D. B., Hill, N. S., Burgess, G., Rubin, L. J., Barst, R. J., Galiè, N., Simonneau, G., & Badesch, D. B. (2007). Personal non-commercial use only. *The Journal of Rheumatology*. www.jrheum.org

Badesch, D. B., Tapson, V. F., McGoon, M. D., Brundage, B. H., Rubin, L. J., Wigley, F. M., Rich, S., Barst, R. J., Barrett, P. S., Kral, K. M., Jöbsis, M. M., Loyd, J. E., Murali, S., Frost, A., Girgis, R., Bourge, R. C., Ralph, D. D., Elliott, C. G., Hill, N. S., … Knobil, K. (2000). Continuous intravenous epoprostenol for pulmonary hypertension due to the scleroderma spectrum of disease. A randomized, controlled trial. *Annals of Internal Medicine*, *132*(6), 425–434. https://doi.org/10.7326/0003-4819-132-6-200003210-00002

Balasubbramanian, D., Goodlett, B. L., & Mitchell, B. M. (2019). Is IL-12 pro-inflammatory or anti-inflammatory? Depends on the blood pressure.

Cardiovascular Research, *115*, 998–999. https://doi.org/10.1093/cvr/cvz028

Baran, P., Hansen, S., Waetzig, G. H., Akbarzadeh, M., Lamertz, L., Huber, H. J., Reza Ahmadian, M., Moll, J. M., & Scheller, J. (2018a). The balance of interleukin (IL)-6, IL-6soluble IL-6 receptor (sIL-6R), and IL-6sIL-6Rsgp130 complexes allows simultaneous classic and trans-signaling. *Journal of Biological Chemistry*, *293*(18), 6762–6775. https://doi.org/10.1074/jbc.RA117.001163

Baran, P., Hansen, S., Waetzig, G. H., Akbarzadeh, M., Lamertz, L., Huber, H. J., Reza Ahmadian, M., Moll, J. M., & Scheller, J. (2018b). The balance of interleukin (IL)-6, IL-6·soluble IL-6 receptor (sIL-6R), and IL-6·sIL-6R·sgp130 complexes allows simultaneous classic and trans-signaling. *The Journal of Biological Chemistry*, *293*(18), 6762. https://doi.org/10.1074/JBC.RA117.001163

Baraut, J., Farge, D., Jean-Louis, F., Masse, I., Ivan Grigore, E., M Arruda, L. C., Lamartine, J., Verrecchia, F., & Michel, L. (2011). *Transforming growth factor-β increases interleukin-13 synthesis via GATA-3 transcription factor in T-lymphocytes from patients with systemic sclerosis*. https://doi.org/10.1186/s13075-015-0708-0

Barratt, S. L., Creamer, A., Hayton, C., & Chaudhuri, N. (2018). Idiopathic Pulmonary Fibrosis (IPF): An Overview. *Journal of Clinical Medicine*, *7*(8). https://doi.org/10.3390/JCM7080201

Bauer, Y., de Bernard, S., Hickey, P., Ballard, K., Cruz, J., Cornelisse, P., Chadha-Boreham, H., Distler, O., Rosenberg, D., Doelberg, M., Roux, S., Nayler, O., & Lawrie, A. (2021). Identifying early pulmonary arterial hypertension biomarkers in systemic sclerosis: machine learning on proteomics from the DETECT cohort. *European Respiratory Journal*, *57*(6). https://doi.org/10.1183/13993003.02591-2020

Beesley, C. F., Goldman, N. R., Taher, T. E., Denton, C. P., Abraham, D. J., Mageed, R. A., & Ong, V. H. (2023). Dysregulated B cell function and

disease pathogenesis in systemic sclerosis. In *Frontiers in Immunology* (Vol. 13). Frontiers Media S.A. https://doi.org/10.3389/fimmu.2022.999008

Biernacka, A., Dobaczewski, M., & Frangogiannis, N. G. (2011). TGF-β signaling in fibrosis. *Growth Factors (Chur, Switzerland)*, *29*(5), 196. https://doi.org/10.3109/08977194.2011.595714

Bihlet, A. R., Balchen, T., Goteti, K., Sonne, J., Ladel, C., Karsdal, M. A., Ona, V., Moreau, F., Waterhouse, R., Bay-Jensen, A. C., & Guehring, H. (2024). Safety, Tolerability, and Pharmacodynamics of the ADAMTS-5 Nanobody M6495: Two Phase 1, Single-Center, Double-Blind, Randomized, Placebo-Controlled Studies in Healthy Subjects and Patients With Osteoarthritis. *ACR Open Rheumatology*, *6*(4), 205–213. https://doi.org/10.1002/ACR2.11610

Blair, H. A. (2022). Spesolimab: First Approval. *Drugs*, *82*(17), 1681. https://doi.org/10.1007/S40265-022-01801-4

Brigstock, D. R. (2010). Connective tissue growth factor (CCN2, CTGF) and organ fibrosis: Lessons from transgenic animals. *Journal of Cell Communication and Signaling*, *4*(1), 1–4. https://doi.org/10.1007/S12079-009-0071-5/FIGURES/1

Brown, M. C., Cary, L. A., Jamieson, J. S., Cooper, J. A., & Turner, C. E. (2005). Src and FAK Kinases Cooperate to Phosphorylate Paxillin Kinase Linker, Stimulate Its Focal Adhesion Localization, and Regulate Cell Spreading and Protrusiveness. *Molecular Biology of the Cell*, *16*(9), 4316. https://doi.org/10.1091/MBC.E05-02-0131

Brown, M., & O'Reilly, S. (2019). The immunopathogenesis of fibrosis in systemic sclerosis. In *Clinical and Experimental Immunology* (Vol. 195, Issue 3, pp. 310–321). Blackwell Publishing Ltd. https://doi.org/10.1111/cei.13238

Buechler, C., Krautbauer, S., & Eisinger, K. (2015). Adipose tissue fibrosis. *World Journal of Diabetes*, *6*(4), 548. https://doi.org/10.4239/wjd.v6.i4.548

Butterfield, T. A., Best, T. M., & Merrick, M. A. (2006). The Dual Roles of Neutrophils and Macrophages in Inflammation: A Critical Balance Between Tissue Damage and Repair. *Journal of Athletic Training*, *41*(4), 457. https://doi.org/10.1016/s0162-0908(08)79217-1

Cai, Z., Tian, S., Klein, T., Tu, L., Geenen, L. W., Koudstaal, T., van den Bosch, A. E., de Rijke, Y. B., Reiss, I. K. M., Boersma, E., van der Ley, C., Van Faassen, M., Kema, I., Duncker, D. J., Boomars, K. A., Tran-Lundmark, K., Guignabert, C., & Merkus, D. (2022). Kynurenine metabolites predict survival in pulmonary arterial hypertension: A role for IL-6/IL-6Rα. *Scientific Reports 2022 12:1*, *12*(1), 1–14. https://doi.org/10.1038/s41598-022-15039-3

Callewaert, F., Roodt, J., Ulrichts, H., Stohr, T., Van Rensburg, W. J., Lamprecht, S., Rossenu, S., Priem, S., Willems, W., & Holz, J. B. (2012). Evaluation of efficacy and safety of the anti-VWF Nanobody ALX-0681 in a preclinical baboon model of acquired thrombotic thrombocytopenic purpura. *Blood*, *120*(17), 3603–3610. https://doi.org/10.1182/BLOOD-2012-04-420943

Castelli, M. S., McGonigle, P., & Hornby, P. J. (2019). The pharmacology and therapeutic applications of monoclonal antibodies. *Pharmacology Research & Perspectives*, *7*(6), e00535. https://doi.org/10.1002/PRP2.535

Cayrol, C., & Girard, J. P. (2018). Interleukin-33 (IL-33): A nuclear cytokine from the IL-1 family. In *Immunological Reviews* (Vol. 281, Issue 1, pp. 154–168). Blackwell Publishing Ltd. https://doi.org/10.1111/imr.12619

Chaisson, N. F., & Hassoun, P. M. (2013a). Systemic sclerosis-associated pulmonary arterial hypertension. *Chest*, *144*(4), 1346–1356. https://doi.org/10.1378/CHEST.12-2396

Chaisson, N. F., & Hassoun, P. M. (2013b). Systemic Sclerosis-Associated Pulmonary Arterial Hypertension. *Chest*, *144*(4), 1346. https://doi.org/10.1378/CHEST.12-2396

Chaqour, B., & Karrasch, C. (2020). Eyeing the Extracellular Matrix in Vascular Development and Microvascular Diseases and Bridging the Divide between Vascular Mechanics and Function. *International Journal of Molecular Sciences 2020, Vol. 21, Page 3487*, *21*(10), 3487. https://doi.org/10.3390/IJMS21103487

Chekol Abebe, E., Yibeltal Shiferaw, M., Tadele Admasu, F., & Asmamaw Dejenie, T. (2022). Ciltacabtagene autoleucel: The second anti-BCMA CAR T-cell therapeutic armamentarium of relapsed or refractory multiple myeloma. *Frontiers in Immunology*, *13*, 991092. https://doi.org/10.3389/FIMMU.2022.991092

Chen, L., Deng, H., Cui, H., Fang, J., Zuo, Z., Deng, J., Li, Y., Wang, X., & Zhao, L. (2017). Inflammatory responses and inflammation-associated diseases in organs. *Oncotarget*, *9*(6), 7204. https://doi.org/10.18632/ONCOTARGET.23208

Chen, M. M., Lam, A., Abraham, J. A., Schreiner, G. F., & Joly, A. H. (2000). CTGF Expression is Induced by TGF- β in Cardiac Fibroblasts and Cardiac Myocytes: a Potential Role in Heart Fibrosis. *Journal of Molecular and Cellular Cardiology*, *32*(10), 1805–1819. https://doi.org/10.1006/JMCC.2000.1215

Chen, Y., Zhou, J., Xu, S., & Nie, J. (2023). Role of Interleukin-6 Family Cytokines in Organ Fibrosis. *Kidney Diseases*, *9*(4), 239–253. https://doi.org/10.1159/000530288

Chen, Z., Zhang, N., Chu, H. Y., Yu, Y., Zhang, Z. K., Zhang, G., & Zhang, B. T. (2020a). Connective Tissue Growth Factor: From Molecular Understandings to Drug Discovery. *Frontiers in Cell and Developmental Biology*, *8*, 593269. https://doi.org/10.3389/FCELL.2020.593269/BIBTEX

Chen, Z., Zhang, N., Chu, H. Y., Yu, Y., Zhang, Z. K., Zhang, G., & Zhang, B. T. (2020b). Connective Tissue Growth Factor: From Molecular Understandings to Drug Discovery. *Frontiers in Cell and Developmental Biology*, *8*(October), 1–17. https://doi.org/10.3389/fcell.2020.593269

Cheong, F. Y., Gower, A. C., & Farber, H. W. (2017). Changes in gene expression profiles in patients with pulmonary arterial hypertension associated with scleroderma treated with tadalafil. *Seminars in Arthritis and Rheumatism*, *46*(4), 465–472. https://doi.org/10.1016/J.SEMARTHRIT.2016.05.015

Chujo, S., Shirasaki, F., Kawara, S., Inagaki, Y., Kinbara, T., Inaoki, M., Takigawa, M., & Takehara, K. (2005). Connective Tissue Growth Factor Causes Persistent Proa2(I) Collagen Gene Expression Induced by Transforming Growth Factor-b in a Mouse Fibrosis Model. *JOURNAL OF CELLULAR PHYSIOLOGY*, *203*, 447–456. https://doi.org/10.1002/jcp.20251

Cipriani, P., Benedetto, P. Di, Ruscitti, P., Campese, A. F., Liakouli, V., Carubbi, F., Pantano, I., Berardicurt, O., Screpanti, I., & Giacomelli, R. (2014). *Impaired Endothelium-Mesenchymal Stem Cells Cross-talk in Systemic Sclerosis: a Link Between Vascular and Fibrotic Features*. 1–12. https://doi.org/10.1186/s13075-014-0442-z

Conway, B., & Hughes, J. (2012). Review Cellular orchestrators of renal fibrosis. *Q J Med* , *105*, 611–615. https://doi.org/10.1093/qjmed/hcr235

Costa-Pereira, A. P. (2014). Regulation of IL-6-type cytokine responses by MAPKs. *Biochemical Society Transactions*, *42*(1), 59–62. https://doi.org/10.1042/BST20130267

Cruikshank, W. W., Kornfeld, H., & Center, D. M. (2000). Interleukin-16. In *Journal of Leukocyte Biology* (Vol. 67, Issue 6, pp. 757–766). Federation of American Societies for Experimental Biology. https://doi.org/10.1002/jlb.67.6.757

Cunningham, S., Piedra, P. A., Martinon-Torres, F., Szymanski, H., Brackeva, B., Dombrecht, E., Detalle, L., Fleurinck, C., Piedra, P. A., Verhulst, S., Matthijs, I., Proesmans, M., Goetghebuer, T., Bosheva, M., Dosev, S., Nikolova, O., Chakarova, P., Wu Hupat, E., Mesa Monsalve, J., … Warachit, B. (2021). Nebulised ALX-0171 for respiratory syncytial virus

lower respiratory tract infection in hospitalised children: a double-blind, randomised, placebo-controlled, phase 2b trial. *The Lancet. Respiratory Medicine*, *9*(1), 21–32. https://doi.org/10.1016/S2213-2600(20)30320-9

Daoussis, D., & Liossis Mediterr, S.-N. J. (2019). Treatment of systemic sclerosis associated fibrotic manifestations: Current options and future directions. *MEDITERRANEAN JOURNAL OF RHEUMATOLOGY* , *30*(1), 33–40. https://doi.org/10.31138/mjr.30.1.33

De Genst, E., Saerens, D., Muyldermans, S., & Conrath, K. (2005). *Antibody repertoire development in camelids*. https://doi.org/10.1016/j.dci.2005.06.010

De Vries, J. E. (1995). Immunosuppressive and anti-inflammatory properties of interleukin 10. *Annals of Medicine*, *27*(5), 537–541. https://doi.org/10.3109/07853899509002465

Deidda, M., Piras, C., Cadeddu Dessalvi, C., Locci, E., Barberini, L., Orofino, S., Musu, M., Mura, M. N., Manconi, P. E., Finco, G., Atzori, L., & Mercuro, G. (2017). Distinctive metabolomic fingerprint in scleroderma patients with pulmonary arterial hypertension. *International Journal of Cardiology*, *241*, 401–406. https://doi.org/10.1016/J.IJCARD.2017.04.024

Del Galdo, F., Shaw, M. A., & Jimenez, S. A. (2010). Proteomic analysis identification of a pattern of shared alterations in the secretome of dermal fibroblasts from systemic sclerosis and nephrogenic systemic fibrosis. *The American Journal of Pathology*, *177*(4), 1638–1646. https://doi.org/10.2353/AJPATH.2010.091095

Deng, L., Huang, T., & Zhang, L. (2023). T cells in idiopathic pulmonary fibrosis: crucial but controversial. *Cell Death Discovery 2023 9:1*, *9*(1), 1–6. https://doi.org/10.1038/s41420-023-01344-x

Denton, C. P., & Abraham, D. J. (2001). Transforming growth factor-beta and connective tissue growth factor: key cytokines in scleroderma pathogenesis. *Current Opinion in Rheumatology*, *13*(6), 505–511. https://doi.org/10.1097/00002281-200111000-00010

Denton, C. P., & Black, C. M. (2004). Scleroderma—clinical and pathological advances. *Best Practice & Research Clinical Rheumatology*, *18*(3), 271–290. https://doi.org/10.1016/J.BERH.2004.03.001

Denton, C. P., & Khanna, D. (2017). Systemic sclerosis. *Lancet (London, England)*, *390*(10103), 1685–1699. https://doi.org/10.1016/S0140-6736(17)30933-9

Derynck, R., Akhurst, R. J., & Balmain, A. (2001). TGF-β signaling in tumor suppression and cancer progression. *Nature Genetics 2001 29:2*, *29*(2), 117–129. https://doi.org/10.1038/ng1001-117

Derynck, R., & Zhang, Y. E. (2003). Smad-dependent and Smad-independent pathways in TGF-beta family signalling. *Nature*, *425*(6958), 577–584. https://doi.org/10.1038/NATURE02006

Deshmane, S. L., Kremlev, S., Amini, S., & Sawaya, B. E. (2009). Monocyte Chemoattractant Protein-1 (MCP-1): An Overview. *Journal of Interferon & Cytokine Research*, *29*(6), 313. https://doi.org/10.1089/JIR.2008.0027

Dhillon, S. (2014). Intravenous tocilizumab: A review of its use in adults with rheumatoid arthritis. *BioDrugs*, *28*(1), 75–106. https://doi.org/10.1007/S40259-013-0076-8/TABLES/8

D'Huyvetter, M., De Vos, J., Caveliers, V., Vaneycken, I., Heemskerk, J., Duhoux, F. P., Fontaine, C., Vanhoeij, M., Windhorst, A. D., van der Aa, F., Harry Hendrikse, N., Eersels, J. L. E., Everaert, H., Gykiere, P., Devoogdt, N., Raes, G., Lahoutte, T., & Keyaerts, M. (2021a). Phase I Trial of 131I-GMIB-Anti-HER2-VHH1, a New Promising Candidate for HER2-Targeted Radionuclide Therapy in Breast Cancer Patients. *Journal of Nuclear Medicine : Official Publication, Society of Nuclear Medicine*, *62*(8), 1097–1105. https://doi.org/10.2967/JNUMED.120.255679

D'Huyvetter, M., De Vos, J., Caveliers, V., Vaneycken, I., Heemskerk, J., Duhoux, F. P., Fontaine, C., Vanhoeij, M., Windhorst, A. D., van der Aa, F., Harry Hendrikse, N., Eersels, J. L. E., Everaert, H., Gykiere, P., Devoogdt, N., Raes, G., Lahoutte, T., & Keyaerts, M. (2021b). Phase I Trial of 131I-

GMIB-Anti-HER2-VHH1, a New Promising Candidate for HER2-Targeted Radionuclide Therapy in Breast Cancer Patients. *Journal of Nuclear Medicine : Official Publication, Society of Nuclear Medicine*, *62*(8), 1097–1105. https://doi.org/10.2967/JNUMED.120.255679

Distler, O., Assassi, S., Cottin, V., Cutolo, M., Danoff, S. K., Denton, C. P., Distler, J. H. W., Hoffmann-Vold, A.-M., Johnson, S. R., Ladner, U. M., Smith, V., Volkmann, E. R., & Maher, T. M. (2020). *Predictors of progression in systemic sclerosis patients with interstitial lung disease*. https://doi.org/10.1183/13993003.02026-2019

Distler, O., Pap, T., Kowal-Bielecka, O., Meyringer, R., Guiducci, S., Landthaler, M., Schölmerich, J., Michel, B. A., Gay, R. E., Matucci-Cerinic, M., Gay, S., & Müller-Ladner, U. (2001). Overexpression of Monocyte Chemoattractant Protein 1 in Systemic Sclerosis Role of Platelet-Derived Growth Factor and Effects on Monocyte Chemotaxis and Collagen Synthesis. *ARTHRITIS & RHEUMATISM*, *44*(11), 2665–2678. https://doi.org/10.1002/1529-0131(200111)44:11<2665::AID-ART446>3.0.CO;2-S

Donath, M. Y., & Shoelson, S. E. (2011). Type 2 diabetes as an inflammatory disease. *Nature Reviews Immunology 2011 11:2*, *11*(2), 98–107. https://doi.org/10.1038/nri2925

Dorn, L. E., Petrosino, J. M., Wright, P., & Accornero, F. (2018). CTGF/CCN2 is an autocrine regulator of cardiac fibrosis. *Journal of Molecular and Cellular Cardiology*, *121*, 205–211. https://doi.org/10.1016/J.YJMCC.2018.07.130

Duggan, S. (2018). Caplacizumab: First Global Approval. *Drugs*, *78*(15), 1639–1642. https://doi.org/10.1007/S40265-018-0989-0

Dumoulin, M., Conrath, K., Van Meirhaeghe, A., Meersman, F., Heremans, K., Frenken, L. G. J., Muyldermans, S., Wyns, L., & Matagne, A. (2002). Single-domain antibody fragments with high conformational stability. *Protein Science : A Publication of the Protein Society*, *11*(3), 500–515. https://doi.org/10.1110/PS.34602

Elhai, M., Meune, C., Boubaya, M., Avouac, J., Hachulla, E., Balbir-Gurman, A., Riemekasten, G., Airò, P., Joven, B., Vettori, S., Cozzi, F., Ullman, S., Czirják, L., Tikly, M., Müller-Ladner, U., Caramaschi, P., Distler, O., Iannone, F., Ananieva, L. P., … Allanore, Y. (2017). Mapping and predicting mortality from systemic sclerosis. *Annals of the Rheumatic Diseases*, *76*(11), 1897–1905. https://doi.org/10.1136/annrheumdis-2017-211448

England, E., Rees, D. G., Scott, I. C., Carmen, S., Chan, D. T. Y., Chaillan Huntington, C. E., Houslay, K. F., Erngren, T., Penney, M., Majithiya, J. B., Rapley, L., Sims, D. A., Hollins, C., Hinchy, E. C., Strain, M. D., Kemp, B. P., Corkill, D. J., May, R. D., Vousden, K. A., … Cohen, E. S. (2023). Tozorakimab (MEDI3506): an anti-IL-33 antibody that inhibits IL-33 signalling via ST2 and RAGE/EGFR to reduce inflammation and epithelial dysfunction. *Scientific Reports*, *13*(1). https://doi.org/10.1038/s41598-023-36642-y

Eser, P., & Jänne, P. A. (2018). TGFβ pathway inhibition in the treatment of non-small cell lung cancer. *Pharmacology & Therapeutics*, *184*, 112–130. https://doi.org/10.1016/J.PHARMTHERA.2017.11.004

Fan, W. H., Pech, M., & Karnovsky, M. J. (2000). Connective tissue growth factor (CTGF) stimulates vascular smooth muscle cell growth and migration in vitro. *European Journal of Cell Biology*, *79*(12), 915–923. https://doi.org/10.1078/0171-9335-00122

Ferreira, A. M., Takagawa, S., Fresco, R., Zhu, X., Varga, J., & DiPietro, L. A. (2006). Diminished Induction of Skin Fibrosis in Mice with MCP-1 Deficiency. *Journal of Investigative Dermatology*, *126*(8), 1900–1908. https://doi.org/10.1038/SJ.JID.5700302

Fishman, D., Faulds, G., Jeffey, R., Mohamed-Ali, V., Yudkin, J. S., Humphries, S., & Woo, P. (1998). The effect of novel polymorphisms in the interleukin-6 (IL-6) gene on IL-6 transcription and plasma IL-6 levels, and an association with systemic-onset juvenile chronic arthritis. *The Journal of Clinical Investigation*, *102*(7), 1369–1376. https://doi.org/10.1172/JCI2629

Fowell, D. J. (2016). Modification of T Cell Functions at Sites of Infection and Inflammation. *Encyclopedia of Immunobiology*, *3*, 336–343. https://doi.org/10.1016/B978-0-12-374279-7.08019-X

Fraile, J. M., Palliyil, S., Barelle, C., Porter, A. J., & Kovaleva, M. (2021). Non-Alcoholic Steatohepatitis (NASH) - A Review of a Crowded Clinical Landscape, Driven by a Complex Disease. *Drug Design, Development and Therapy*, *15*, 3997–4009. https://doi.org/10.2147/DDDT.S315724

Gabay, C. (2006). Interleukin-6 and chronic inflammation. *Arthritis Research and Therapy*, *8*(SUPPL. 2), 1–6. https://doi.org/10.1186/AR1917/TABLES/1

Gabay, C., Eidlen, D., & Arend, W. P. (1997). Interleukin 1 receptor antagonist (IL-1Ra) is an acute-phase protein. *J Clin Invest*, *99*(12), 2930–2940. https://doi.org/10.1172/JCI119488

Garbers, C., Thaiss, W., Jones, G. W., Waetzig, G. H., Lorenzen, I., Guilhot, F., Lissilaa, R., Ferlin, W. G., Grötzinger, J., Jones, S. A., Rose-John, S., & Scheller, J. (2011). Inhibition of Classic Signaling Is a Novel Function of Soluble Glycoprotein 130 (sgp130), Which Is Controlled by the Ratio of Interleukin 6 and Soluble Interleukin 6 Receptor. *The Journal of Biological Chemistry*, *286*(50), 42959. https://doi.org/10.1074/JBC.M111.295758

García Rodríguez, L. A., González-Pérez, A., Michel, A., & Sáez, M. E. (2019). Contemporary epidemiology of systemic sclerosis: A population-based cohort study in the United Kingdom. *Seminars in Arthritis and Rheumatism*, *49*(1), 105–111. https://doi.org/10.1016/J.SEMARTHRIT.2018.11.002

Gauldie, J., Richards, C., Harnish, D., Lansdorp, P., & Baumann, H. (1987). Interferon beta 2/B-cell stimulatory factor type 2 shares identity with monocyte-derived hepatocyte-stimulating factor and regulates the major acute phase protein response in liver cells. *Proceedings of the National Academy of Sciences of the United States of America*, *84*(20), 7251–7255. https://doi.org/10.1073/PNAS.84.20.7251

Ginès, P., Krag, A., Abraldes, J. G., Solà, E., Fabrellas, N., & Kamath, P. S. (2021). Liver cirrhosis. *The Lancet*, *398*(10308), 1359–1376. https://doi.org/10.1016/S0140-6736(21)01374-X

Gondry, O., Caveliers, V., Xavier, C., Raes, L., Vanhoeij, M., Verfaillie, G., Fontaine, C., Glorieus, K., De Grève, J., Joris, S., Luyten, I., Zwaenepoel, K., Vandenbroucke, F., Waelput, W., Thyparambil, S., Vaneycken, I., Cousaert, J., Bourgeois, S., Devoogdt, N., … Keyaerts, M. (2024a). Phase II Trial Assessing the Repeatability and Tumor Uptake of [68Ga]Ga-HER2 Single-Domain Antibody PET/CT in Patients with Breast Carcinoma. *Journal of Nuclear Medicine*, *65*(2), 178–184. https://doi.org/10.2967/JNUMED.123.266254

Gondry, O., Caveliers, V., Xavier, C., Raes, L., Vanhoeij, M., Verfaillie, G., Fontaine, C., Glorieus, K., De Grève, J., Joris, S., Luyten, I., Zwaenepoel, K., Vandenbroucke, F., Waelput, W., Thyparambil, S., Vaneycken, I., Cousaert, J., Bourgeois, S., Devoogdt, N., … Keyaerts, M. (2024b). Phase II Trial Assessing the Repeatability and Tumor Uptake of [68Ga]Ga-HER2 Single-Domain Antibody PET/CT in Patients with Breast Carcinoma. *Journal of Nuclear Medicine*, *65*(2), 178–184. https://doi.org/10.2967/JNUMED.123.266254

Gong, Y., Liang, S., Zeng, L., Ni, Y., Zhou, S., & Yuan, X. (2019). Effects of blood sample handling procedures on measurable interleukin 6 in plasma and serum. *Journal of Clinical Laboratory Analysis*, *33*(7). https://doi.org/10.1002/JCLA.22924

Good, R. B., Gilbane, A. J., Trinder, S. L., Denton, C. P., Coghlan, G., Abraham, D. J., & Holmes, A. M. (2015). *SHORT COMMUNICATION Endothelial to Mesenchymal Transition Contributes to Endothelial Dysfunction in Pulmonary Arterial Hypertension*. https://doi.org/10.1016/j.ajpath.2015.03.019

Guignabert, C., Avouac, J., Hoffmann-Vold, A.-M., Olschewski, H., Odler, B., Foris, V., Gungl, A., Müller, V., Hassoun, P. M., Kwapiszewska, G., & Kovacs, G. (2018). Biomarkers for Pulmonary Vascular Remodeling in

Systemic Sclerosis: A Pathophysiological Approach. *Frontiers in Physiology* | *Www.Frontiersin.Org*, *1*, 587. https://doi.org/10.3389/fphys.2018.00587

Gulhar, R., Ashraf, M. A., & Jialal, I. (2023). Physiology, Acute Phase Reactants. *StatPearls*. https://www.ncbi.nlm.nih.gov/books/NBK519570/

Hamers-Casterman, C., Atarhouch, T., Muyldermans, S., Robinson, G., Hammers, C., Songa, E. B., Bendahman, N., & Hammers, R. (1993a). Naturally occurring antibodies devoid of light chains. *Nature*, *363*(6428), 446–448. https://doi.org/10.1038/363446A0

Hamers-Casterman, C., Atarhouch, T., Muyldermans, S., Robinson, G., Hammers, C., Songa, E. B., Bendahman, N., & Hammers, R. (1993b). Naturally occurring antibodies devoid of light chains. *Nature*, *363*(6428), 446–448. https://doi.org/10.1038/363446A0

Hannoodee, S., & Nasuruddin, D. N. (2022). Acute Inflammatory Response. *Nature*, *206*(4979), 20. https://doi.org/10.1038/206020a0

Hansel, T. T., Kropshofer, H., Singer, T., Mitchell, J. A., & George, A. J. T. (2010). The safety and side effects of monoclonal antibodies. In *Nature Reviews Drug Discovery* (Vol. 9, Issue 4, pp. 325–338). https://doi.org/10.1038/nrd3003

Hao, J., He, L., Tredget, E., Zhang, S., & Zhang, M. (2020). T Cells in Fibrosis and Fibrotic Diseases. *Front. Immunol*, *11*, 1142. https://doi.org/10.3389/fimmu.2020.01142

Hasegawa, M., Sato, S., Fujimoto, M., Ihn, H., Kikuchi, K., & Takehara, K. (1998). Serum levels of interleukin 6 (IL-6), oncostatin M, soluble IL-6 receptor, and soluble gp130 in patients with systemic sclerosis. *The Journal of Rheumatology*, *25*(2), 308–313. https://europepmc.org/article/med/9489824

Hasegawa, M., Sato, S., Ihn, H., & Takehara, K. (1999). Enhanced production of interleukin-6 (IL-6), oncostatin M and soluble IL-6 receptor by cultured

peripheral blood mononuclear cells from patients with systemic sclerosis. *Rheumatology*, *38*(7), 612–617. https://doi.org/10.1093/RHEUMATOLOGY/38.7.612

Hasegawa, M., Sato, S., Ihn1, H., & Takehara, K. (1999). Enhanced production of interleukin-6 (IL-6), oncostatin M and soluble IL-6 receptor by cultured peripheral blood mononuclear cells from patients with systemic sclerosis. *Rheumatology*, *38*, 612–617.

Hasegawa, M., Sato, S., & Takehara, K. (2001). Augmented production of chemokines (monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein-1α (MIP-1α) and MIP-1β) in patients with systemic sclerosis: MCP-1 and MIP-1α may be involved in the development of pulmonary fibrosis. *Clinical and Experimental Immunology*, *117*(1), 159–165. https://doi.org/10.1046/J.1365-2249.1999.00929.X

Henderson, N. C., Rieder, F., & Wynn, T. A. (2020). Fibrosis: from mechanisms to medicines. *Nature 2020 587:7835*, *587*(7835), 555–566. https://doi.org/10.1038/s41586-020-2938-9

Heresi, G. A., & Minai, O. A. (2008). Bosentan in systemic sclerosis. In *Drugs of Today* (Vol. 44, Issue 6, pp. 415–428). https://doi.org/10.1358/dot.2008.44.6.1220138

Herrero-Cervera, A., Soehnlein, O., & Kenne, E. (2022). *Neutrophils in chronic inflammatory diseases*. https://doi.org/10.1038/s41423-021-00832-3

Herzog, E. L., Mathur, A., Tager, A. M., Feghali-Bostwick, C., Schneider, F., & Varga, J. (2014). *Interstitial Lung Disease Associated With Systemic Sclerosis and Idiopathic Pulmonary Fibrosis: How Similar and Distinct?* https://doi.org/10.1002/art.38702

Hinz, B., Phan, S. H., Thannickal, V. J., Prunotto, M., Desmoulire, A., Varga, J., De Wever, O., Mareel, M., & Gabbiani, G. (2012). Recent developments in myofibroblast biology: paradigms for connective tissue remodeling. *The American Journal of Pathology*, *180*(4), 1340–1355. https://doi.org/10.1016/J.AJPATH.2012.02.004

Ho, K. T., & Reveille, J. D. (2003a). The clinical relevance of autoantibodies in scleroderma. In *Arthritis Research and Therapy* (Vol. 5, Issue 2, pp. 80–93). BioMed Central. https://doi.org/10.1186/ar628

Ho, K. T., & Reveille, J. D. (2003b). The clinical relevance of autoantibodies in scleroderma. In *Arthritis Research and Therapy* (Vol. 5, Issue 2, pp. 80–93). BioMed Central. https://doi.org/10.1186/ar628

Ho, Y. Y., Lagares, D., Tager, A. M., & Kapoor, M. (2014). Fibrosis - A lethal component of systemic sclerosis. *Nature Reviews Rheumatology*, *10*(7), 390–402. https://doi.org/10.1038/nrrheum.2014.53

Holbourn, K. P., Acharya, K. R., & Perbal, B. (2008a). The CCN family of proteins: structure–function relationships. *Trends in Biochemical Sciences*, *33*(10), 461. https://doi.org/10.1016/J.TIBS.2008.07.006

Holbourn, K. P., Acharya, K. R., & Perbal, B. (2008b). The CCN family of proteins: structure–function relationships. *Trends in Biochemical Sciences*, *33*(10), 461. https://doi.org/10.1016/J.TIBS.2008.07.006

Holmes, A., Abraham, D. J., Sa, S., Shiwen, X., Black, C. M., & Leask, A. (2001). CTGF and SMADs, Maintenance of Scleroderma Phenotype Is Independent of SMAD Signaling. *Journal of Biological Chemistry*, *276*(14), 10594–10601. https://doi.org/10.1074/jbc.M010149200

Holtmann, H., & Resch, K. (1995). Cytokines. *Die Naturwissenschaften*, *82*(4), 178–187. https://doi.org/10.1007/BF01143192

Holton, S. E., Mitchem, M., Pipavath, S., Morrell, E. D., Bhatraju, P. K., Hamerman, J. A., Speake, C., Malhotra, U., Wurfel, M. M., Ziegler, S., & Mikacenic, C. (2024). *Mediators of monocyte chemotaxis and matrix remodeling are associated with the development of fibrosis in patients with COVID-19*. https://doi.org/10.1101/2023.04.28.23289261

Huang, E., Peng, N., Xiao, F., Hu, D., Wang, X., & Lu, L. (2020). The Roles of Immune Cells in the Pathogenesis of Fibrosis. *Molecular Sciences*. https://doi.org/10.3390/ijms21155203

Huang, X.-L., Wang, Y.-J., Yan, • Jun-Wei, Wan, Y.-N., Chen, B., Li, B.-Z., Yang, • Guo-Jun, & Wang, J. (2015). *Role of anti-inflammatory cytokines IL-4 and IL-13 in systemic sclerosis*. https://doi.org/10.1007/s00011-015-0806-0

Igaz, P., Horváth, A., Horváth, B., Szalai, C., Va Pállinger, E. ´, Lgyi, R., Tó Th, S., Rose-John, S., & Falus, A. (2000). Soluble interleukin-6 receptor (sIL-6R) makes IL-6R negative T cell line respond to IL-6; it inhibits TNF production. *Immunology Letters*, *71*, 143–148. www.elsevier.com/locate/

Ikawa, Y., Ng, P. S., Endo, K., Kondo, M., Chujo, S., Ishida, W., Shirasaki, F., Fujimoto, M., & Takehara, K. (2008). Neutralizing monoclonal antibody to human connective tissue growth factor ameliorates transforming growth factor-β-induced mouse fibrosis. *Journal of Cellular Physiology*, *216*(3), 680–687. https://doi.org/10.1002/JCP.21449

Ismail, M., & Pinzani, M. (2009). Reversal of Liver Fibrosis. *Saudi Journal of Gastroenterology : Official Journal of the Saudi Gastroenterology Association*, *15*(1), 72. https://doi.org/10.4103/1319-3767.45072

Jamialahmadi, H., Nazari, S. E., TanzadehPanah, H., Saburi, E., Asgharzadeh, F., Khojasteh-Leylakoohi, F., Alaei, M., Mirahmadi, M., Babaei, F., Asghari, S. Z., Mansouri, S., Khalili-Tanha, G., Maftooh, M., Fiuji, H., Hassanian, S. M., Ferns, G. A., Khazaei, M., & Avan, A. (2023). Targeting transforming growth factor beta (TGF-β) using Pirfenidone, a potential repurposing therapeutic strategy in colorectal cancer. *Scientific Reports 2023 13:1*, *13*(1), 1–14. https://doi.org/10.1038/s41598-023-41550-2

Jerjen, R., Nikpour, M., Krieg, T., Denton, C. P., & Saracino, A. M. (2022). Systemic sclerosis in adults. Part II: management and therapeutics. *Journal of the American Academy of Dermatology*, *87*(5), 957–978. https://doi.org/10.1016/J.JAAD.2021.10.066

Jia, Q., Lei, Y., Chen, S., Liu, S., Wang, T., & Cheng, Y. (2023). Circulating inflammatory cytokines and risk of idiopathic pulmonary fibrosis: a

Mendelian randomization study. *BMC Pulmonary Medicine*, *23*(1), 1–10. https://doi.org/10.1186/S12890-023-02658-3/FIGURES/4

Jin, B. K., Odongo, S., Radwanska, M., & Magez, S. (2023). NANOBODIES®: A Review of Generation, Diagnostics and Therapeutics. *International Journal of Molecular Sciences*, *24*(6), 5994. https://doi.org/10.3390/IJMS24065994

Jin, W., Zheng, Y., & Zhu, P. (2022). T cell abnormalities in systemic sclerosis. In *Autoimmunity Reviews* (Vol. 21, Issue 11). Elsevier B.V. https://doi.org/10.1016/j.autrev.2022.103185

Jindal, S., Pedersen, D. V., Gera, N., Chandler, J., Patel, R., Neill, A., Cone, J., Zhang, Y., Yuan, C. X., Millman, E. E., Carlin, D., Puffer, B., Sheridan, D., Andersen, G. R., & Tamburini, P. (2024). Characterization of the bispecific VHH antibody gefurulimab (ALXN1720) targeting complement component 5, and designed for low volume subcutaneous administration. *Molecular Immunology*, *165*, 29–41. https://doi.org/10.1016/J.MOLIMM.2023.12.004

Jones, S. A., Takeuchi, T., Aletaha, D., Smolen, J., Choy, E. H., & McInnes, I. (2018). Interleukin 6: The biology behind the therapy. *Considerations in Medicine*, *2*(1), 2–6. https://doi.org/10.1136/CONMED-2018-000005

Kaplanski, G., Marin, V., Montero-Julian, F., Mantovani, A., & Farnarier, C. (2003). IL-6: a regulator of the transition from neutrophil to monocyte recruitment during inflammation. *Trends in Immunology*, *24*(1), 25–29. https://doi.org/10.1016/S1471-4906(02)00013-3

Kaplon, H., Crescioli, S., Chenoweth, A., Visweswaraiah, J., & Reichert, J. M. (2023). *Antibodies to watch in 2023*. *15*(1). https://doi.org/10.1080/19420862.2022.2153410

Katsiari, C. G., Simopoulou, T., Alexiou, I., Lazaros, &, & Sakkas, I. (2018). *Human Vaccines & Immunotherapeutics Immunotherapy of systemic sclerosis*. https://doi.org/10.1080/21645515.2018.1491508

Kaul, B., Cottin, V., Collard, H. R., & Valenzuela, C. (2021). Variability in Global Prevalence of Interstitial Lung Disease. *Frontiers in Medicine*, *8*, 751181. https://doi.org/10.3389/FMED.2021.751181

Keam, S. J. (2023a). Ozoralizumab: First Approval. *Drugs*, *83*(1), 87–92. https://doi.org/10.1007/S40265-022-01821-0

Keam, S. J. (2023b). Ozoralizumab: First Approval. *Drugs*, *83*(1), 87–92. https://doi.org/10.1007/S40265-022-01821-0

Kendall, R. T., Renaud, L., Baatz, J. E., Malaab, M., Nguyen, X.-X., & Feghali-Bostwick, C. A. (2021). Systemic sclerosis biomarkers detection in the secretome of TGFβ1-activated primary human lung fibroblasts. *Journal of Proteomics*, *242*, 1874–3919. https://doi.org/10.1016/j.jprot.2021.104243

Keyaerts, M., Xavier, C., Heemskerk, J., Devoogdt, N., Everaert, H., Ackaert, C., Vanhoeij, M., Duhoux, F. P., Gevaert, T., Simon, P., Schallier, D., Fontaine, C., Vaneycken, I., Vanhove, C., De Greve, J., Lamote, J., Caveliers, V., & Lahoutte, T. (2016). Phase I Study of 68Ga-HER2-Nanobody for PET/CT Assessment of HER2 Expression in Breast Carcinoma. *Journal of Nuclear Medicine : Official Publication, Society of Nuclear Medicine*, *57*(1), 27–33. https://doi.org/10.2967/JNUMED.115.162024

Khan, K., Xu, S., Nihtyanova, S., Derrett-Smith, E., Abraham, D., Denton, C. P., & Ong, V. H. (2012). Clinical and pathological significance of interleukin 6 overexpression in systemic sclerosis. *Annals of the Rheumatic Diseases*, *71*(7), 1235–1242. https://doi.org/10.1136/ANNRHEUMDIS-2011-200955

Khanna, D., Lin, C. J. F., Furst, D. E., Goldin, J., Kim, G., Kuwana, M., Allanore, Y., Matucci-Cerinic, M., Distler, O., Shima, Y., van Laar, J. M., Spotswood, H., Wagner, B., Siegel, J., Jahreis, A., Denton, C. P., Lucero, E., Pons-Estel, B., Rivero, M., … Steen, V. D. (2020). Tocilizumab in systemic sclerosis: a randomised, double-blind, placebo-controlled, phase 3 trial. *The Lancet Respiratory Medicine*, *8*(10), 963–974. https://doi.org/10.1016/S2213-2600(20)30318-

0/ATTACHMENT/30E7561D-256B-4A2D-8E99-27B01E62D634/MMC1.PDF

Khanna, D., Lin, C. J. F., Furst, D. E., Wagner, B., Zucchetto, M., Raghu, G., Martinez, F. J., Goldin, J., Siegel, J., & Denton, C. P. (2022). Long-Term Safety and Efficacy of Tocilizumab in Early Systemic Sclerosis-Interstitial Lung Disease: Open-Label Extension of a Phase 3 Randomized Controlled Trial. *American Journal of Respiratory and Critical Care Medicine*, *205*(6), 674–684. https://doi.org/10.1164/RCCM.202103-0714OC

Kimura, A., & Kishimoto, T. (2010). IL-6: regulator of Treg/Th17 balance. *European Journal of Immunology*, *40*(7), 1830–1835. https://doi.org/10.1002/EJI.201040391

Kitaba, S., Murota, H., Terao, M., Azukizawa, H., Terabe, F., Shima, Y., Fujimoto, M., Tanaka, T., Naka, T., Kishimoto, T., & Katayama, I. (2012). Blockade of Interleukin-6 Receptor Alleviates Disease in Mouse Model of Scleroderma. *The American Journal of Pathology*, *180*(1), 165–176. https://doi.org/10.1016/J.AJPATH.2011.09.013

Kodama, T., Takehara, T., Hikita, H., Shimizu, S., Shigekawa, M., Tsunematsu, H., Li, W., Miyagi, T., Hosui, A., Tatsumi, T., Ishida, H., Kanto, T., Hiramatsu, N., Kubota, S., Takigawa, M., Tomimaru, Y., Tomokuni, A., Nagano, H., Doki, Y., … Hayashi, N. (2011). Increases in p53 expression induce CTGF synthesis by mouse and human hepatocytes and result in liver fibrosis in mice. *The Journal of Clinical Investigation*, *121*(8), 3343–3356. https://doi.org/10.1172/JCI44957

Köhler, G., & Milstein, C. (1976). Derivation of specific antibody-producing tissue culture and tumor lines by cell fusion. *European Journal of Immunology*, *6*(7), 511–519. https://doi.org/10.1002/EJI.1830060713

Korn, T., & Hiltensperger, M. (2021). Role of IL-6 in the commitment of T cell subsets. *Cytokine*, *146*, None. https://doi.org/10.1016/J.CYTO.2021.155654

Koudstaal, T., Funke-Chambour, M., Kreuter, M., Molyneaux, P. L., & Wijsenbeek, M. S. (2023). *Pulmonary fibrosis: from pathogenesis to clinical decision-making*. https://doi.org/10.1016/j.molmed.2023.08.010

Lafyatis, R., & York, M. (2009). INNATE IMMUNITY AND INFLAMMATION IN SYSTEMIC SCLEROSIS. *Current Opinion in Rheumatology*, *21*(6), 617. https://doi.org/10.1097/BOR.0B013E32832FD69E

Landén, N. X., Li, D., & Ståhle, M. (2016). Transition from inflammation to proliferation: a critical step during wound healing. *Cellular and Molecular Life Sciences: CMLS*, *73*(20), 3861. https://doi.org/10.1007/S00018-016-2268-0

Landi, C., Bargagli, E., Carleo, A., Refini, R. M., Bennett, D., Bianchi, L., Cillis, G., Prasse, A., Bini, L., & Rottoli, P. (2019). Bronchoalveolar lavage proteomic analysis in pulmonary fibrosis associated with systemic sclerosis: S100A6 and 14-3-3ε as potential biomarkers. *Rheumatology (Oxford, England)*, *58*(1), 165–178. https://doi.org/10.1093/rheumatology/key223

Leask, A. (2004). Transcriptional profiling of the scleroderma fibroblast reveals a potential role for connective tissue growth factor (CTGF) in pathological fibrosis. *The Keio Journal of Medicine*, *53*(2), 74–77. https://doi.org/10.2302/KJM.53.74

Leask, A. (2015). Matrix remodeling in systemic sclerosis. *Seminars in Immunopathology*, *37*(5), 559–563. https://doi.org/10.1007/S00281-015-0508-2

Lech, M., & Anders, H. J. (2013). Macrophages and fibrosis: How resident and infiltrating mononuclear phagocytes orchestrate all phases of tissue injury and repair. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, *1832*(7), 989–997. https://doi.org/10.1016/J.BBADIS.2012.12.001

Lei, P. P., Qu, Y. Q., Shuai, Q., Tao, S. M., Bao, Y. X., Wang, Y., Wang, S. W., & Wang, D. H. (2013). Fibrocytes are associated with the fibrosis of

coronary heart disease. *Pathology Research and Practice*, *209*(1), 36–43. https://doi.org/10.1016/j.prp.2012.09.012

Li, D., Guabiraba, R., Elle Besnard, A.-G., Komai-Koma, M., Jabir, M. S., Zhang, L., Graham, G. J., Kurowska-Stolarska, M., Liew, F. Y., Mcsharry, C., & Xu, D. (2014). *IL-33 promotes ST2-dependent lung fibrosis by the induction of alternatively activated macrophages and innate lymphoid cells in mice*. https://doi.org/10.1016/j.jaci.2014.05.011

Liu, S. C., Hsu, C. J., Chen, H. Te, Tsou, H. K., Chuang, S. M., & Tang, C. H. (2012). CTGF Increases IL-6 Expression in Human Synovial Fibroblasts through Integrin-Dependent Signaling Pathway. *PLOS ONE*, *7*(12), e51097. https://doi.org/10.1371/JOURNAL.PONE.0051097

Liu, S.-C., Hsu, C.-J., Fong, Y.-C., Chuang, S.-M., & Tang, C.-H. (2013). CTGF induces monocyte chemoattractant protein-1 expression to enhance monocyte migration in human synovial fibroblasts. *BBA - Molecular Cell Research*, *1833*, 1114–1124. https://doi.org/10.1016/j.bbamcr.2012.12.014

López, A. L., Chiurchiù, V., Rea, I. M., Gibson, D. S., Mcgilligan, V., Mcnerlan, S. E., Alexander, H. D., & Ross, O. A. (2018). *Age and Age-Related Diseases: Role of inflammation Triggers and Cytokines*. *9*, 1. https://doi.org/10.3389/fimmu.2018.00586

Lovisa, S. (2021). Epithelial-to-Mesenchymal Transition in Fibrosis: Concepts and Targeting Strategies. *Frontiers in Pharmacology*, *12*. https://doi.org/10.3389/FPHAR.2021.737570

Lu, R. M., Hwang, Y. C., Liu, I. J., Lee, C. C., Tsai, H. Z., Li, H. J., & Wu, H. C. (2020). Development of therapeutic antibodies for the treatment of diseases. *Journal of Biomedical Science 2020 27:1*, *27*(1), 1–30. https://doi.org/10.1186/S12929-019-0592-Z

Lust, J. A., Donovan, K. A., Kline, M. P., Greipp, P. R., Kyle, R. A., & Maihle, N. J. (1992a). Isolation of an mRNA encoding a soluble form of the human interleukin-6 receptor. *Cytokine*, *4*(2), 96–100. https://doi.org/10.1016/1043-4666(92)90043-Q

Lust, J. A., Donovan, K. A., Kline, M. P., Greipp, P. R., Kyle, R. A., & Maihle, N. J. (1992b). Isolation of an mRNA encoding a soluble form of the human interleukin-6 receptor. *Cytokine*, *4*(2), 96–100. https://doi.org/10.1016/1043-4666(92)90043-Q

Mack, M. (2018). Inflammation and fibrosis. *Matrix Biology*, *68–69*, 106–121. https://doi.org/10.1016/J.MATBIO.2017.11.010

Maeda, K., Mehta, H., Drevets, D. A., & Coggeshall, K. M. (2010). IL-6 increases B-cell IgG production in a feed-forward proinflammatory mechanism to skew hematopoiesis and elevate myeloid production. *Blood*, *115*(23), 4699. https://doi.org/10.1182/BLOOD-2009-07-230631

Maehara, T., Kaneko, N., Perugino, C. A., Mattoo, H., Kers, J., Allard-Chamard, H., Mahajan, V. S., Liu, H., Murphy, S. J. H., Ghebremichael, M., Fox, D., Payne, A. S., Lafyatis, R., Stone, J. H., Khanna, D., & Pillai, S. (2020). Cytotoxic CD4+ T lymphocytes may induce endothelial cell apoptosis in systemic sclerosis. *Journal of Clinical Investigation*, *130*(5), 2451–2464. https://doi.org/10.1172/JCI131700

Makino, K., Makino, T., Stawski, L., Lipson, K. E., Leask, A., & Trojanowska, M. (2017). Anti-connective tissue growth factor (CTGF/CCN2) monoclonal antibody attenuates skin fibrosis in mice models of systemic sclerosis. *Arthritis Research and Therapy*, *19*(1), 1–10. https://doi.org/10.1186/s13075-017-1356-3

Mancini, M., Ishida, Y., Kuninaka, Y., Mukaida, N., & Kondo, T. (2023). *Immune Mechanisms of Pulmonary Fibrosis with Bleomycin*. https://doi.org/10.3390/ijms24043149

Marino, M., Scuderi, F., Provenzano, C., Scheller, J., Rose-John, S., & Bartoccioni, E. (2008). IL-6 regulates MCP-1, ICAM-1 and IL-6 expression in human myoblasts. *Journal of Neuroimmunology*, *196*(1–2), 41–48. https://doi.org/10.1016/J.JNEUROIM.2008.02.005

Markham, A. (2022). Envafolimab: First Approval. *Drugs*, *82*(2), 235–240. https://doi.org/10.1007/S40265-022-01671-W

Martín-López, M., & Carreira, P. E. (2023). The Impact of Progressive Pulmonary Fibrosis in Systemic Sclerosis–Associated Interstitial Lung Disease. *Journal of Clinical Medicine*, *12*(20), 6680. https://doi.org/10.3390/JCM12206680

Masi, A. T. (1980). Preliminary criteria for the classification of systemic sclerosis (scleroderma). *Arthritis & Rheumatism*, *23*(5), 581–590. https://doi.org/10.1002/ART.1780230510

Matsushita, T., Hamaguchi, Y., Hasegawa, M., Takehara, K., & Fujimoto, M. (2016). Decreased levels of regulatory B cells in patients with systemic sclerosis: association with autoantibody production and disease activity. *Rheumatology (Oxford, England)*, *55*(2), 263–267. https://doi.org/10.1093/RHEUMATOLOGY/KEV331

Matucci Cerinic, M., Valentini, G., Sorano, G., Cuomo, G., Fenu, L., Generini, S., Cinotti, S., Morfini, M., Pignone, A., Guiducci, S., Del Rosso, A., Kalfin, R., Das, D., Marongiu, F., of Medicine, P., in Rheumatology, F., & Professor of Medicine, A. (2003). Blood Coagulation, Fibrinolysis, and Markers of Endothelial Dysfunction in Systemic Sclerosis. *Seminars in Arthritis and Rheumatism*, *32*(5), 285–295. https://doi.org/10.1053/sarh.2002.50011

Mavropoulos, A., Liaskos, C., Simopoulou, T., Bogdanos, D. P., & Sakkas, L. I. (2017). IL-10-producing regulatory B cells (B10 cells), IL-17 + T cells and autoantibodies in systemic sclerosis. *Clinical Immunology*, *184*, 26–32. https://doi.org/10.1016/j.clim.2017.04.013

Mavropoulos, A., Simopoulou, T., Varna, A., Liaskos, C., Katsiari, C. G., Bogdanos, D. P., & Sakkas, L. I. (2016). Breg Cells Are Numerically Decreased and Functionally Impaired in Patients with Systemic Sclerosis. *Arthritis and Rheumatology*, *68*(2), 494–504. https://doi.org/10.1002/art.39437

Mayes, M. D. (2003). Scleroderma epidemiology. *Rheumatic Disease Clinics of North America*, *29*(2), 239–254. https://doi.org/10.1016/S0889-857X(03)00022-X

McCafferty, J., Griffiths, A. D., Winter, G., & Chiswell, D. J. (1990). Phage antibodies: filamentous phage displaying antibody variable domains. *Nature*, *348*(6301), 552–554. https://doi.org/10.1038/348552A0

Melton, E., & Qiu, H. (2020). *Molecular Sciences Interleukin-36 Cytokine/Receptor Signaling: A New Target for Tissue Fibrosis*. https://doi.org/10.3390/ijms21186458

Mercurio, S., Latinkic, B., Itasaki, N., Krumlauf, R., & Smith, J. C. (2004). Connective-tissue growth factor modulates WNT signalling and interacts with the WNT receptor complex. *Development (Cambridge, England)*, *131*(9), 2137–2147. https://doi.org/10.1242/DEV.01045

Mittal, M., Siddiqui, M. R., Tran, K., Reddy, S. P., & Malik, A. B. (2014). Reactive Oxygen Species in Inflammation and Tissue Injury. *Antioxidants & Redox Signaling*, *20*(7), 1126. https://doi.org/10.1089/ARS.2012.5149

Mogensen, T. H. (2009). Pathogen Recognition and Inflammatory Signaling in Innate Immune Defenses. *Clinical Microbiology Reviews*, *22*(2), 240. https://doi.org/10.1128/CMR.00046-08

Mohamed-Ali, V., Flower, L., Sethi, J., Hotamisligil, G., Gray, R., Humphries, S. E., York, D. A., & Pinkney, J. (2001a). β-Adrenergic Regulation of IL-6 Release from Adipose Tissue: In Vivo and in Vitro Studies. *The Journal of Clinical Endocrinology & Metabolism*, *86*(12), 5864–5869. https://doi.org/10.1210/JCEM.86.12.8104

Mohamed-Ali, V., Flower, L., Sethi, J., Hotamisligil, G., Gray, R., Humphries, S. E., York, D. A., & Pinkney, J. (2001b). β-adrenergic regulation of IL-6 release from adipose tissue: In vivo and in vitro studies. *Journal of Clinical Endocrinology and Metabolism*, *86*(12), 5864–5869. https://doi.org/10.1210/jc.86.12.5864

Mohamed-ali, V., Goodrick, S., Bulmer, K., P Holly, J. M., Yudkin, J. S., Coppack, S. W., & Bul-mer, K. (1999). *Production of soluble tumor necrosis factor receptors by human subcutaneous adipose tissue in vivo*. http://www.ajpendo.org

Montero-Julian, F. A. (2001). The soluble IL-6 receptors: serum levels and biological function. *Cellular and Molecular Biology (Noisy-Le-Grand, France)*, *47*(4), 583–597. https://europepmc.org/article/med/11502067

Muangchan, C., & Pope, J. E. (2012). Interleukin 6 in Systemic Sclerosis and Potential Implications for Targeted Therapy. *The Journal of Rheumatology*, *39*(6), 1120–1124. https://doi.org/10.3899/JRHEUM.111423

Mülberg, J., Schooltink, H., Stoyan, T., Günther, M., Graeve, L., Buse, G., Mackiewicz, A., Heinrich, P. C., & Rose-John, S. (1993). The soluble interleukin-6 receptor is generated by shedding. *European Journal of Immunology*, *23*(2), 473–480. https://doi.org/10.1002/EJI.1830230226

Munger, J. S., Huang, X., Kawakatsu, H., Griffiths, M. J. D., Dalton, S. L., Wu, J., Pittet, J. F., Kaminski, N., Garat, C., Matthay, M. A., Rifkin, D. B., & Sheppard, D. (1999). A Mechanism for Regulating Pulmonary Inflammation and Fibrosis: The Integrin αvβ6 Binds and Activates Latent TGF β1. *Cell*, *96*(3), 319–328. https://doi.org/10.1016/S0092-8674(00)80545-0

Murakami, M., & Hirano, T. (2012). The molecular mechanisms of chronic inflammation development. *Frontiers in Immunology*, *3*(NOV). https://doi.org/10.3389/FIMMU.2012.00323

Mutsaers, H. A. M., Merrild, C., Nørregaard, R., & Plana-Ripoll, O. (2023). The impact of fibrotic diseases on global mortality from 1990 to 2019. *Journal of Translational Medicine*, *21*, 818. https://doi.org/10.1186/s12967-023-04690-7

Muyldermans, S. (2013a). Nanobodies: natural single-domain antibodies. *Annual Review of Biochemistry*, *82*, 775–797. https://doi.org/10.1146/ANNUREV-BIOCHEM-063011-092449

Muyldermans, S. (2013b). Nanobodies: natural single-domain antibodies. *Annual Review of Biochemistry*, *82*, 775–797. https://doi.org/10.1146/ANNUREV-BIOCHEM-063011-092449

Muyldermans, S., Baral, T. N., Retamozzo, V. C., De Baetselier, P., De Genst, E., Kinne, J., Leonhardt, H., Magez, S., Nguyen, V. K., Revets, H., Rothbauer, U., Stijlemans, B., Tillib, S., Wernery, U., Wyns, L., Hassanzadeh-Ghassabeh, G., & Saerens, D. (2009). Camelid immunoglobulins and nanobody technology. *Veterinary Immunology and Immunopathology*, *128*(1–3), 178–183. https://doi.org/10.1016/J.VETIMM.2008.10.299

Navaratnam, V., Fleming, K. M., West, J., Smith, C. J. P., Jenkins, R. G., Fogarty, A., & Hubbard, R. B. (2011). The rising incidence of idiopathic pulmonary fibrosis in the UK. *Thorax*, *66*(6), 462–467. https://doi.org/10.1136/THX.2010.148031

Nie, Y.-J., Wu, S.-H., Xuan, Y.-H., & Yan, G. (2022). Role of IL-17 family cytokines in the progression of IPF from inflammation to fibrosis. *Military Medical Research*, *9*(21). https://doi.org/10.1186/s40779-022-00382-3

Nihtyanova, S. I., & Denton, C. P. (2010). Autoantibodies as predictive tools in systemic sclerosis. *Nature Reviews Rheumatology 2010 6:2*, *6*(2), 112–116. https://doi.org/10.1038/nrrheum.2009.238

Nurbhai, S., Roberts, K. J., Carlton, T. M., Maggiore, L., Cubitt, M. F., Ray, K. P., Reckless, J., Mohammed, H., Irving, P., MacDonald, T. T., Vossenkämper, A., West, M. R., Parkes, G. C., & Crowe, J. S. (2019). Oral Anti-Tumour Necrosis Factor Domain Antibody V565 Provides High Intestinal Concentrations, and Reduces Markers of Inflammation in Ulcerative Colitis Patients. *Scientific Reports*, *9*(1). https://doi.org/10.1038/S41598-019-50545-X

Oberg, H. H., Wesch, D., Grüssel, S. S., Rose-John, S., & Kabelitz, D. (2006). Differential expression of CD126 and CD130 mediates different STAT-3 phosphorylation in CD4+CD25- and CD25high regulatory T cells.

International Immunology, *18*(4), 555–563. https://doi.org/10.1093/INTIMM/DXH396

Oh, J. H., & Jun, D. W. (2023). The latest global burden of liver cancer: A past and present threat. *Clinical and Molecular Hepatology*, *29*(2), 355. https://doi.org/10.3350/CMH.2023.0070

Opal, S. M., & DePalo, V. A. (2000). Anti-Inflammatory Cytokines. *Chest*, *117*(4), 1162–1172. https://doi.org/10.1378/CHEST.117.4.1162

O'Reilly, S., Ciechomska, M., Cant, R., Hügle, T., & van Laar, J. M. (2012). Interleukin-6, its role in fibrosing conditions. In *Cytokine and Growth Factor Reviews* (Vol. 23, Issue 3, pp. 99–107). https://doi.org/10.1016/j.cytogfr.2012.04.003

Osafo-Addo, A. D., & Herzog, E. L. (2017). CCL2 and T cells in pulmonary fibrosis: an old player gets a new role. *Thorax*, *72*(11), 967–968. https://doi.org/10.1136/THORAXJNL-2017-210517

pADL-22c Phagemid. (n.d.).

Papp, K. A., Weinberg, M. A., Morris, A., & Reich, K. (2021). IL17A/F nanobody sonelokimab in patients with plaque psoriasis: a multicentre, randomised, placebo-controlled, phase 2b study. *The Lancet*, *397*(10284), 1564–1575. https://doi.org/10.1016/S0140-6736(21)00440-2

Pardon, E., Laeremans, T., Triest, S., F Rasmussen, ren G., Wohlk, A., Ruf, A., Muyldermans, S., J Hol, W. G., Kobilka, B. K., & Steyaert, J. (2014). A general protocol for the generation of Nanobodies for structural biology. *Nature Protocols*. https://doi.org/10.1038/nprot.2014.039

Parikh, V., Bhardwaj, A., & Nair, A. (2019). Pharmacotherapy for pulmonary arterial hypertension. In *Journal of Thoracic Disease* (Vol. 11, pp. S1767–S1781). AME Publishing Company. https://doi.org/10.21037/jtd.2019.09.14

Paulin, R., & Michelakis, E. D. (2014). The Metabolic Theory of Pulmonary Arterial Hypertension. *Circulation Research*, *115*(1), 148–164. https://doi.org/10.1161/CIRCRESAHA.115.301130

Peng, W. J., Yan, J. W., Wan, Y. N., Wang, B. X., Tao, J. H., Yang, G. J., Pan, H. F., & Wang, J. (2012). Matrix metalloproteinases: A review of their structure and role in systemic sclerosis. *Journal of Clinical Immunology*, *32*(6), 1409–1414. https://doi.org/10.1007/s10875-012-9735-7

Peoples, C., Medsger, T. A., Lucas, M., Rosario, B. L., & Feghali-Bostwick, C. A. (2016). Gender differences in systemic sclerosis: Relationship to clinical features, serologic status and outcomes. *Journal of Scleroderma and Related Disorders*, *1*(2), 204–212. https://doi.org/10.5301/jsrd.5000209

Piera-Velazquez, S., Mendoza, F. A., Jimenez, S. A., Brenner, D. A., Kisseleva, T., & Fuxe, J. (2016). Endothelial to Mesenchymal Transition (EndoMT) in the Pathogenesis of Human Fibrotic Diseases. *Journal of Clinical Medicine 2016, Vol. 5, Page 45*, *5*(4), 45. https://doi.org/10.3390/JCM5040045

Ponticos, M. (2013). Connective tissue growth factor (CCN2) in blood vessels. *Vascular Pharmacology*, *58*(3), 189–193. https://doi.org/10.1016/j.vph.2013.01.004

Pothin, E., Lesuisse, D., & Lafaye, P. (2020). Brain Delivery of Single-Domain Antibodies: A Focus on VHH and VNAR. *Pharmaceutics 2020, Vol. 12, Page 937*, *12*(10), 937. https://doi.org/10.3390/PHARMACEUTICS12100937

Puglisi, S., & Vancheri, C. (2018). Pathobiology of Novel Approaches to Treatment. *Interstitial Lung Disease*, 25–37. https://doi.org/10.1016/B978-0-323-48024-6.00002-1

Rajagopal, S., & Yu, Y. R. A. (2022). The Pathobiology of Pulmonary Arterial Hypertension. *Cardiology Clinics*, *40*(1), 1–12. https://doi.org/10.1016/J.CCL.2021.08.001

Ramazani, Y., Knops, N., Elmonem, M. A., Nguyen, T. Q., Arcolino, F. O., van den Heuvel, L., Levtchenko, E., Kuypers, D., & Goldschmeding, R. (2018). Connective tissue growth factor (CTGF) from basics to clinics. *Matrix Biology*, *68–69*, 44–66. https://doi.org/10.1016/J.MATBIO.2018.03.007

Reeh, H., Rudolph, N., Billing, U., Christen, H., Streif, S., Bullinger, E., Schliemann-Bullinger, M., Findeisen, R., Schaper, F., Huber, H. J., & Dittrich, A. (2019). Response to IL-6 trans- A nd IL-6 classic signalling is determined by the ratio of the IL-6 receptor α to gp130 expression: Fusing experimental insights and dynamic modelling. *Cell Communication and Signaling*, *17*(1). https://doi.org/10.1186/s12964-019-0356-0

Rehman, K., & Akash, M. S. H. (2016). Mechanisms of inflammatory responses and development of insulin resistance: how are they interlinked? *Journal of Biomedical Science 2016 23:1*, *23*(1), 1–18. https://doi.org/10.1186/S12929-016-0303-Y

Rice, L. M., Mantero, J. C., Stratton, E. A., Warburton, R., Roberts, K., Hill, N., Simms, R. W., Domsic, R., Farber, H. W., & Layfatis, R. (2018). Serum biomarker for diagnostic evaluation of pulmonary arterial hypertension in systemic sclerosis. *Arthritis Research and Therapy*, *20*(1). https://doi.org/10.1186/s13075-018-1679-8

Rice, L. M., Padilla, C. M., McLaughlin, S. R., Mathes, A., Ziemek, J., Goummih, S., Nakerakanti, S., York, M., Farina, G., Whitfield, M. L., Spiera, R. F., Christmann, R. B., Gordon, J. K., Weinberg, J., Simms, R. W., & Lafyatis, R. (2015). Fresolimumab treatment decreases biomarkers and improves clinical symptoms in systemic sclerosis patients. *The Journal of Clinical Investigation*, *125*(7), 2795–2807. https://doi.org/10.1172/JCI77958

Richeldi, L., Fernández Pérez, E. R., Costabel, U., Albera, C., Lederer, D. J., Flaherty, K. R., Ettinger, N., Perez, R., Scholand, M. B., Goldin, J., Peony Yu, K. H., Neff, T., Porter, S., Zhong, M., Gorina, E., Kouchakji, E., & Raghu, G. (2020). Pamrevlumab, an anti-connective tissue growth factor therapy, for idiopathic pulmonary fibrosis (PRAISE): a phase 2,

randomised, double-blind, placebo-controlled trial. *The Lancet Respiratory Medicine*, *8*(1), 25–33. https://doi.org/10.1016/S2213-2600(19)30262-0

Rockey, D. C., Bell, P. D., & Hill, J. A. (2015). Fibrosis--a common pathway to organ injury and failure. *New England Journal of Medicine*, *372*(12), 1138–1149. https://doi.org/10.1056/NEJMRA1300575

Romano, M., Sironi, M., Toniatti, C., Polentarutti, N., Fruscella, P., Ghezzi, P., Faggioni, R., Luini, W., Van Hinsbergh, V., Sozzani, S., Bussolino, F., Poli, V., Ciliberto, G., & Mantovani, A. (1997). Role of IL-6 and its soluble receptor in induction of chemokines and leukocyte recruitment. *Immunity*, *6*(3), 315–325. https://doi.org/10.1016/S1074-7613(00)80334-9

Roofeh, D., Lin, C. J. F., Goldin, J., Kim, G. H., Furst, D. E., Denton, C. P., Huang, S., & Khanna, D. (2021). Tocilizumab Prevents Progression of Early Systemic Sclerosis Associated Interstitial Lung Disease. *Arthritis & Rheumatology (Hoboken, N.J.)*, *73*(7), 1301. https://doi.org/10.1002/ART.41668

Roovers, R. C., Laeremans, T., Huang, L., De Taeye, S., Verkleij, A. J., Revets, H., De Haard, H. J., & Van Bergen En Henegouwen, P. M. P. (2007). Efficient inhibition of EGFR signaling and of tumour growth by antagonistic anti-EFGR Nanobodies. *Cancer Immunology, Immunotherapy : CII*, *56*(3), 303–317. https://doi.org/10.1007/S00262-006-0180-4

Rose-John, S. (2012a). Il-6 trans-signaling via the soluble IL-6 receptor: Importance for the proinflammatory activities of IL-6. In *International Journal of Biological Sciences* (Vol. 8, Issue 9, pp. 1237–1247). https://doi.org/10.7150/ijbs.4989

Rose-John, S. (2012b). Il-6 trans-signaling via the soluble IL-6 receptor: Importance for the proinflammatory activities of IL-6. In *International Journal of Biological Sciences* (Vol. 8, Issue 9, pp. 1237–1247). https://doi.org/10.7150/ijbs.4989

Rose-John, S., Jenkins, B. J., Garbers, C., Moll, J. M., & Scheller, J. (2023). Targeting IL-6 trans-signalling: past, present and future prospects. *Nature*

Reviews Immunology 2023 23:10, *23*(10), 666–681. https://doi.org/10.1038/s41577-023-00856-y

Roth, M., Nauck, M., Tamm, M., Perruchoud, A. P., Ziesche, R., & Block, L. H. (1995). Intracellular interleukin 6 mediates platelet-derived growth factor-induced proliferation of nontransformed cells. *Proceedings of the National Academy of Sciences*, *92*(5), 1312–1316. https://doi.org/10.1073/PNAS.92.5.1312

Rottoli, P., Magi, B., Perari, M. G., Liberatori, S., Nikiforakis, N., Bargagli, E., Cianti, R., Bini, L., & Pallini, V. (2005). Cytokine profile and proteome analysis in bronchoalveolar lavage of patients with sarcoidosis, pulmonary fibrosis associated with systematic sclerosis and idiopathic pulmonary fibrosis. *Proteomics*, *5*(5), 1423–1430. https://doi.org/10.1002/pmic.200301007

Sakkas, L. I., & Simopoulou, T. (2023). T cells in the pathogenesis of systemic sclerosis. *Translational Autoimmunity: Volume 6: Advances in Autoimmune Rheumatic Diseases*, 447–474. https://doi.org/10.1016/B978-0-323-85831-1.00022-X

Sanges, S., Rice, L., Tu, L., Valenzi, E., Cracowski, J. L., Montani, D., Mantero, J. C., Ternynck, C., Marot, G., Bujor, A. M., Hachulla, E., Launay, D., Humbert, M., Guignabert, C., & Lafyatis, R. (2023). Biomarkers of haemodynamic severity of systemic sclerosis-associated pulmonary arterial hypertension by serum proteome analysis. *Annals of the Rheumatic Diseases*, *82*(3), 365–373. https://doi.org/10.1136/ard-2022-223237

Sanjabi, S., Oh, S. A., & Li, M. O. (2024). *Regulation of the Immune Response by TGF-b: From Conception to Autoimmunity and Infection*. https://doi.org/10.1101/cshperspect.a022236

Sanjabi, S., Zenewicz, L. A., Kamanaka, M., & Flavell, R. A. (2009). Anti-inflammatory and pro-inflammatory roles of TGF-beta, IL-10, and IL-22 in immunity and autoimmunity. *Current Opinion in Pharmacology*, *9*(4), 447–453. https://doi.org/10.1016/J.COPH.2009.04.008

Sargent, J. L., Milano, A., Bhattacharyya, S., Varga, J., Connolly, M. K., Chang, H. Y., & Whitfield, M. L. (2010). *A TGFb-Responsive Gene Signature Is Associated with a Subset of Diffuse Scleroderma with Increased Disease Severity*. https://doi.org/10.1038/jid.2009.318

Sarker, S. A., Jäkel, M., Sultana, S., Alam, N. H., Bardhan, P. K., Chisti, M. J., Salam, M. A., Theis, W., Hammarström, L., & Frenken, L. G. J. (2013). Anti-rotavirus protein reduces stool output in infants with diarrhea: a randomized placebo-controlled trial. *Gastroenterology*, *145*(4). https://doi.org/10.1053/J.GASTRO.2013.06.053

Sato, S., Nagaoka, T., Hasegawa, M., Tamatani, T., Nakanishi, T., Takigawa, M., & Takehara, K. (2000). Serum levels of connective tissue growth factor are elevated in patients with systemic sclerosis: association with extent of skin sclerosis and severity of pulmonary fibrosis. *The Journal of Rheumatology*, *27*(1), 149–154. https://europepmc.org/article/med/10648031

Schaper, F., & Rose-John, S. (2015). Interleukin-6: Biology, signaling and strategies of blockade. *Cytokine & Growth Factor Reviews*, *26*(5), 475–487. https://doi.org/10.1016/J.CYTOGFR.2015.07.004

Scheibe, K., Kersten, C., Schmied, A., Vieth, M., Primbs, T., Carlé, B., Knieling, F., Claussen, J., Klimowicz, A. C., Zheng, J., Baum, P., Meyer, S., Schürmann, S., Friedrich, O., Waldner, M. J., Rath, T., Wirtz, S., Kollias, G., Ekici, A. B., … Neufert, C. (2019). *Inhibiting Interleukin 36 Receptor Signaling Reduces Fibrosis in Mice With Chronic Intestinal Inflammation*. https://doi.org/10.1053/j.gastro.2018.11.029

Scheller, J., Chalaris, A., Schmidt-Arras, D., & Rose-John, S. (2011). The pro- and anti-inflammatory properties of the cytokine interleukin-6. In *Biochimica et Biophysica Acta - Molecular Cell Research* (Vol. 1813, Issue 5, pp. 878–888). https://doi.org/10.1016/j.bbamcr.2011.01.034

Schermuly, R. T., Ghofrani, H. A., Wilkins, M. R., & Grimminger, F. (2011). Mechanisms of disease: pulmonary arterial hypertension. *Nature Reviews. Cardiology*, *8*(8), 443. https://doi.org/10.1038/NRCARDIO.2011.87

Schneider, C. A., Rasband, W. S., & Eliceiri, K. W. (2012). NIH Image to ImageJ: 25 years of image analysis. *Nature Methods 2012 9:7*, *9*(7), 671–675. https://doi.org/10.1038/nmeth.2089

Schoenfeld, S. R., & Castelino, F. V. (2015). Interstitial lung disease in Scleroderma. *Rheumatic Disease Clinics of North America*, *41*(2), 237–248. https://doi.org/10.1016/j.rdc.2014.12.005

Sgonc, R., Gruschwitz, M. S., Boeck, G., Sepp, N., Gruber, J., & Wick, G. (2000). Endothelial cell apoptosis in systemic sclerosis is induced by antibody-dependent cell-mediated cytotoxicity via CD95. *Arthritis and Rheumatism*, *43*(11), 2550–2562. https://doi.org/10.1002/1529-0131(200011)43:11<2550::AID-ANR24>3.0.CO;2-H

Shah, P. V, Balani, P., Lopez, A. R., Nobleza, C. M. N., Siddiqui, M., & Khan, S. (2021). A Review of Pirfenidone as an Anti-Fibrotic in Idiopathic Pulmonary Fibrosis and Its Probable Role in Other Diseases. *Cureus*, *13*(1). https://doi.org/10.7759/CUREUS.12482

She, Y. X., Yu, Q. Y., & Tang, X. X. (2021). Role of interleukins in the pathogenesis of pulmonary fibrosis. *Cell Death Discovery*, *7*(1). https://doi.org/10.1038/s41420-021-00437-9

Sherwood, E. R., & Toliver-Kinsky, T. (2004). Mechanisms of the inflammatory response. *Best Practice & Research Clinical Anaesthesiology*, *18*(3), 385–405. https://doi.org/10.1016/J.BPA.2003.12.002

Shima, Y. (2021). Cytokines Involved in the Pathogenesis of SSc and Problems in the Development of Anti-Cytokine Therapy. *Cells*, *10*(5). https://doi.org/10.3390/CELLS10051104

Shi-wen, X., Pennington, D., Holmes, A., Leask, A., Bradham, D., Beauchamp, J. R., Fonseca, C., Du Bois, R. M., Martin, G. R., Black, C. M., & Abraham,

D. J. (2000). Autocrine overexpression of CTGF maintains fibrosis: RDA analysis of fibrosis genes in systemic sclerosis. *Experimental Cell Research*, *259*(1), 213–224. https://doi.org/10.1006/EXCR.2000.4972

Shi-Wen, X., Renzoni, E. A., Kennedy, L., Howat, S., Chen, Y., Pearson, J. D., Bou-Gharios, G., Dashwood, M. R., du Bois, R. M., Black, C. M., Denton, C. P., Abraham, D. J., & Leask, A. (2007). Endogenous endothelin-1 signaling contributes to type I collagen and CCN2 overexpression in fibrotic fibroblasts. *Matrix Biology*, *26*(8), 625–632. https://doi.org/10.1016/j.matbio.2007.06.003

Singh, R. P., Hasan, S., Sharma, S., Nagra, S., Yamaguchi, D. T., Wong, D. T. W., Hahn, B. H., & Hossain, A. (2014). Th17 cells in inflammation and autoimmunity. *Autoimmunity Reviews*, *13*(12), 1174–1181. https://doi.org/10.1016/J.AUTREV.2014.08.019

Smith, S., Wu, P. W., Seo, J. J., Fernando, T., Jin, M., Contreras, J., Montano, E. N., Gabhann, J. N., Cunningham, K., Widaa, A., McCarthy, E. M., Molloy, E. S., Kearns, G., Murphy, C. C., Kong, W., Björkbacka, H., Kornfeld, H., Forbess, L., Venuturupalli, S., … Jefferies, C. A. (2018). IL-16/miR-125a axis controls neutrophil recruitment in pristane-induced lung inflammation. *JCI Insight*, *3*(15). https://doi.org/10.1172/JCI.INSIGHT.120798

Snick, J. Van. (1990). INTERLEUKIN-6: AN OVERVIEW. *Annu. Rev. Lmmunol*, *8*, 253–278. www.annualreviews.org

Sobolewski, P., Maślińska, M., Wieczorek, M., Łagun, Z., Malewska, A., Roszkiewicz, M., Nitskovich, R., Szymańska, E., & Walecka, I. (2019a). Systemic sclerosis - Multidisciplinary disease: Clinical features and treatment. In *Reumatologia* (Vol. 57, Issue 4, pp. 221–233). Termedia Publishing House Ltd. https://doi.org/10.5114/reum.2019.87619

Sobolewski, P., Maślińska, M., Wieczorek, M., Łagun, Z., Malewska, A., Roszkiewicz, M., Nitskovich, R., Szymańska, E., & Walecka, I. (2019b). Systemic sclerosis – multidisciplinary disease: clinical features and

treatment. *Reumatologia*, *57*(4), 221. https://doi.org/10.5114/REUM.2019.87619

Solomon, J. J., Olson, A. L., Fischer, A., Bull, T., Brown, K. K., & Raghu, G. (2013). Scleroderma lung disease. *European Respiratory Review*, *22*(127), 6. https://doi.org/10.1183/09059180.00005512

Song, I. W., Nagamani, S. C. S., Nguyen, D., Grafe, I., Sutton, V. R., Gannon, F. H., Munivez, E., Jiang, M. M., Tran, A., Wallace, M., Esposito, P., Musaad, S., Strudthoff, E., McGuire, S., Thornton, M., Shenava, V., Rosenfeld, S., Huang, S., Shypailo, R., … Lee, B. (2022). Targeting TGF-β for treatment of osteogenesis imperfecta. *The Journal of Clinical Investigation*, *132*(7). https://doi.org/10.1172/JCI152571

Song, Z.-M., Liu, F., Chen, Y.-M., Liu, Y.-J., Wang, X.-D., & Du, S.-Y. (2018). *CTGF-mediated ERK signaling pathway influences the inflammatory factors and intestinal flora in ulcerative colitis*. https://doi.org/10.1016/j.biopha.2018.12.063

Steeland, S., Vandenbroucke, R. E., & Libert, C. (2016). Nanobodies as therapeutics: big opportunities for small antibodies. *Drug Discovery Today*, *21*(7), 1076–1113. https://doi.org/10.1016/J.DRUDIS.2016.04.003

Steen, V. (2003). Predictors of end stage lung disease in systemic sclerosis. *Annals of the Rheumatic Diseases*, *62*(2), 97–99. https://doi.org/10.1136/ARD.62.2.97

Stenmark, K. R., Frid, M., & Perros, F. (2016). Endothelial-to-Mesenchymal transition: An evolving paradigm and a promising therapeutic target in PAH. *Circulation*, *133*(18), 1734–1737. https://doi.org/10.1161/CIRCULATIONAHA.116.022479

Szklarczyk, D., Franceschini, A., Wyder, S., Forslund, K., Heller, D., Huerta-Cepas, J., Simonovic, M., Roth, A., Santos, A., Tsafou, K. P., Kuhn, M., Jensen, L. J., & Von Mering, C. (2014). STRING v10: protein-protein interaction networks, integrated over the tree of life. *Nucleic Acids Research*, *43*, 447–452. https://doi.org/10.1093/nar/gku1003

Taga, T., Hibi, M., Hirata, Y., Yamasaki, K., Yasukawa, K., Matsuda, T., Hirano, T., & Kishimoto, T. (1989). Interleukin-6 Triggers the Association of Its Receptor with a Possible Signal Transducer, gp130. *Cell*, *58*, 573–581.

Tam, A. Y. Y., Horwell, A. L., Trinder, S. L., Khan, K., Xu, S., Ong, V., Denton, C. P., Norman, J. T., Holmes, A. M., Bou-Gharios, G., & Abraham, D. J. (2021a). Selective deletion of connective tissue growth factor attenuates experimentally-induced pulmonary fibrosis and pulmonary arterial hypertension. *International Journal of Biochemistry and Cell Biology*, *134*. https://doi.org/10.1016/J.BIOCEL.2021.105961

Tam, A. Y. Y., Horwell, A. L., Trinder, S. L., Khan, K., Xu, S., Ong, V., Denton, C. P., Norman, J. T., Holmes, A. M., Bou-Gharios, G., & Abraham, D. J. (2021b). Selective deletion of connective tissue growth factor attenuates experimentally-induced pulmonary fibrosis and pulmonary arterial hypertension. *The International Journal of Biochemistry & Cell Biology*, *134*. https://doi.org/10.1016/J.BIOCEL.2021.105961

Tanaka, T., & Kishimoto, T. (2014). *The Biology and Medical Implications of Interleukin-6*. https://doi.org/10.1158/2326-6066.CIR-14-0022

Tanaka, T., Ogata, A., & Narazaki, M. (2013). Tocilizumab: An updated review of its use in the treatment of rheumatoid arthritis and its application for other immune-Mediated diseases. In *Clinical Medicine Insights: Therapeutics* (Vol. 5, pp. 33–52). Libertas Academica Ltd. https://doi.org/10.4137/CMT.S9282

Tilg, H., Trehu, E., Atkins, M. B., Dinarello, C. A., & Mier, J. W. (1994). Interleukin-6 (IL-6) as an Anti-inflammatory Cytokine: Induction of Circulating IL-1 Receptor Antagonist and Soluble Tumor Necrosis Factor Receptor p55. *Blood*, *83*(1), 113–118. https://doi.org/10.1182/BLOOD.V83.1.113.113

Toda, N., Mukoyama, M., Yanagita, M., & Yokoi, H. (2018). CTGF in kidney fibrosis and glomerulonephritis. *Inflammation and Regeneration*, *38*(1), 1–8. https://doi.org/10.1186/S41232-018-0070-0/FIGURES/3

Tsai, C.-C., Wu, S.-B., Kau, H.-C., & Wei, Y.-H. (2018). *Essential role of connective tissue growth factor (CTGF) in transforming growth factor-β1 (TGF-β1)-induced myofibroblast transdifferentiation from Graves' orbital fibroblasts*. https://doi.org/10.1038/s41598-018-25370-3

Turner, M., Chantry, D., & Feldmann, M. (1990). Transforming growth factor beta induces the production of interleukin 6 by human peripheral blood mononuclear cells. *Cytokine*, *2*(3), 211–216. https://doi.org/10.1016/1043-4666(90)90018-O

Van Caam, A., Aarts, J., Van Ee, T., Vitters, E., Koenders, M., Van De Loo, F., Van Lent, P., Van Den Hoogen, F., Thurlings, R., Vonk, M. C., & Van Der Kraan, P. M. (2020). TGFβ-mediated expression of TGFβ-activating integrins in SSc monocytes: Disturbed activation of latent TGFβ? *Arthritis Research and Therapy*, *22*(1), 1–9. https://doi.org/10.1186/S13075-020-2130-5/FIGURES/3

Van Den Hoogen, F., Khanna, D., Fransen, J., Johnson, S. R., Baron, M., Tyndall, A., Matucci-Cerinic, M., Naden, R. P., Medsger, T. A., Carreira, P. E., Riemekasten, G., Clements, P. J., Denton, C. P., Distler, O., Allanore, Y., Furst, D. E., Gabrielli, A., Mayes, M. D., Van Laar, J. M., … Radboud, : (2013). 2013 Classification Criteria for Systemic Sclerosis An American College of Rheumatology/European League Against Rheumatism Collaborative Initiative Arthritis & Rheumatism. *ARTHRITIS & RHEUMATISM*, *65*(11), 2737–2747. https://doi.org/10.1002/art.38098

Van Der Linden, R. H. J., Frenken, L. G. J., De Geus, B., Harmsen, M. M., Ruuls, R. C., Stok, W., De Ron, L., Wilson, S., Davis, P., & Verrips, C. T. (1999). Comparison of physical chemical properties of llama VHH antibody fragments and mouse monoclonal antibodies. *Biochimica et Biophysica Acta*, *1431*(1), 37–46. https://doi.org/10.1016/S0167-4838(99)00030-8

Van Roy, M., Ververken, C., Beirnaert, E., Hoefman, S., Kolkman, J., Vierboom, M., Breedveld, E., 't Hart, B., Poelmans, S., Bontinck, L., Hemeryck, A., Jacobs, S., Baumeister, J., & Ulrichts, H. (2015). The preclinical pharmacology of the high affinity anti-IL-6R Nanobody® ALX-0061

supports its clinical development in rheumatoid arthritis. *Arthritis Research & Therapy*, *17*(1). https://doi.org/10.1186/S13075-015-0651-0

Vancheri, C., Cottin, V., Kreuter, M., & Hilberg, O. (2015). IPF, comorbidities and management implications. *Sarcoidosis, Vasculitis, and Diffuse Lung Diseases : Official Journal of WASOG*, *32 Suppl 1*, 17–23. https://europepmc.org/article/med/26237439

Vanneaux, V., Farge-Bancel, D., Lecourt, S., Baraut, J., Cras, A., Jean-Louis, F., Brun, C., Verrecchia, F., Larghero, J., & Michel, L. (2013). Expression of transforming growth factor β receptor II in mesenchymal stem cells from systemic sclerosis patients. *BMJ Open*, *3*, 1890. https://doi.org/10.1136/bmjopen-2012

Vignali, D. A. A., & Kuchroo, V. K. (2012). IL-12 family cytokines: immunological playmakers. In *Nature Immunology* (Vol. 13, Issue 8, pp. 722–728). https://doi.org/10.1038/ni.2366

Vincke, C., & Muyldermans, S. (2012). Introduction to heavy chain antibodies and derived Nanobodies. *Methods in Molecular Biology (Clifton, N.J.)*, *911*, 15–26. https://doi.org/10.1007/978-1-61779-968-6_2

Volkmann, E. R., & Fischer, A. (2021). Update on morbidity and mortality in systemic sclerosis–related interstitial lung disease. *Journal of Scleroderma and Related Disorders*, *6*(1), 11. https://doi.org/10.1177/2397198320915042

W Distler, J. H., Akhmetshina, A., Schett, G., & Distler, O. (2009). *Monocyte chemoattractant proteins in the pathogenesis of systemic sclerosis*. https://doi.org/10.1093/rheumatology/ken401

Wasinger, V. C., Cordwell, S. J., Poljak, A., Yan, J. X., Gooley, A. A., Wilkins, M. R., Duncan, M. W., Harris, R., Williams, K. L., & Humphery-Smith, I. (1995). Progress with gene-product mapping of the Mollicutes: Mycoplasma genitalium. *ELECTROPHORESIS*, *16*(1), 1090–1094. https://doi.org/10.1002/elps.11501601185

Wells, R. G. (2013). Tissue mechanics and fibrosis. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, *1832*(7), 884–890. https://doi.org/10.1016/J.BBADIS.2013.02.007

Wesolowski, J., Alzogaray, V., Reyelt, J., Unger, M., Juarez, K., Urrutia, M., Cauerhff, A., Danquah, W., Rissiek, B., Scheuplein, F., Schwarz, N., Adriouch, S., Boyer, O., Seman, M., Licea, A., Serreze, D. V., Goldbaum, F. A., Haag, F., & Koch-Nolte, F. (2009). Single domain antibodies: promising experimental and therapeutic tools in infection and immunity. *Medical Microbiology and Immunology*, *198*(3), 157–174. https://doi.org/10.1007/S00430-009-0116-7

What is NPX? - Olink. (n.d.). Retrieved 8 November 2022, from https://www.olink.com/faq/what-is-npx/

Wilkins, M. R., Pasquali, C., Appel, R. D., Ou, K., Golar, O., Sanchez, J.-C., Yan, J. X., Gooley, A. A., Hughes, G., Humphery-Smith, I., Wllliams, K. L., & Hochstrasser, D. F. (1996). From Proteins to Proteomes: Large Scale Protein Identification by Two-Dimensional Electrophoresis and Amino Acid Analysis 6-a. In *BIOTECHNOLOGY* (Vol. 14). http://www.nature.com/naturebiotechnology

Wu, W., Jordan, S., Graf, N., de Oliveira Pena, J., Curram, J., Allanore, Y., Matucci-Cerinic, M., Pope, J. E., Denton, C. P., Khanna, D., Distler, O., Guiducci, S., Walker, U., Jaeger, V., Bannert, B., Lapadula, G., Becvarare, R., Cutolo, M., Valentini, G., … Sarzi Puttini, P. (2019). Progressive skin fibrosis is associated with a decline in lung function and worse survival in patients with diffuse cutaneous systemic sclerosis in the european scleroderma trials and research (eustar) cohort. *Annals of the Rheumatic Diseases*, *78*(5), 648–656. https://doi.org/10.1136/annrheumdis-2018-213455

Wu, X., Ming, B., Wu, T., Gao, R., Hu, P., Tang, J., Zhong, J., & Dong, L. (2022). *IL-33/ST2 axis contributes to the dermal fibrosis of systemic sclerosis via promoting fibroblasts activation*. https://doi.org/10.1016/j.jdermsci.2022.07.009

Wynn, T. A. (2008). Cellular and molecular mechanisms of fibrosis. *Journal of Pathology*, *214*(2), 199–210. https://doi.org/10.1002/PATH.2277

Xiao, L. Z., Topley, N., Ito, T., & Phillips, A. (2005). Interleukin-6 regulation of transforming growth factor (TGF)-β receptor compartmentalization and turnover enhances TGF-β1 signaling. *Journal of Biological Chemistry*, *280*(13), 12239–12245. https://doi.org/10.1074/jbc.M413284200

Xing, Z., Gauldie, J., Cox, G., Baumann, H., Jordana, M., Lei, X. F., & Achong, M. K. (1998a). IL-6 is an antiinflammatory cytokine required for controlling local or systemic acute inflammatory responses. *Journal of Clinical Investigation*, *101*(2), 311. https://doi.org/10.1172/JCI1368

Xing, Z., Gauldie, J., Cox, G., Baumann, H., Jordana, M., Lei, X. F., & Achong, M. K. (1998b). IL-6 is an antiinflammatory cytokine required for controlling local or systemic acute inflammatory responses. *Journal of Clinical Investigation*, *101*(2), 311–320. https://doi.org/10.1172/JCI1368

Xu, B., Xu, G., Yu, Y., & Lin, J. (2021). The role of TGF-β or BMPR2 signaling pathway-related miRNA in pulmonary arterial hypertension and systemic sclerosis. *Arthritis Research & Therapy*, *23*(1). https://doi.org/10.1186/S13075-021-02678-6

Yanaba, K., Komura, K., Kodera, M., Matsushita, T., Hasegawa, M., Takehara, K., & Sato, S. (2006). Serum levels of monocyte chemotactic protein-3/CCL7 are raised in patients with systemic sclerosis: association with extent of skin sclerosis and severity of pulmonary fibrosis. *Annals of the Rheumatic Diseases*, *65*(1), 124–126. https://doi.org/10.1136/ARD.2005.040782

Yanaba, K., Yoshizaki, A., Muroi, E., Hara, T., Ogawa, F., Shimizu, K., Hasegawa, M., Fujimoto, M., Takehara, K., Sato, S., & Sato, S. (2010). CCL13 is a promising diagnostic marker for systemic sclerosis. *British Journal of Dermatology*. https://doi.org/10.1111/j.1365-2133.2009.09507.x

Ye, J., Que, B., Huang, Y., Lin, Y., Chen, J., Liu, L., Shi, Y., Wang, Y., Wang, M., Zeng, T., Wang, Z., Hu, H., Xu, Y., Shi, L., Ye, D., Liu, J., Jiang, H.,

Wan, J., & Ji, Q. (2019). Interleukin-12p35 knockout promotes macrophage differentiation, aggravates vascular dysfunction, and elevates blood pressure in angiotensin II-infused mice. *Cardiovascular Research*, *115*(6), 1102–1113. https://doi.org/10.1093/CVR/CVY263

Yip, R. M. L., & Yim, C. W. (2019). Role of Interleukin 6 Inhibitors in the Management of Rheumatoid Arthritis. *Journal of Clinical Rheumatology*, *27*(8), e516. https://doi.org/10.1097/RHU.0000000000001293

Younossi, Z., Anstee, Q. M., Marietti, M., Hardy, T., Henry, L., Eslam, M., George, J., & Bugianesi, E. (2017). Global burden of NAFLD and NASH: trends, predictions, risk factors and prevention. *Nature Publishing Group*, *14*. https://doi.org/10.1038/nrgastro.2017.109

Younossi, Z. M., Golabi, P., de Avila, L., Paik, J. M., Srishord, M., Fukui, N., Qiu, Y., Burns, L., Afendy, A., & Nader, F. (2019). The global epidemiology of NAFLD and NASH in patients with type 2 diabetes: A systematic review and meta-analysis. *Journal of Hepatology*, *71*(4), 793–801. https://doi.org/10.1016/J.JHEP.2019.06.021

Yudkin, J. S., Kumari, M., Humphries, S. E., & Mohamed-Ali, V. (2000a). Inflammation, obesity, stress and coronary heart disease. *Atherosclerosis*, 209–214.

Yudkin, J. S., Kumari, M., Humphries, S. E., & Mohamed-Ali, V. (2000b). Inflammation, obesity, stress and coronary heart disease: is interleukin-6 the link? *Atherosclerosis*, *148*(2), 209–214. https://doi.org/10.1016/S0021-9150(99)00463-3

Zamanian, R. T., Badesch, D., Chung, L., Domsic, R. T., Medsger, T., Pinckney, A., Keyes-Elstein, L., D'Aveta, C., Spychala, M., White, R. J., Hassoun, P. M., Torres, F., Sweatt, A. J., Molitor, J. A., Khanna, D., Maecker, H., Welch, B., Goldmuntz, E., Nicolls, M. R., … Sood, N. (2021). Safety and efficacy of B-cell depletion with rituximab for the treatment of systemic sclerosis–associated pulmonary arterial hypertension: A multicenter, double-blind, randomized, placebo-controlled trial. *American*

Journal of Respiratory and Critical Care Medicine, *204*(2), 209–221. https://doi.org/10.1164/rccm.202009-3481OC

Zeisberg, M., & Kalluri, R. (2013). Cellular Mechanisms of Tissue Fibrosis. 1. Common and organ-specific mechanisms associated with tissue fibrosis. *Am J Physiol Cell Physiol*, *304*, 216–225. https://doi.org/10.1152/ajpcell.00328.2012.-Fi

Zhang, S., Yang, T., Xu, X., Wang, M., Zhong, L., Yang, Y., Zhai, Z., Xiao, F., & Wang, C. (2015). Oxidative stress and nitric oxide signaling related biomarkers in patients with pulmonary hypertension: a case control study. *BMC Pulmonary Medicine*, *15*(1). https://doi.org/10.1186/S12890-015-0045-8

Zhang, X., & Mosser, D. M. (2008). Macrophage activation by endogenous danger signals. *The Journal of Pathology*, *214*(2), 161. https://doi.org/10.1002/PATH.2284

Zhao, M., Wu, J., Wu, H., Sawalha, A. H., & Lu, Q. (2016). *Clinical Treatment Options in Scleroderma: Recommendations and Comprehensive Review*. *1*, 3. https://doi.org/10.1007/s12016-020-08831-4

Zhao, W. H., Liu, J., Wang, B. Y., Chen, Y. X., Cao, X. M., Yang, Y., Zhang, Y. L., Wang, F. X., Zhang, P. Y., Lei, B., Gu, L. F., Wang, J. L., Yang, N., Zhang, R., Zhang, H., Shen, Y., Bai, J., Xu, Y., Wang, X. G., … Zhang, W. G. (2018). A phase 1, open-label study of LCAR-B38M, a chimeric antigen receptor T cell therapy directed against B cell maturation antigen, in patients with relapsed or refractory multiple myeloma. *Journal of Hematology and Oncology*, *11*(1), 1–8. https://doi.org/10.1186/S13045-018-0681-6/FIGURES/2

Zheng, Q., Cox, I. A., Campbell, J. A., Xia, Q., Otahal, P., De Graaff, B., Corte, T. J., Teoh, A. K. Y., Walters, E. H., & Palmer, A. J. (2022). Mortality and survival in idiopathic pulmonary fibrosis: a systematic review and meta-analysis. *ERJ Open Research*. https://doi.org/10.1183/23120541.00591-2021

Zhu, B., Ma, A.-Q., Yang, L., & Dang, X.-M. (2013). Atorvastatin Attenuates Bleomycin-Induced Pulmonary Fibrosis via Suppressing iNOS Expression and the CTGF (CCN2)/ERK Signaling Pathway. *OPEN ACCESS Int. J. Mol. Sci*, *14*, 14. https://doi.org/10.3390/ijms141224476

Appendix I

Analytes in the Cardiovascular II and Immuno-Oncology panels

Cardiovascular II panel (V. 5006)	Immuno-Oncology panel (v.3111)
BMP-6	IL8
ANGPT1	TNFRSF9
ADM	TIE2
CD40-L	MCP-3
SLAMF7	CD40-L
PGF	IL-1alpha
ADAM-TS13	CD244
BOC	EGF
IL-4RA	ANGPT1
SRC	IL7
IL-1ra	PGF
IL6	IL6
TNFRSF10A	ADGRG1
STK4	MCP-1
IDUA	CRTAM
TNFRSF11A	CXCL11
PAR-1	MCP-4
TRAIL-R2	TRAIL
PRSS27	FGF2
TIE2	CXCL9
TF	CD8A
IL1RL2	CAIX
PDGF subunit B	MUC-16
IL-27	ADA
IL-17D	CD4
CXCL1	NOS3
LOX-1	IL2
Gal-9	Gal-9
GIF	VEGFR-2
SCF	CD40
IL18	IL18
FGF-21	GZMH
PIgR	KIR3DL1
RAGE	LAP TGF-beta-1

SOD2	CXCL1
CTRC	TNFSF14
FGF-23	IL33
SPON2	TWEAK
GH	PDGF subunit B
FS	PDCD1
GLO1	FASLG
CD84	CD28
PAPPA	CCL19
SERPINA12	MCP-2
REN	CCL4
DECR1	IL15
MERTK	Gal-1
KIM1	PD-L1
THBS2	CD27
TM	CXCL5
VSIG2	IL5
AMBP	HGF
PRELP	GZMA
HO-1	HO-1
XCL1	CX3CL1
IL16	CXCL10
SORT1	CD70
CEACAM8	IL10
PTX3	TNFRSF12A
PSGL-1	CCL23
CCL17	CD5
CCL3	CCL3
MMP7	MMP7
IgG	Fc receptor II-b ARG1
ITGB1BP2	NCR1
DCN	DCN
Dkk-1	TNFRSF21
LPL	TNFRSF4
PRSS8	MIC-A/B
AGRP	CCL17
HB-EGF	ANGPT2
GDF-2	PTN
FABP2	CXCL12
THPO	IFN-gamma
MARCO	LAMP3
GT	CASP-8
BNP	ICOSLG

MMP12	MMP12
ACE2	CXCL13
PD-L2	PD-L2
CTSL1	VEGFA
hOSCAR	IL4
TNFRSF13B	LAG3
TGM2	IL12RB1
LEP	IL13
CA5A	CCL20
HSP 27	TNF
CD4	KLRD1
NEMO	GZMB
VEGFD	CD83
PARP-1	IL12
HAOX1	CSF-1