Investigating the pathways involved in the progression of fibrosis in tuberculous pericarditis

by

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FKMSIY001

SUBMITTED TO THE UNIVERSITY OF CAPE TOWN

In fulfilment of the requirements for the degree

MASTER OF SCIENCE IN CHEMICAL AND SYSTEMS BIOLOGY

FACULTY OF HEALTH SCIENCES

UNIVERSITY OF CAPE TOWN

NOVEMBER 2022

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Abstract

Extrapulmonary tuberculosis (EPTB) accounts for about 15% of all cases of tuberculosis (TB) globally. Tuberculous pericarditis (TBP) is a severe form of EPTB involving the pericardium that is present in 1-2% of TB cases, and poses diagnostic and therapeutic challenges, with mortality rates ranging between 8-34% despite anti-tuberculous therapy. TBP is the leading cause of pericardial constriction in Africa which is a life-threatening TBP complication.

There has been significant progress towards understanding the pathogenesis of TBP. However, the mechanisms leading to the fibrotic phenotype are poorly understood, and the definitive pathophysiology is not clear.

Various immune-modulatory and fibrotic cytokines, peptides, and pathways have been studied to improve our understanding of the mechanism of TBP progression.

These findings prompted the investigation of the pathogenesis of TBP by identifying proteins and novel pathways that may contribute to TBP disease progression. This thesis aimed to investigate a) the molecular mechanisms involved in the development of fibrosis in TBP, and b) the potential of ACE inhibitors (ACEi) for managing fibrosis progression.

Pericardial fluid was obtained from patients with TBP and patients undergoing coronary artery bypass surgery (non-infectious controls). Label-free quantitative discovery mass spectrometry (MS) was employed to investigate the TBP proteome. Proteins involved in fibrotic pathways were identified, with proteins of interest involved in wound healing, extracellular matrix (ECM) organisation, and regulation of TGF-β production.

Autoantibody profiling was used to investigate whether the post-translational modification citrullination is involved in autoantibody production. Thirteen autoantibodies were expressed in the TBP pericardial fluid samples. The levels of leucine-rich repeat flightless-interacting protein 2 (LRRFIP2) were increased in TBP samples compared to in control samples. LRRFIP2 functions as part of a checkpoint connecting innate and adaptive immunity in autoimmune diseases.

Finally, a systematic review was conducted to investigate the antifibrotic potential of ACEi in myocardial fibrosis. ACEi results in a significant reduction in the progression of fibrosis as monotherapy, and shows an even greater reduction in fibrosis when used in combination with other renin aldosterone angiotensin (RAAS). These findings form

the basis for future studies, including pharmacological studies, on the prevention and management of fibrotic conditions, in particular TBP.

The research presented in the present thesis forms a foundation for ongoing work on the pathophysiology, diagnosis, and treatment of TBP and its progression, and has implications for the reduction of the burden of disease on both patients and the health care system.

Acknowledgements

I wish to express my gratitude to my supervisors, Prof Ed Sturrock and Prof Mpiko Ntsekhe providing me the opportunity to work on this project and in.

My heartfelt thanks also go to Vinasha for her patience, support, guidance, and friendship throughout the years. Your contribution to my growth holds a special place in my heart.

I would also like to thank every member of Sturrock lab for their support, warmth, and patience during the years. Thanks to Sylva for adding colour to the lab and for being always willing to help. Many thanks to Tariq for running the mass spectrometric samples, and Tobz and Muneerah who were helpful with the citrullination work.

My heartful gratitude also goes out to my friends and family for their ongoing support. A special mention to my community in Cape Town for their endless support during this challenging period.

The work reported herein was made possible through partial funding support by the National Research Foundation. The content hereof is the sole responsibility of the authors and does not necessarily represent the official views of the NRF or the funders.

Lastly I would like to pay homage to God for his steady guidance in this journey.

Abbreviations and Acronyms

ACPA	Anti-citrullinated peptide antibodies
ADA	Adenosine deaminase
Ac-SDKP	N-acetyl-seryl-aspartyl-lysyl-proline
ACE	Angiotensin converting enzyme
ACE2	Angiotensin converting Enzyme 2
ACEi	ACE inhibitor
ACN	Acetonitrile
AGT	Angiotensinogen
Ang 1-9	Angiotensin 1-9
Ang I	Angiotensin I
Anti-CCP	Anti-cyclic citrullinated peptide
ARB	Angiotensin Receptor Blocker
AT_1R	Angiotensin Type 1 Receptor
AT_2R	Angiotensin Type 2 Receptor
BSA	Bovine serum albumin
CSF	Cerebrospinal fluid
DTT	Dithiothreitol
ECM	Extracellular matrix
EPTB	Extrapulmonary tuberculosis
FA	Formic acid
FASP	Filter-aided sample preparation
FGF	Fibroblast growth factor
GSH	Groote Schuur Hospital
HCL	Hydrochloric acid
HIV	Human Immunodeficiency Virus
IAA	Iodoacetamide
IFN-γ	interferon-γ
IGHG1	Human Immunoglobulin Heavy Constant Gamma 1
KRT19	Keratin19
LC	Liquid chromatography
LC-MS/S	MS/MS Tandem Liquid chromatography tandem
LRRFIP2	Leucine-rich repeat flightless-interacting protein 2

MAGEB2	Melanoma antigen family B2
MasR	Mas receptor
MRA	Mineralocorticoid receptor antagonist
Mtb	*Mycobacterium* Tuberculosis
MS	Mass spectrometry
NaOH	Sodium hydroxide
Ni	Neprilysin inhibitor
PCFF	Pericardial cell-free fluid
PCR	Polymerase chain reaction
PDFGF	Platelet-derived growth factor
RA	Rheumatoid Arthritis
RAS	Renin-angiotensin system
RBPJ	Recombination signal binding protein for immunoglobulin kappa J region
RCT	Randomised Controlled Trials
RFU	Relative Fluorescence Units
siRNA	Small interfering RNA
SLE	Systemic Lupus Erythematosus
Smad	Son of a mother against decapentaplegic
TCA	Trichloroacetic acid
TGF-β	Transforming growth factor-β
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor-α
TRC	Transmembrane domain recognition complex
NLRP3	NACHT, leucine-rich repeat, and pyrin domain-containing protein 3

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1. Introduction

The Pericardium and its response to insult

The normal pericardium

The pericardium is a fibrous structure surrounding the heart that is made up of two layers: the outer layer, or parietal pericardium, and the inner layer, or visceral pericardium. These two layers form a cavity containing 10 to 35 ml of pericardial fluid under normal physiological conditions (Little and Freeman 2006). The pericardium has multiple functions, including reducing friction during cardiac motion, anchoring, shock absorption, prevention of adhesion formation, and the spread of inflammation or neoplasia.

Pericarditis

Pericarditis is a clinical syndrome resulting from the pathophysiological response to injury to the pericardium. Pericarditis is a result of inflammation of the pericardium from various causes. The common causes of pericarditis are, but not limited to:

- Infectious causes, e.g., viral, bacterial, fungal, parasitic.
- Autoimmune causes, e.g., systemic lupus erythematosus (SLE), and rheumatoid arthritis (RA)
- Malignant causes, e.g., lung and breast cancer metastasis to the pericardium
- Metabolic causes, e.g., uraemic pericarditis
- Traumatic pericarditis(Imazio, Gaita et al. 2015).

Inflammation of the pericardium is accompanied by an accumulation of pericardial fluid rich in inflammatory cells and fibrin within the pericardium that can compromise cardiac function with resultant haemodynamic instability. Chronic pericarditis can result in the thickening, adhesion, and fibrosis of the pericardium with resultant obliteration of the pericardial space, and constrictive pericarditis(Ramasamy, Mayosi et al. 2018).

TBP

Mycobacterium tuberculosis (Mtb) is the pathogenic bacteria that is the causative agent of tuberculosis, pulmonary and extrapulmonary. Mtb is the most frequent cause of all pericardial diseases across Sub-Saharan Africa (Noubiap, Agbor et al. 2019). TBP diseases causes 4-13% of heart failure cases in Sub-Saharan Africa (Agbor, Essouma et al. 2018).

Mtb spreads from the primary site of infection, which is usually the lungs, to other organs via haematogenous or lymphatic spread. Common extrapulmonary sites are the brain, gastrointestinal system and pericardium (Rodriguez-Takeuchi, Renjifo et al. 2019).

Mtb spreads to the pericardial sac, where pericardial fluid is located, resulting in an inflammation, and eventually causing TBP.

TBP occurs following a severe immune response to Mtb bacilli in the pericardium, or as an inflammatory response upon retrograde lymphatic node spread of Mtb bacilli into the pericardium (Mayosi, Burgess et al. 2005). Immune system activation results in an early fibrinous exudate which progresses to granuloma formation that is predominantly composed of specialized epithelioid macrophages and phagocytes (Ramasamy, Mayosi et al. 2018). Thereafter a lymphocyte-rich serosanguineous exudate forms and the granuloma architecture is reorganised with the deposition of extracellular matrix (ECM), fibrin and collagen. Pro-inflammatory cytokines are significantly higher in pericardial tuberculosis compared to in viral, malignant, or other bacterial causes of pericarditis *in vitro.* Tumour necrosis factor-α (TNF-α) plays a major role in the regulation of inflammation and recruitment of macrophages and natural killer cells due to its pleiotropic nature. Other upregulated cytokines associated with TBP are interferon gamma (INF-γ) and interleukin-8 (IL8). Elevated IL10 levels are also associated with TBP, during which it functions as an immune regulator and not as a pro-inflammatory cytokine (Pankuweit, Wädlich et al. 2000, Burgess, Reuter et al. 2002, Karatolios, Moosdorf et al. 2012, Marakalala, Raju et al. 2016). Much of the morbidity of TBP is thought to be mediated through these proinflammatory cytokines and intrapericardial immune and inflammatory responses.

On a clinical level, TB pericarditis is classified into four stages which are summarized in Table 1-1

Table 1-1 Stages of TB pericarditis. Adapted from Ntsekhe and Mayosi, 2013.

Stage	Pathological manifestation	Clinical manifestation
One	Dry stage (least common)	Acute pericarditis (chest pain, pericardial friction rub and widespread ST elevation without effusion)
Two	Effusive stage (most common)	(1) Moderate to large pericardial effusion with symptoms and signs of heart failure and/or cardiac tamponade
		(2) Effusive constrictive pericarditis with evidence of simultaneous compressive pericardial fluid and visceral constrictive pericarditis
Three	Adsorptive stage	Symptoms and signs compatible with constrictive pericarditis but radiological and echocardiographic evidence of thick fibrinous fluid around the heart
Four	Constrictive stage	Symptoms, signs and echocardiography compatible with constrictive pericarditis with no residual fluid in the pericardium

Epidemiology of TBP

Developing countries have a high prevalence and incidence of tuberculosis (TB). South Africa is one of the 30 high burden TB countries that contribute to 87% of TB cases worldwide. Within the setting of the human immunodeficiency virus (HIV) pandemic and poor socioeconomic conditions, there is a large population susceptible to Mtb in South Africa. The HIV co-infection rate among notified TB cases in South Africa was reported to be 60% in the 2019 Global report (WHO 2019). The high prevalence of TB is associated with poor outcomes and increased incidence of extrapulmonary TB.

Estimated TB incidence rates, 2017

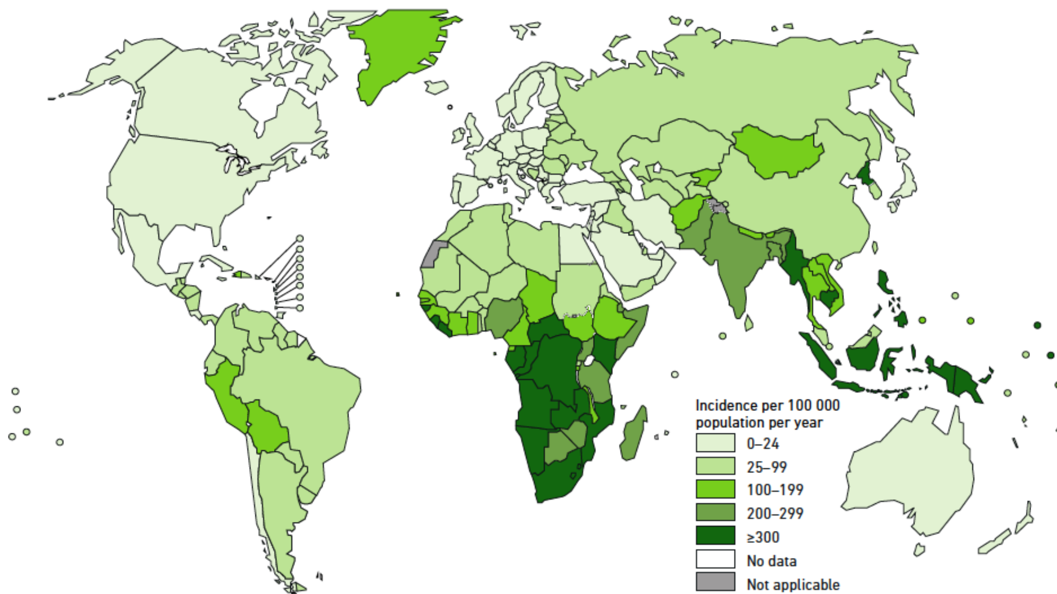

Figure 1-1 World map showing the incidence of TB cases as of 2017. Figure adapted from WHO global tuberculosis report, 2018

TB is the cause of up to 80% of cases with pericardial effusion in TB endemic regions. The effusion is characterised by exudation, leucocytosis, and dominant lymphocytes and monocytes (Ntsekhe and Mayosi 2013). The major complications of TB pericarditis include cardiac tamponade, constrictive pericarditis, and death.

Diagnosis and management of TBP

TB pericarditis is diagnosed using a biomarker-based approach by measuring unstimulated interferon-γ (IFN-γ), adenosine deaminase (ADA), and performing polymerase chain reaction (PCR) on pericardial fluid (Mutyaba and Ntsekhe 2017). The treatment strategy for TB pericarditis is to treat the Mtb pathogen using an anti-TB regimen (rifampicin, isoniazid, pyrazinamide, and ethambutol). Rifampicin and isoniazid have a poor penetration into the pericardium, often necessitating dosage or drug regimen alterations in patients with TB pericarditis (Shenje, Ifeoma Adimora-Nweke et al. 2015).

Constrictive pericarditis

Constrictive pericarditis is characterised by a thick fibrinous exudative pericardial sac compressing the heart (Ntsekhe and Mayosi 2013). Constrictive pericarditis is a life-threatening sequelae of TB pericarditis, occurring in 30 to 60% of patients, regardless of anti-tuberculosis treatment (Ntsekhe, Matthews et al. 2013). Constriction occurs when the pericardium layers lose compliance due to fibrous scarring and calcification and follows from excessive ECM fibrin and collagen deposition.

Although the precise mechanisms underlying the progression from inflammation to constriction are not known, various immune-modulatory cytokines and peptides have been studied to understand the mechanism of fibrosis. For example, significantly higher expression of transforming growth factor-β (TGF-β) is attributed to its major role in ECM deposition through the son of a mother against decapentaplegic (Smad) signalling pathway and fibrosis-related gene expression (Burgess, Reuter et al. 2002, Liu, Tan et al. 2012, Ristić, Pankuweit et al. 2013). A similar pattern of pro-fibrotic cytokines are seen and described in the literature (Figure 1-2)(Ramasamy, Mayosi et al. 2018). Careful studies of these cytokines and other dysregulated proteins in TB pericarditis are likely to herald the identification of potential novel biomarkers of fibrosis.

Background and rationale for Investigating the pathways involved in the progression of fibrosis TBP.

Molecular mechanisms of fibrosis

The precise mechanism that leads from infection to inflammation then fibrosis of the pericardium is unknown. This has led to various immune-modulatory cytokines and peptides being studied to understand the mechanism of fibrosis. In addition, prediction of high-risk TB pericarditis patients who go on to develop tuberculous pericardial

constriction is lacking, showing the need for work to be done to identify deregulated proteins which may be key factors to the mechanism of fibrosis.

Although there has been little progress, to date studies have shown that -inflammatory cytokines are significantly raised in TB pericardial inflammation as compared to viral, malignant or other *in vitro* bacterial causes of pericarditis(Pankuweit, Wädlich et al. 2000). Of interest is tumour necrosis factor-α (TNF-α) which plays a major role in the regulation of inflammation and recruitment of macrophages to the site of infection. Other upregulated cytokines are interferon-γ and interleukin-8 (IL8) (Pankuweit, Wädlich et al. 2000, Burgess, Reuter et al. 2002, Karatolios, Moosdorf et al. 2012, Ntsekhe, Matthews et al. 2013).

A similar pattern is seen in pro-fibrotic cytokines across studies in pericarditis. Significant expression of TGF-β is attributed to its major role in ECM deposition through the son of a mother against decapentaplegic (Smad) signalling pathway and fibrosis-related gene expression (Liu, Tan et al. 2012, Ntsekhe, Matthews et al. 2013, Ristić, Pankuweit et al. 2013).

More recently, a proof of concept study designed to assess the potential role of the major antifibrotic peptide, N-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP) showed decreased Ac-SDKP levels in patients with TBP (Ntsekhe, Matthews et al. 2012).

Ac-SDKP is an immune-modulatory and pro-angiogenic peptide that is released following the cleavage of thymosin-β4 by meprin-α and prolyl-oligopeptidase. Ac-SDKP is present in plasma and mononuclear cells and is ubiquitously distributed *in vivo*. Ac-SDKP may inhibit cardiac fibroblast proliferation (Rhaleb, Peng et al. 2001), mesangial cell proliferation (Castoldi, di Gioia et al. 2013), and epithelial cell to mesenchymal cell transition through the inhibition of the TGF-β/ Smad signalling pathway, thus preventing inflammation and fibrosis (Rhaleb, Peng et al. 2001, Ntsekhe, Matthews et al. 2012, Castoldi, di Gioia et al. 2013, Kumar and Yin 2018).

Ac-SDKP is preferentially hydrolysed to inactive fragments by the N-domain catalytic site angiotensin-converting enzyme (ACE). ACE produced in macrophages and phagocytes, and levels of ACE are higher under inflammatory conditions (Okamura, Rakugi et al. 1999). ACE is a key enzyme of the renin-angiotensin system (RAS) which

in addition to its role in blood pressure and electrolyte regulation, has well-described roles in the fibrotic process.

The RAA system

The renin-angiotensin aldosterone system (RAAS) is traditionally known for its role in blood pressure and electrolyte regulation through the activation of the classical RAS pathway (Figure 1-2). The pathway starts with the production of renin from the kidneys which cleaves the N-terminal peptide angiotensin I (Ang I) from the liver prohormone angiotensinogen (AGT)(Inagami 1994). Ang I is then converted by ACE to angiotensin II (Ang II), a potent vasoconstrictor (Skeggs, Marsh et al. 1954, Skeggs, Kahn et al. 1956). Ang II exerts its effects by acting on angiotensin type 1 and 2 receptors (AT_1R and AT_2R). AT_1R activation results in vasoconstriction, aldosterone and anti-diuretic hormone release, and central nervous system stimulation for blood pressure regulation (Timmermans, Benfield et al. 1992). AT_2R activation counters the effects mediated by AT_1R; however, AT_2R is only abundant during fetal development (Lazard, Briend-Sutren et al. 1994, Horiuchi 1996, Santos, Oudit et al. 2019).

1.4.1 RAAS in fibrosis

Ang II, the main effector peptide of the RAS, is a profibrotic peptide that is involved in pathologic fibrosis in diseased organs (Weber, Brilla et al. 1993, Brilla, Zhou et al. 1994). Ang II directly acts on fibroblasts inducing fibroblast proliferation and collagen synthesis in a dose-dependent manner through the activation of the canonical and non-canonical fibrotic pathways which ultimately result in TGF-β translation, the major profibrotic cytokine (Bader, Peters et al. 2001, Morihara, Takai et al. 2006).

Angiotensin converting enzyme 2 (ACE 2) converts Ang I to Angiotensin 1-9 (Ang 1-9) through the hydrolysis of the carboxy terminal leucine from Ang I (Donoghue, Hsieh et al. 2000). ACE2 also cleaves a single leucine residue from the carboxyl terminal of Ang II converting it to the heptapeptide Angiotensin 1-7 (Ang 1-7) (Ferrario 2006). Ang 1-7 has opposing effects to those of Ang II which it by binding to Mas receptors (MasR) thus mediating vasodilatory, anti-hyperproliferative and antifibrotic effects (Ferrario 2006, Ferreira, Shenoy et al. 2011).

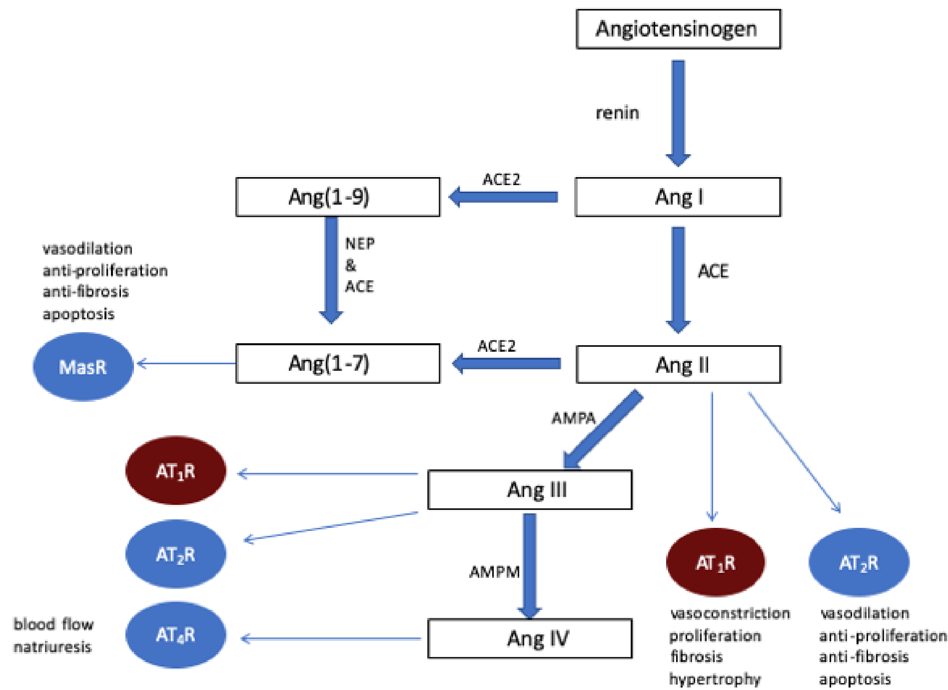

Figure 1-2 The Renin-angiotensin system,. Neutral endopeptidase (NEP); aminopeptidase (AMPA). Adapted from Fyhquist and Saijonmaa 2008.

Angiotensin-converting enzyme inhibitors in fibrosis

ACE inhibitors (ACEi) are a first-line therapy for the management of hypertension. Additionally, there is an increasing body of evidence to support the therapeutic protective roles of ACEi in fibrosis. Antifibrotic ACEi effects have been seen in myocardial fibrosis (Brilla, Rupp et al. 2003), diabetic nephropathy (Sharma, Eltayeb et al. 1999), pulmonary fibrosis (Kreuter, Lederer et al. 2019) and muscular dystrophies (Silva, Magalhães et al. 2017) with functional analysis showing improved organ function, which is associated with improved prognosis. Molecular studies of the fibrotic pathway under ACE inhibition have shown results of decreased Smad protein phosphorylation and MAP kinase activation (Fang, Wang et al. 2018). ACEi simultaneously inhibit the signal transduction pathway of TGF-β (Kuno, Yamada et al. 2003).

In addition to the inhibition of Ang II production, ACEi increase Ac-SDKP levels by inhibiting its hydrolysis (Mnguni, Engel et al. 2015). Elevated levels of Ac-SDKP have been associated with antifibrotic effects in numerous studies (Azizi, Rousseau et al. 1996, Rasoul, Carretero et al. 2004, Peng, Carretero et al. 2005). ACEi also increase Ang I, which is converted to Ang 1-7 by ACE2. Ang 1-7 upregulation by ACEi has been suggested to have antifibrotic effects (Kocks, Lely et al. 2005, Ferreira, Shenoy et al. 2011). However, it is not known whether ACEi penetrate the pericardium, which warrant further investigation.

This antifibrotic effect of ACEi has not been studied in the pericardium and pericardial tissue fibrosis which would be required to determine whether ACEi can be used as potential antifibrotics in TBP. In addition, understanding the mechanisms by which ACEi act to inhibit and reverse fibrosis could open avenues for the development of diagnostic tools and therapeutic strategies in patients with constrictive pericarditis to reduce the frequency of pericardiotomies, thus reducing the associated morbidity.

In summary, Mtb is the most frequent cause of pericardial diseases in Sub-Saharan Africa with complicated sequalae that are associated with high morbidity and mortality (tamponade and constrictive pericarditis) and poor treatment options once these complications occur. The only definite treatment for tuberculous constriction is a pericardiectomy, the surgical removal of the pericardium. This surgical procedure is not widely available to the patients who need it. Moreover, pericardiectomy has a reported perioperative mortality rate of 14%(Mutyaba, Balkaran et al. 2014)

Given preliminary data which suggests the presence of significant Ang II and ACE activity in the pericardial fluid but not serum of TBP patients (own, unpublished), it is likely that RAAS dysregulation and RAAS related pathway may play a significant mechanistic role in post TBP fibrosis. better understanding of RAAS in TBP progression is needed particularly given the potential for ACEi use antifibrotic agents in TBP.

This lays the foundation for the investigation of changes at a proteomic level in patients with TBP. Mass spectrometry-based discovery proteomics allows for the identification of deregulated peptides in TBP to identify drivers of the mechanisms leading to constriction. Through protein and protein network identification and

quantification, understanding the events leading up to pericardial scarring can prove beneficial in understanding the mechanisms involved and the identification of potential therapeutic interventions for pericardial constriction.

Lastly, studying the possibility of the presence of citrullination aims to open avenues to determine whether there exists an autoimmune component in the pathogenesis of tuberculous pericardial constriction.

Protein citrullination is a post-translational modification that occurs when the enzyme, protein arginine deiminase enzyme, PAD2, catalyses the calcium-dependent hydrolytic conversion of peptidyl arginine to peptidyl citrulline (ROGERS and SIMMONDS 1958). The immune system mounts an antibody response to the citrullinated proteins resulting in autoimmunity.

Patients with TB have been found to test positive for anti-citrullinated peptide antibodies (ACPA) antibodies (Lima, Oliveira et al. 2013). In addition, the positive CCP results are caused by TB and not anti-TB treatment which has been shown to induce anti-histone and antinuclear antibodies.

An unchecked auto-antibody component driving constriction might explain the persistent inflammatory and fibrotic events in the pericardium despite the absence of a heavy mycobacterial load. This has resulted in interest to investigate auto-antibody formation in patients with TB pericarditis through the study of protein citrullination.

Aims and objectives.

The overall aims are to identify proteins which could be used as predictive markers for pericardial constriction in high-risk patients with TBP, and changes in the TBP proteome which could warrant potential treatment. This will be achieved through the following objectives:

1. Perform discovery proteomic analysis of TB pericardial cell free fluid (PCFF) to identify proteins involved in the pathophysiological processes of pericardial fibrosis by:
    a) Immunoaffinity depletion of proteins such as such as albumins and immunoglobulins to identify less-abundant proteins.

    b) Functional pathway analysis to identify fibrosis-specific processes.

2. To investigate dysregulation of RAS metabolites in TBP using mass spectrometry-based RAS fingerprinting.
3. To conduct a systematic review to investigate the antifibrotic potential of ACEi in myocardial fibrosis.
4. To determine the levels of citrullinated proteins in TBP patients by
    a. autoantibody detection in citrullinated protein microarray technology.

2. Proteomic analysis of TBP pericardial fluid

2.1 Background

2.1.1 Discovery proteomics

Discovery proteomics, untargeted proteomics, is the use of liquid chromatography/tandem mass spectrometry (LC-MS/MS) to comprehensively identify proteins in a sample without prior knowledge of what proteins may be present. The complex biology of the proteome is traditionally analysed with various technical disciplines, such as microscopy and antibody-based techniques such as western blotting. However, these methods are often limited by the complexity of the proteome, low-abundance proteins, and the dynamic state of the proteins within any given system. This highlights the need for proteome analysis with highly sensitive techniques (Aebersold and Mann 2003).

Mass spectrometry (MS) is the method of choice for the analysis of the proteome. MS-based technologies can identify and quantify thousands of proteins, enabling the study of their composition, structure, function, and control (Aebersold and Mann 2003). MS-based techniques have thus played a significant role in understanding and predicting the individual nature of disease progression, drug response, biomarker discovery and diagnostics.

Mass spectrometers consist of three components; an ion source to produce gas phase ions, a mass analyser that separates the ions according to their mass-charge ratio (m/z) and an ion detector that records the ions per their m/z value (Aebersold and Mann 2003).

Two methods of ionisation are widely used in MS, namely Matrix-Assisted Laser Desorption Ionisation (MALDI) and Electrospray Ionisation (ESI). ESI, used in this experiment, requires the sample to be liquid form and is subsequently transferred to a gas phase. Thus, ESI is often coupled to liquid chromatography (LC). The buffer containing the peptides is sprayed from an electrospray needle with a high voltage (2-5kV) releasing droplets containing solvated ions towards the MS. The ions, once exposed to the environment, evaporate, and become increasingly unstable due to increasing density of positively charged ions in one droplet. This results in coulombic explosions ultimately resulting in smaller droplets, this process continues at an

exponential rate until the ions reach the analyser in the gas phase (Konermann, Ahadi et al. 2013).

The orbitrap mass analyser (Fig. 2-1), maintains ions in orbital motion due to electrostatic attraction between an outer barrel and an inner electrode. The ions cycle around the inner electrode on elliptical trajectories (Scigelova and Makarov 2006). The oscillating ions induce an image current that are simultaneously detected by a differential amplifier in the outer electrode (Hu, Noll et al. 2005).

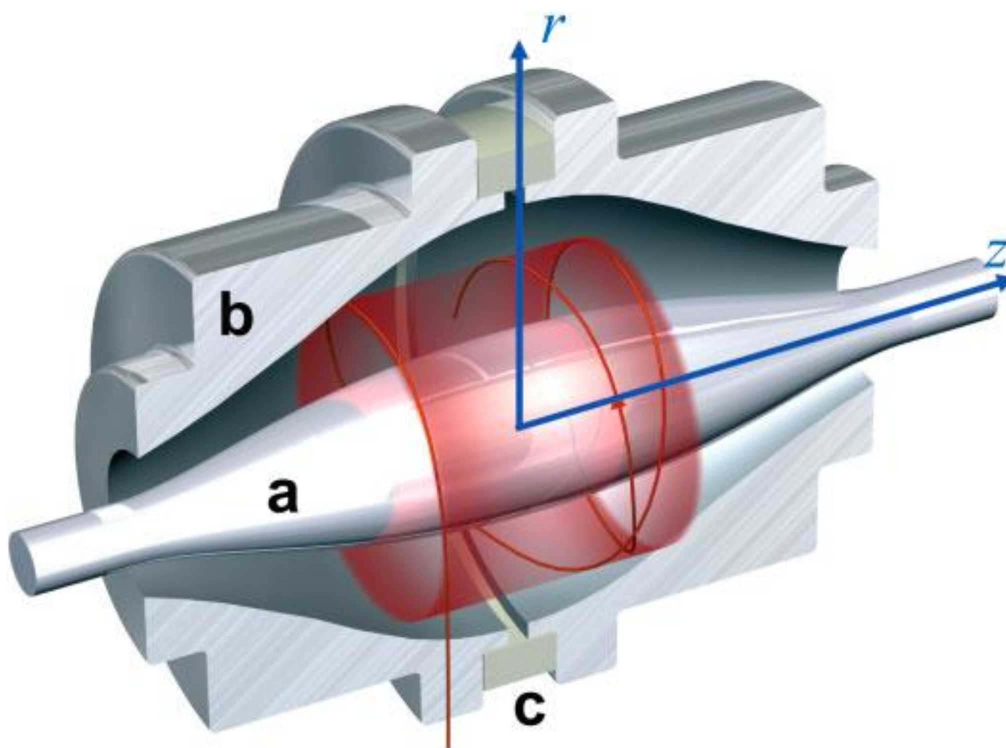

Figure 2-1 Orbitrap mass analyser. ***Ions move around the central electrode (a). the outer electrode (b) is split by an insulating ceramic ring (c) which has a differential amplifier for the detection of induced image currents (adapted from Scigelova and Makarov 2006).***

Two commonly used MS-based methods are top-down and bottom-up proteomics. Top-down proteomics involves the analysis of proteins as intact entities (Tran, Zamdborg et al. 2011). In this work, a bottom-up proteomics workflow was adapted (Fig 2-2) which involves the analysis of protein-derived peptides. Bottom-up

proteomics experiments tend to be more reproducible and capable of higher throughput than top-down experiments. As a result, bottom-up proteomics are more frequently used in mass spectrometry-based proteomics studies (Aebersold and Mann 2016).

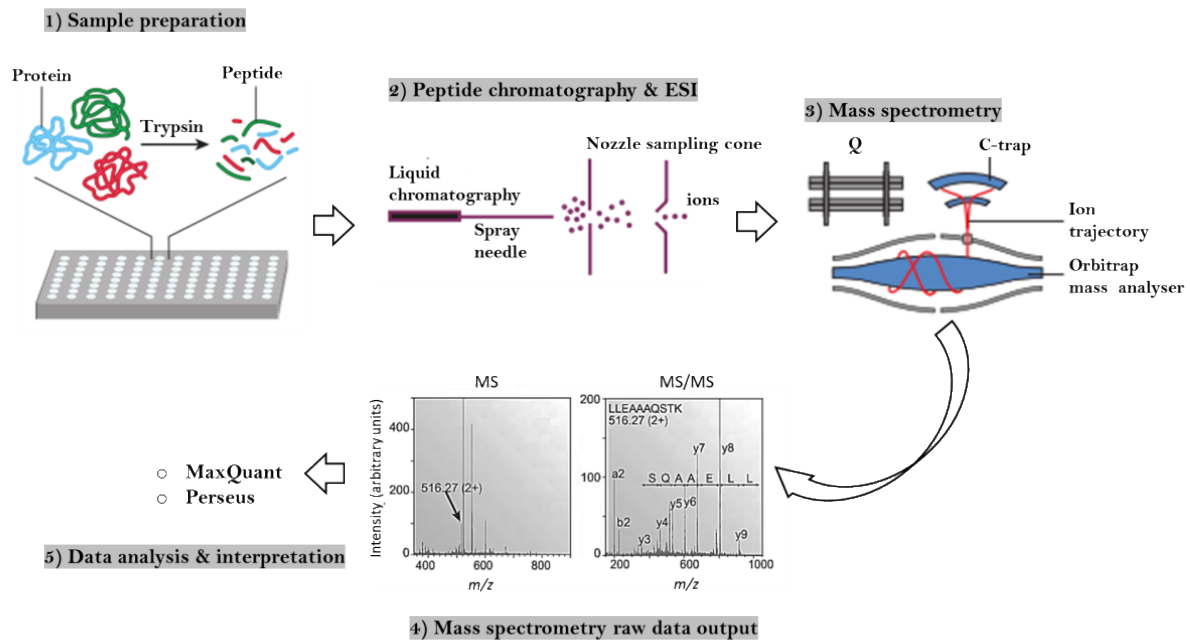

Figure 2-2 General proteomics workflow ***[adapted from (Aebersold and Mann 2003; Aebersold and Mann 2016)]***

Study objectives

Mass spectrometry-based discovery proteomics allows for the identification of deregulated peptides in TBP to identify drivers of fibrosis by:

- Performing discovery proteomic analysis of TB pericardial cell free fluid (PCFF) to identify proteins involved in the pathophysiological processes of pericardial fibrosis by:
    c) Immunoaffinity depletion of proteins such as such as albumins and immunoglobulins to identify low-abundance proteins
    d) Functional pathway analysis to identify fibrosis-specific processes, and protein and protein network identification and quantification, towards a more complete understanding of the events leading up to pericardial scarring.

2.3 Materials and methods

Patient recruitment

For the purpose of this study, participants with TBP were recruited from the IMPI TBP REGISTRY- a prospective study of patients with TBP referred to Groote Schuur Hospital designed to investigate the microbiological, immunological, molecular and other mechanisms involved in its complications. Control samples were obtained from patients without TB who underwent cardiac surgery for coronary artery disease. The study has ethical approval under the license HREC REF NO: RO36/2019.

Sample collection and processing

TB pericardial fluid samples were collected during pericardiocentesis at Groote Schuur Hospital (GSH) and centrifuged in a biosafety level 3 facility to obtain pericardial cell-free fluid (PCFF). PCFF was filtered, then cryopreserved at -80°C until further use.

Sample preparation

Protein Quantification

A Bradford colorimetric assay was used for protein quantification. Briefly, 5 µl of PCFF samples were added to 250 µl diluted dye reagent (1 in 4 dilution) (Bio-Rad) and incubated in a 96 well-plate in triplicate at room temperature for 5 minutes. The absorbance was measured at 595 nm in a plate reader. A bovine serum albumin (BSA) standard curve was generated to convert absorbance into micrograms per microlitre (µg/µl).

10 diseased (TBP) and 10 control samples were prepared for MS analysis (Fig 2-3). 5 from the diseased group, and 5 from the control group underwent depletion.

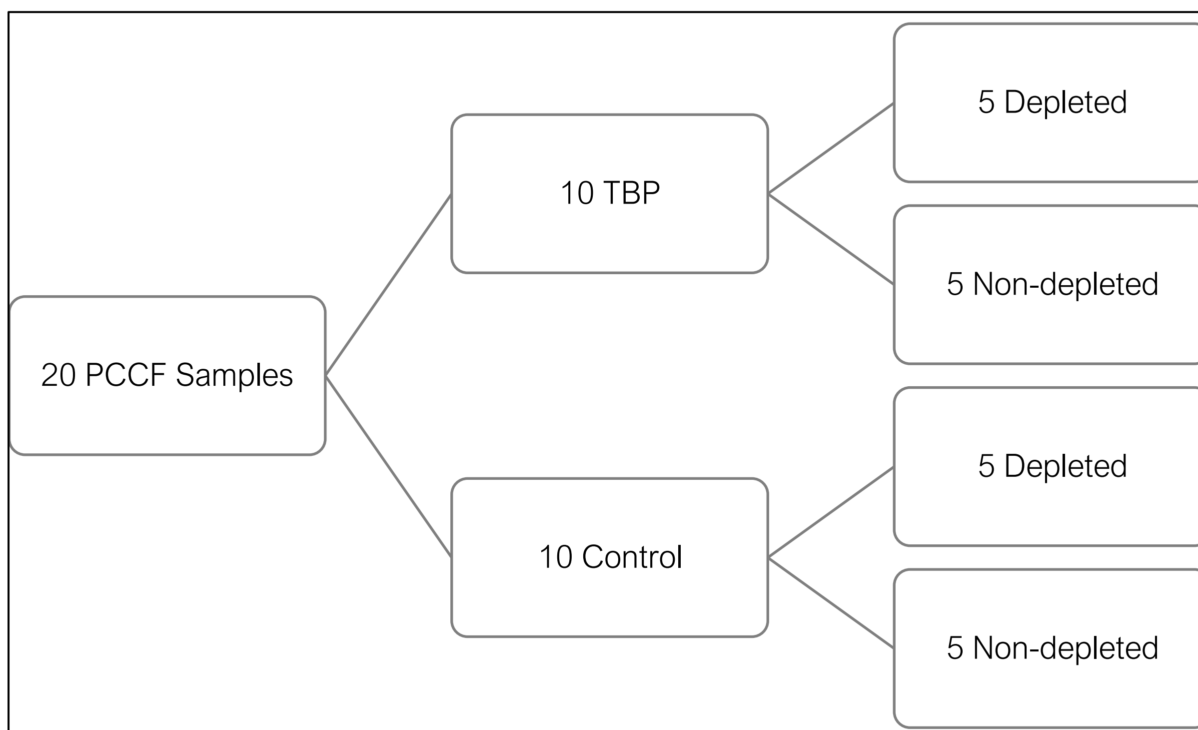

Figure 2-3 PCFF MS preparation workflow

Depletion of highly abundant proteins was performed using Thermo Scientific High Select^{TM} Top14 Abundant Protein Depletion Mini Spin Columns. The depletion columns use immobilised antibodies to remove human serum albumin, albumin, IgG, IgA, IgM, IgD, IgE, kappa and lambda light chains, alpha-1-1acidgylcoprotein, alpha-1-antitrypsin, alpha2-macroglobulin, apolipoprotein A1, fibrinogen, haptoglobin, and transferrin from serum and plasma. Aliquots of 400 µg of protein were added to the columns for depletion, incubated for 10 minutes, and then centrifuged at 14000 x *g* for 1 minute.

In-solution digestion

Prior to in-solution digestion, the depleted PCFF samples were quantified by Bradford assay, and protein precipitation was performed by adding 120µl of ice-cold acetone to a 30 µl of depleted PCFF sample, and incubating on ice for 60 minutes. Samples were then centrifuged at 14 500 x *g*, 4˚C for 30 min. The supernatant was discarded, and the precipitate was retained for tryptic digest.

Mass spectrometry

LC-MS/MS was performed on a Q-Exactive Quadrupole Orbitrap mass spectrometer (Thermo Fischer Scientific) coupled to a Dionex ultra HPLC (Thermo Fischer Scientific). The samples were loaded on a 30 cm C18 analytical column (packed in-house, 3.6µm beads Aeris C18 packing material) and eluted during a 120-minute gradient (6-40% of 95% ACN and 0.1% FA) at a constant flowrate of 300nl/min. The peptides were ionised by ESI (~2.5 kV) prior to entering the mass spectrometer. Each sample run was preceded by a 30-minute wash cycle. The full mass spectrometry parameters are summarized in Table 2-1.

Table 2-1 Mass spectrometry parameter settings

MS LEVEL	PARAMETER	SETTINGS
MS1	Run time	120 minutes
	Polarity	positive
	Default charge state	2
	Scan range	300-1750 m/z
	Resolution	70000
	Automatic gain control target	3e6
	Maximum injection time	250ms
MS2	Resolution	17500
	Automatic gain control target	5e4
	Maximum injection time	80ms
	TopN	10
	Isolation window	2 m/z
	Scan range	200-2000 m/z
	Intensity threshold	6.3e3

	Dynamic exclusion	30s

Data processing and analysis

The raw data files were analysed in MaxQuant (version 1.5.7.4) using MaxQuant's in-built search engine, Andromeda by searching against the Human Uniprot 2019 database for protein identification and label free quantification. The raw files were analysed simultaneously as separate experiments on MaxQuant. MaxQuant settings were on default, and the datasets generated were filtered by mapping to a reverse decoy database with variable oxidation modifications (M) and Acetyl (protein N-term) and a fixed modification of carbamidomethyl (C) with a fixed discovery rate (FDR) set at 1%. For quantification, only unique peptides were selected to improve the confidence of identified proteins. The data was then analysed statistically with Perseus (version 1.6.1.3).

Functional enrichment and interaction analysis

Peptides that were identified as contaminants, reverse hits, or only identified by site were excluded using Perseus. To assess the biological significance of the resulting identified proteins, Panther and STRING-DB were used to determine protein interactions and perform gene ontology (GO) analysis.

2.4 Results

Protein extraction

Previous work in our lab had applied different protein precipitation methods to three PCFF samples to optimise the extraction for subsequent proteomic analysis. These methods included acetone, methanol-chloroform, TCA and a modified ACN method for protein precipitation. The protein concentrations were then determined with a Bradford assay. Figure 2-3 shows the comparison of the protein concentrations achieved using the different methods on three PCFF samples. Each method, except TCA precipitation, yielded a comparable amount of protein in each of the three samples (labelled PCFF1, PCFF2 and PCFF3). TCA precipitation resulted in poor yields. Acetone precipitation yielded the most protein(2.5mg/ml) and was therefore chosen for subsequent proteomic analyses. The other precipitation methods had poorer resuspension in water, particularly the TCA method, which may have contributed to the observed lower protein yields.

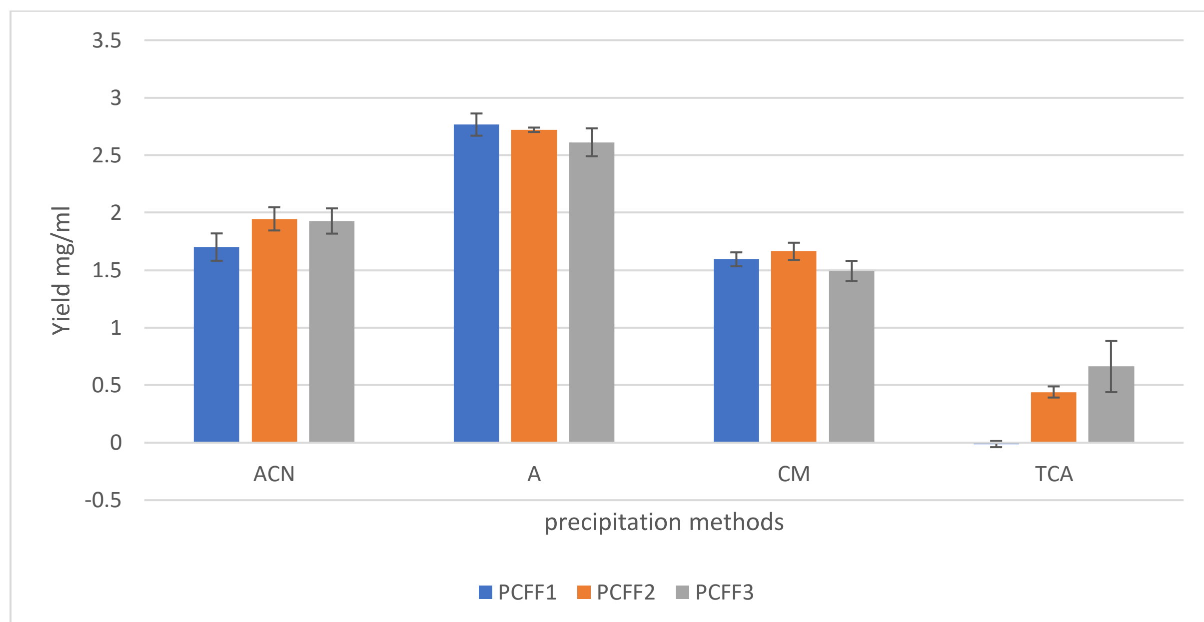

Figure 2-4Comparison of protein precipitation methods. ACN- modified acetonitrile, A- acetone, CM- chloroform methanol, TCA- trichloroacetic acid.

PCCF MS data analysis

PCFF samples were precipitated using acetone, 10 TBP and 10 control. 10 PCCF samples, then prepared for proteomic analysis using the in solution tryptic digest protocol, and analysed with a Q-Exactive mass spectrometer.

After analysing 15 PCCF samples,127066 MS /MS spectra were submitted and 90010 (70.8%) were identified by mapping to the human Uniprot protein database. This corresponded to the identification of 180 protein groups. There was a low percentage of matches to the decoy database (0.56% of the total protein groups) and potential contaminants (4,44% of total protein groups), indicative of good data.

Data analysis and processing were carried out in Perseus for comparison of the identified proteins from the different PCFF samples. Principal component analysis demonstrated that this pericardial fluid proteome could distinguish between controls and patients with TBP (Fig 2-5).

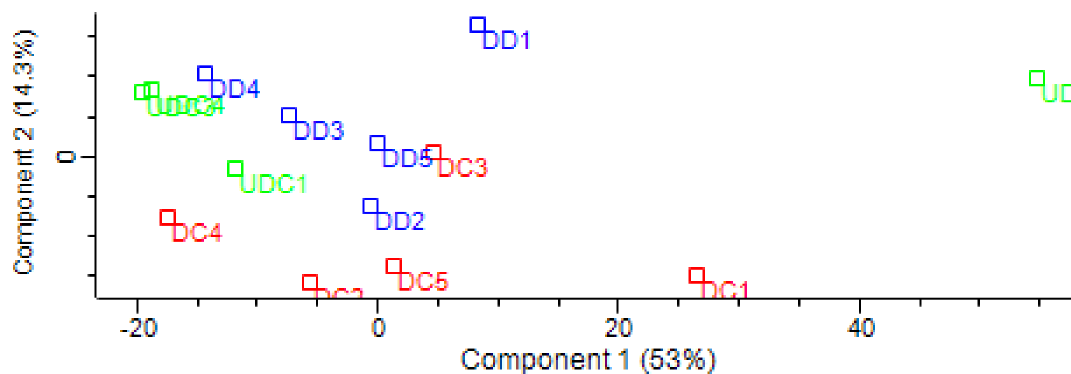

Figure 2-5 Principal Component Analysis (PCA) of PCCF samples. DD- Depleted diseased. DC- Depleted control. UDC- Undepleted control.

Fig 2-5 shows the efficacy of the depletion as the samples are not clustered together as previously seen, and additionally seen in the undepleted samples compared to the depleted samples. Further, the top 5 proteins post depletion are metalloprotease inhibitor 1, zinc finger protein 587B, Ig alpha-1 chain C region, complement factor H, and Coagulation factor XII. The top 5 proteins ae Ig Kappa chain C region, serum albumin, Ig gamma-1 chain C, alpha-1-antitrypsin, and apolipoprotein A-I. T

The identified protein groups from the disease arm were analysed using Panther and STRING-DB to identify the enriched GO terms associated with specific cellular components and biological processes (Table 2-2 and 3). The Interpro tool was used to classify the data into protein families. The sushi/SCR/CCP superfamily, membrane

attack complex (MAC), and serpin superfamily were enriched in the TBP patient samples, implying that these pathways are active in the pathophysiology of TBP. Disease enrichment analysis in Panther did not identify pericarditis as the pathology from which the data was sourced. The proteins APOH, APOB, TTR, APOE, ALB, LPA, RYR2, and SERPINC1 associated with cardiovascular disease.

Table 2-2 Gene ontology analysis showing the enriched cellular component terms of significantly expressed proteins in the pericardial fluid of patients with TBP.

GO ID	Cellular component	Identified proteins (p<0.01)
GO:0062023	Collagen-containing extracellular matrix	APOH,TIMP1,APOC3,HRG,APOE,SERPINF1,ORM1,LGALS3BP,A1BG,HPX,LUM,ITIH1,SERPING1,AZGP1,C1QB,SERPINF2,A2M,FBLN1,FGG,APOA4,ITIH2,SERPINC1,C1QC,CST3,ORM2,TGFBI
GO:0005577	Fibrinogen complex	SERPINF2,FGG
GO:0005604	Basement membrane	TIMP1,SERPINF1,FBLN1,CST3,TGFBI

Table 2-3 Gene ontology analysis showing the biological processes of the significantly expressed proteins in the pericardial fluid of patients with TBP.

GO ID		
GO:0006954	Inflammatory response	LBP,TIMP1,ORM1,SAA4,SERPINF2,HP,SERPINC1,CD5L,ORM2
GO:0006959	Humoral immune response	C8G,HRG,IGJ,C6,SERPING1,C1QB,CFHR1,C7,ITLN1,PGLYRP2,C8A,C4BPA,CFH,C1QC,CFB
GO:0042060	Wound healing	APOH,SERPIND1,TIMP1,HRG,SERPING1,A2M,FBLN1,FGG,SERPINC1,HBD

GO:0030198	Extracellular matrix organization	TIMP1,TTR,LUM,SERPINF2,A2M,FBLN1,FGG,TGFBI
GO:0071634	Regulation of transforming growth factor beta production	LUM,SERPINF2,FBLN1

A volcano plot was done to provide visual identification of proteins with large fold changes that are also statistically significant using a volcano plot (Fig 2-6). Figure 2-7 shows the heatmap of the significant proteins from the volcano plot. The disease group shows high heat signals from ceruloplasmin, Ig alpha-2- chain C region, Alpha-2-macroglobulin (A2M), Ig heavy chain V-III, and Ig kappa V-III region VG as seen in Figure 2-6.

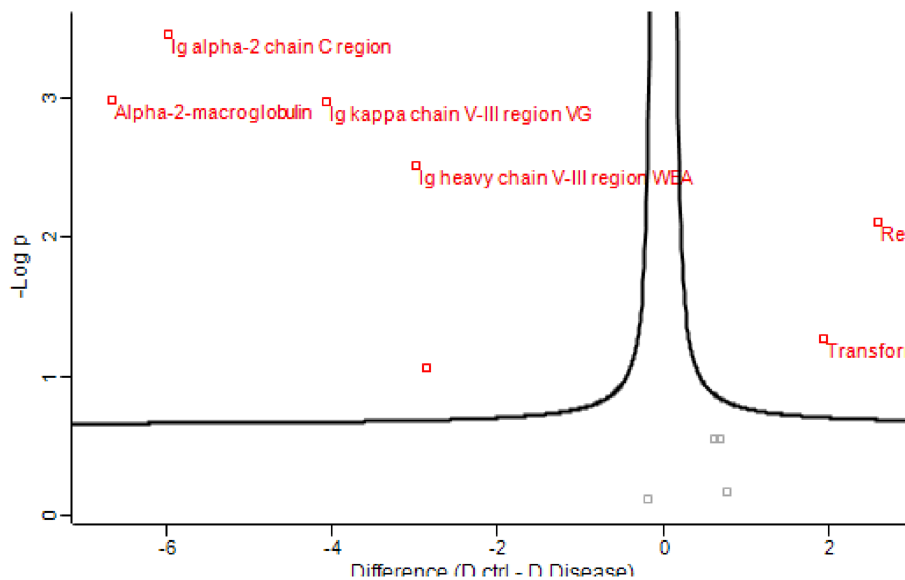

Figure 2-6 Volcano plot of proteins with large fold changes between control and disease.

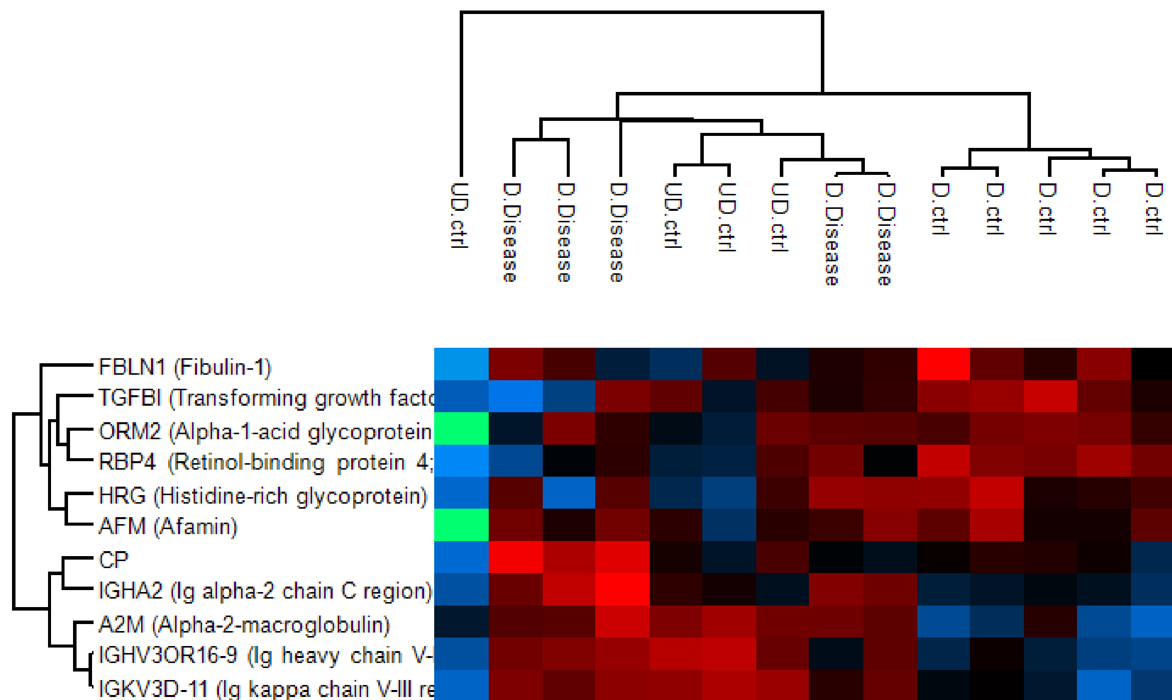

Figure 2-7 Heatmap of the significant proteins between the disease and control (ctrl) group. UD- undepleted. D-depleted. CP- ceruloplasmin.

2.5 Discussion

To the best of our knowledge, this is the first study to perform discovery proteomics on pericardial fluid from patients with TBP. Proteins are attractive diagnostic markers given their phenotypic relevance and stability. Discovery proteomics was applied to pericardial fluid in this study to explore in the deregulated proteins associated with TB pericarditis. This is, to the best of our knowledge, the first study to perform discovery proteomics on pericardial fluid from TB pericarditis patients. Potential disease biomarkers are likely to be enriched in proximal fluids such as pericardial fluid, as it serves as a 'sink' into which proteins are secreted in pericarditis. Thus, direct analysis of the proximal fluid offers a unique opportunity for the identification of novel biomarkers. Furthermore, a protein present in proximal fluids can serve as a surrogate for its availability in systemic circulation (Rifai, Gillette et al. 2006). In the present study, we identified 180 proteins in pericardial fluid and subjected these to functional enrichment analysis to gain understanding of the molecular basis of TBP.

Interestingly, disease enrichment of the data did not identify pericarditis as one of the pathologies from which the data could have been extrapolated. This speaks to the paucity of data on the TBP proteome. KEGG enrichment analysis of the data from the disease group showed that lipid metabolism and the MAC were enriched. The role of lipids and cholesterol in TB immunopathology is poorly characterised. However, cholesterol uptake and catabolism are central to the maintenance of Mtb in the host, contributing to pathogenesis and virulence.

Pulmonary TB granulomas enable Mtb progression to continue despite a robust host immune response. One of the important mechanisms for this survival despite a strong anti-mycobacterial immune response is through pathogen-induced dysregulation of host lipid synthesis (Russell, Cardona et al. 2009). Mtb bacilli are harboured in foamy macrophages within necrotic lesions (Peyron, Vaubourgeix et al. 2008). Foamy macrophages are characterised by lipid bodies that are generated via the dysregulation of host lipid synthesis (influx and efflux of low density lipoprotein particles in plasma), resulting in the conversion of macrophages to foam cells (Galkina and Ley 2009).

Enrichment analysis also showed the serine proteinase (serpin) family is strongly expressed family of proteins that are activators of the acute phase and inflammatory response in TB (Song, Han et al. 2014), and play a critical role in the development of fibrosis (Jiang, Liu et al. 2017). In fibrotic lung disease, it is postulated that the fibrosis develops because of continuous insults to alveolar type II cells, resulting in the activation of myofibroblasts with resultant fibrosis. Studies in serpin-1 knockout animal models have shown a decrease in collagen deposition, hydroxyproline accumulation, and procollagen expression (Jiang, Liu et al. 2017). Of interest, ACEi exert their antifibrotic actions through an increase in fibrinolysis and ECM degradation, which are mediated by serpin-1 expression inhibition. (Oikawa, Freeman et al. 1997)

Ceruloplasmin (CP) is an acute-phase plasma protein activated by monocytes and macrophages. The plasma levels of ceruloplasmin doubles in response to inflammation, trauma, and infectio**n (Bakhautdin, Febbraio et al. 2013)**. CP is an indicator of systemic inflammation in systemic inflammation in irritable bowel syndrome (Bilooka, Fediv et al. 2021). Additionally, CP can be used to significant fibrosis, advanced fibrosis, and cirrhosis in patient with hepatitis B viral infection {Kang, 2020, Serum ceruloplasmin can predict liver fibrosis in hepatitis B virus-infected

patients.. The presence of ceruloplasmin in pericardial fluid suggests that TBP can be a disease profile onto which ceruloplasmin can be added as a non-specific indicator of fibrosis.

Specific and high-throughput Immunoassays can be used to isolate and quantify CP and other proteins of interest use of antibodies to identify and measure pericardial CP levels in patients with TBP at the different stages of the pathogenesis to assess the levels of CP or other protein of interest to disease progression.

A2M is reported in serum samples of TB patients. It is a positive acute phase protein and a well described carrier protein that binds to numerous growth factor and cytokines. A2M is one of the three most differentially expressed proteins in latent pulmonary TB identified by MS analysis compared to healthy controls. A2M expression is further increases two-fold in patients who develop active disease from latent TB{Bapat, 2015, Differential Levels of Alpha-2-Macroglobulin`, Haptoglobin and Sero-Transferrin as Adjunct Markers for TB Diagnosis and Disease Progression in the Malnourished Tribal Population of Melghat`, India}.

Biochemical interaction studies show that A2M have a strong binding affinity for TGF-β1 (K_D= 80 ± nM). A2M also has affinity for fibroblast growth factor (FGF) (KD= 0.59 ± 0.04 μM)(Vandooren and Itoh 2021).

A2M is also a major IL-1β binding carrier protein. Along with TGF-β1, IL-1β is a proposed role player in the mechanism of pericardial constriction. IL-1β mediates fibroblast activation, and increased IL-1β is linked to reduced Ac-SDKP. This culminates in pericardial fibrosis(Howlett, Du Bruyn et al. 2020).

In Diabetic kidney disease, A2M initiates a profibrotic cascade mediated by the Akt signalling pathway, and TGF- β1. TGF- β1 production is also dependant on A2M activation in patients with established diabetic kidney disease. This results in an overexpression of ECM with resultant kidney fibrosis. A2M gene knockout with small interfering RNA (siRNA) inhibits upregulation of fibronectin, collagen IV, the profibrotic cytokine CTGF, and Akt activation(Trink, Li et al. 2021).

It is thus important to further study the potential role of A2M in TBP to determine its role in the pathophysiology of TBP. Additionally, the inhibition of A2M using SiRNA with subsequent studies of the effects on TGF-β1, IL-1, and Ac-SDKP. Identification

of a significant role of A2M in the progression of fibrosis in TBP would provide additional work to the field of TBP diagnostics and therapeutics.

The main limitation of proteomics is the dominance of the 20 most abundant proteins in some biological fluids, which can present up to 95% of the proteome when analysed by mass spectrometry. These highly abundant proteins mask low abundance proteins, which makes it difficult to determine their potential biological and clinical relevance. Depletion of these abundant proteins is currently recommended to circumvent this issue. However, depletion may result in the inadvertent loss of proteins that are bound to the abundant proteins, which may bias the results of the subsequent analysis.

Future work should include the optimisation of methodology for post-depletion LC-MS/MS analysis of depleted proteins to further characterise the depleted proteins (Garbis, Roumeliotis et al. 2011).

There main findings from this study are the addition of established fibrotic markers that have not yet been described in the pathophysiology of TBP.

The enrichment analysis confirms findings that the pathophysiology of TBP towards constriction is the result of the interplay between the innate and humoral immune systems complicated by exaggerated wound healing from the TIMP1,TTR,LUM,SERPINF2,A2M,FBLN1,FGG, and TGFBI pathways

Depletion aims to enrich the less abundant analytes before MS analysis using affinity-based techniques to remove the most abundant proteins comprising 90-99% of the total protein content to allow a deeper exploration into the pericardial fluid (Adkins, Varnum et al. 2002, Callesen, Madsen et al. 2009), but an alternative strategy might be multidimensional liquid chromatographic hyper fractionation, which would not cause the same issue of potential protein loss.

Enrichment-based methods fractionate serum samples prior to proteomic analysis to increase the chances of identifying low-abundance proteins. Enrichment uses affinity ligands to isolate and enrich proteins of a particular subclass for characterization (Trindade, Bastos et al. 2017). These alternative strategies are expected to enable deeper coverage of the pericardial proteome, which could identify novel biomarkers or diagnostic tools for TBP or fibrosis.

3. The antifibrotic potential of ACEi: A systematic review

Background

Fibrosis of the myocardium and pericardium, occurs as a result of an exaggerated response to tissue injury or damage(Matthews, Deffur et al. 2015, Ramasamy, Mayosi et al. 2018, Zile, O'Meara et al. 2019). The renin-angiotensin-aldosterone system (RAAS) is an implicated proponent of the fibrotic response.

In myocardial fibrosis there is an association between increased angiotensin-converting enzyme (ACE) with subsequent upregulation of the angiotensin type 1 and 2 receptors, and the well documented profibrotic RAAS metabolite, angiotensin II (A-II) (Weber, Brilla et al. 1993).

Additionally, an increase in ACE production is observed in inflammatory and granulomatous environments produced by cells of the reticuloendothelial system, contributing to fibrotic changes. More recently, there is evidence that ACE inhibitors block the ACE-mediated hydrolysis of the antifibrotic tetrapeptide N-acetylseryl-aspartyl-lysyl proline (Ac-SDKP) resulting in increased Ac-SDKP plasma levels (Mnguni, Engel et al. 2015). Ac-SDKP reduces fibrosis through reducing TGF-β transcription and Smad phosphorylation, master regulators of extracellular matrix expression(Inoue, Ikemura et al. 2011). This may have implications for a potential role for ACE inhibitors to reduce post tuberculous pericardial fibrosis.

Whether ACE-inhibitors reduce fibrosis to a greater degree compared to other anti-hypertensive agents in patients with hypertension is not known.

TBP (TBP) is associated with high rates of progression to fibrosis(Pasipanodya, Mubanga et al. 2015). TBP is associated with an influx of pro-inflammatory and pro-fibrotic mediators into the pericardial fluid with a resultant reduction in anti-fibrotic compounds such as Ac-SDKP (Ntsekhe, Matthews et al. 2012). That inhibition of Ac-SDKP hydrolysis by ACEi may reduce myocardial or pericardial fibrosis the, suggesting a novel role for ACE inhibitors (ACEi) in preventing pericardial fibrosis post TBP. A systematic review and meta-analysis by Mnguni et.al has shown that ACEi increase levels of Ac-SDKP in healthy humans(Mnguni, Engel et al. 2015).

Study objectives

The antifibrotic effect of ACEi has not been studied in the pericardium and pericardial tissue fibrosis. This further adds motive to pursuing work to determine whether ACEi can be used as a potential antifibrotic in TBP. In addition, looking into the effects of ACEi in fibrosis, how they can act to inhibit, and reverse fibrosis could open avenues for the development of diagnostic tools and therapeutic strategies in patients with constrictive pericarditis to reduce the frequency of pericardiotomies performed, thus the morbidity associated with it.

The objective was to conduct a systematic review, to address the question: in a population of patients with myocardial fibrosis enrolled in randomized controlled trials, do ACEi, compared to other renin-angiotensin-aldosterone antagonists and or placebo, reduce the outcome of myocardial fibrosis as determined by:

i) the composite of biomarkers of fibrosis,
ii) fibrosis as detected by Cardiac Imaging, and
iii) histological evidence of fibrosis.

This review strives to ensure whether there is justification for further research into ACEi as potential treatment modalities in fibrosis and ultimately as a prophylactic drug in constrictive pericarditis as sequelae of TBP.

Methods

Protocol development

Types of studies
Only randomised control trials (RCT) were included. Only studies with human participants were considered regardless of their publication studies or language of publication.

Type of participants
RCT studies with adult patients aged 18 and above with pericardial or myocardial fibrosis as sequelae of cardiovascular disease occurring in adulthood.

Types of interventions

Experimental interventions

The intervention can be any of the ACEi, as monotherapy or part of other interventions.

Comparator interventions

The intervention to be compared to placebo and/or other RAS modulating drugs.

Types of outcome measures

Primary outcome

The effects of ACEi on fibrosis measured as a continuous variable:

- Fibrosis marker levels such as, TGF-Beta, PIIINP, etc.
- Change in the collagen volume fraction (CVF) after ACEi administration (histology)
- Cardiac imagining Echo reflectivity parameters on ultrasound

Search strategy

Two review authors, Siyavuya Fikamva (SF) and Vinasha Ramasamy (VR), independently undertook a comprehensive systematic literature search in the following databases: PubMed, Web of Science, The Cochrane Library, Medline, Embase, ClinicalTrials.gov and the World Health Organisation (WHO) International Clinical Trials Registry Platform (ICTRP) search portal. Only randomised control trials (RCT) were considered. Only human trials were eligible for inclusion. The participants were adults aged 18-65 who were healthy or with myocardial or pericardial fibrosis on inspection. The intervention could be any ACEi, compared with placebo or another other fibrosis modulating drug. Fibrosis as a primary or secondary outcome in the studies deemed them eligible for inclusion. The different markers of fibrosis had to be measured using validated forms of measurement.

The following combination of search terms was used: angiotensin-converting enzyme inhibitors, myocardial fibrosis, human, cardiovascular, human. The search strategy is outlined in Table 3-1. The reference lists of the identified articles were reviewed, and experts undertaking research in the field of ACEI and cardiovascular markers of fibrosis were also consulted. Only randomised control trials on the effect of ACEI on the myocardium were selected.

Selection process

The studies were screened by title in the databases. Studies eligible for potential inclusion from the were added to the automation tool, Rayyan AI for further selection and review. The studies included in Rayyan were further selected based off title, abstract, and lastly full text on Rayyan(Rayyan- Intelligent Systematic Review). This was done independently by two reviewers, SR and VR, through the blinding feature on Rayyan. Reasons for exclusion were added to the excluded studies as per Rayyan automation feature and can be seen on the appendix (appendix reference).

Table 3-1 PubMed search strategy (adapted for use in other databases)

#1	(“Myocardial Fibrosis [MeSH]”)
#2	(“angiotensin converting enzyme inhibitor [MeSH terms]”)
#3	(#1 AND #2) Filters: human

Data extraction and management

Data was extracted independently by two authors, SF and VR, using a standardised data extraction from template from The Cochrane collaboration(Higgins, Thomas et al. 2021). Data were entered into Review Manager statistical software for meta-analysis. Any disagreements on the eligibility of the of articles for inclusion were with MN.

The outcome that was sought were changes in fibrosis following ACEi administration. This assessed through different measurements of fibrosis at various time points. All results were compatible with the outcome. Validated markers of fibrosis were considered including collagen volume fraction, hydroxyproline concentration, cardiac magnetic resonance, plasma procollagen peptide, myocardial fibrosis, aldosterone, sST2, matrix metalloproteases, and galectin-9. The measures of fibrosis were updated as new validated marker of fibrosis discovered during data extraction.

Quality assessment

All articles were appraised independently by two reviewers, SF and VR, for methodological quality in accordance with the methods of the Cochrane

Collaboration (Higgins, Thomas et al. 2021). The articles were assessed for risk of bias based on randomisation, deviation from intended interventions, missing outcome data, measurement of the outcome, selection of the reported results, and overall bias, using the revised tool for assessing risk of bias (RoB2)(Sterne, Savović et al. 2019).

Data Synthesis and analysis

All the relevant articles identified from the search were reviewed for potential inclusion, subject to reading of the abstracts. The full text of the articles were obtained for final evaluation for inclusion as per inclusion criteria. The outcome (i.e. the effect of ACEi on fibrotic markers) was considered as a continuous variable.

Results

Three-hundred and forty-seven papers were identified by the electronic search, of which 40 were removed before screening due to duplication. Thereafter 307 records were screened and 155 papers were excluded based on title and abstract (fig 3-1). A further 139 were excluded following full review of the text, as they were not related to the outcome (n=94), wrong population (n=65), wrong drug (n=58), wrong study design (n=40), wrong publication type (n=19), and language (n=3). Thus, 7 studies met the inclusion criteria; (Brilla, Funck et al. 2000), (Ciulla, Paliotti et al. 2009), (Hayashi, Tsutamoto et al. 2003),(Modena, Aveta et al. 2001), (Silva, Magalhães et al. 2017), and (Zile, O'Meara et al. 2019).

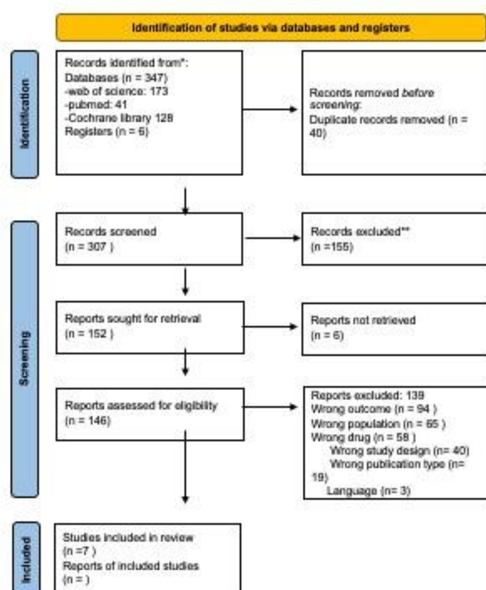

Figure 3-1 Flow diagram of search results.

The included studies are described in table . The reasons for excluding studies that were initially considered relevant are provided in the supplementary material.

Table 3-2 Characteristics of studies included in the review.

Study ID	Participants	Intervention/ Comparator	Diagnostic Method	Outcome
(Brilla, Funck et al. 2000)	35 patients with myocardial fibrosis secondary to primary hypertension	lisinopril (ACEi)(n=18, mean dosage of 11.4±7.2 mg) comparator= hydrochlorothiazide (thiazide) (n=17, Mean dosage of 45.6±9.8mg)	Histology • Collagen volume fraction (%) • Hydroxyproline concentration (µg/mg)	ACE inhibition with lisinopril can regress myocardial fibrosis. No regression of myocardial fibrosis seen with comparator group)
(Ciulla, Paliotti et al. 2009)	84 patients with myocardial fibrosis secondary to primary hypertension	enalapril (n=36, 10-20mg/day) comparator= Candesartan (ARB) (n=48, 8-16mg/day)	Cardiac imagining • Echo reflectivity	Enalapril-based treatment and candesartan treatment induced a moderate but significant reduction in the broadband

				index of myocardial fibrosis.
(Hayashi, Tsutamoto et al. 2003)	134 patients with myocardial fibrosis secondary to acute myocardial infarct	Spironolactone (MRA) 25mg + Enalapril 2.5mg (n=65) Comparator= Enalapril (ACEi)2.5 mg (n=69)	Fibrosis markers • Procollagen type III amino terminal peptide (PIIINP) (pg//ml)	Significant suppression of PIIINP in MRA+ACEi combination than with ACEi alone.
(Modena, Aveta et al. 2001)	46 patients with myocardial fibrosis secondary to acute myocardial infarct	Canreonoate (50mg once daily) (MRA) +ACEi of varying dosages (n=24) Comparator= ACEi (n=22)	Fibrosis markers • PIINP (U/ml)	Canrenoate, combined with an ACEi reduces postinfarction collagen synthesis than with ACEi alone.
(Silva, Magalhães et al. 2017)	42 male patients with myocardial fibrosis secondary to Duchenne Muscular Dystrophy and Becker Muscular Dystrophy	enalapril (ACEi)(n=21, 5-10 mg twice daily) comparator= placebo (n=21)	Histology • Myocardial Fibrosis (MF) mass, g • Fibrosis (% of LV mass)	ACE inhibition was associated with significantly slower progression of MF.
(Zile, O'Meara et al. 2019)	1776 participants with myocardial fibrosis drawn from PARADIGM-HF study	sacubitril/valsartan (Ni/ARB) (n=895, 200mg twice daily) comparator= enalapril (n=881, minimal daily dosage of 10mg, twice daily)	Fibrosis markers • PINP (ng/ml) • PIIINP (ng/ml)	PINP was significantly reduced with sacubitril/valsartan compared with enalapril treatment. There were no statistically significant differences

				between treatment groups with respect to changes in PIIINP.

Change in myocardial fibrosis

Given the high statistical heterogeneity between the studies and variation in outcome measurements (I=), a random effects-model was used to sub analyse outcomes that appeared more than once across the studies.

Three studies, assessed the effect of ACE inhibitor therapy on PIIINP, the marker for collagen synthesis(Modena, Aveta et al. 2001, Hayashi, Tsutamoto et al. 2003, Zile, O'Meara et al. 2019). The administration of ACE inhibitors as monotherapy and in combination with other treatment modalities, was associated with a decrease in PIINP levels

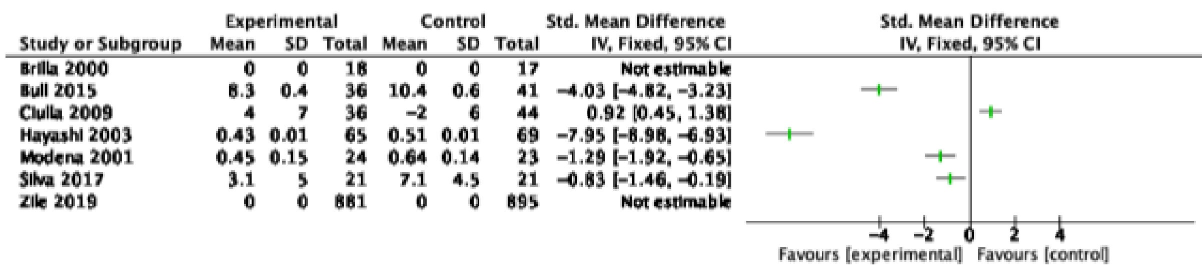

Figure 3-2 Forest plot of included studies

Three studies (1956 participants) assessed the changes in fibrosis by biomarker. Procollagen types II and III is a biomarker used to assess and predict collagen synthesis and turnover. The studies demonstrated a suppression in the procollagens with ACEi administration, used as a monotherapy or in combination with other therapies(Modena, Aveta et al. 2001, Hayashi, Tsutamoto et al. 2003) (. (Zile, O'Meara et al. 2019) was not estimable for the sub analysis due to the data only being available in percentage change.

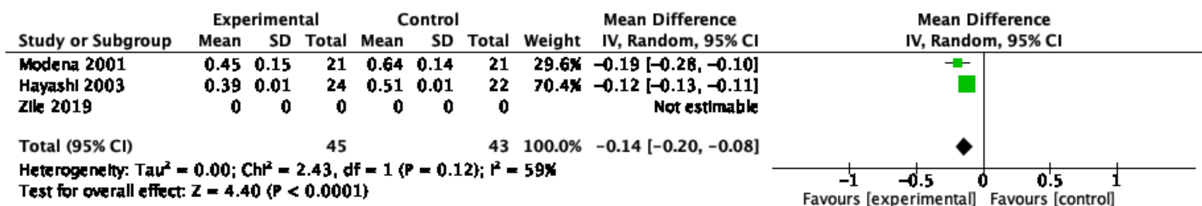

Figure 3-3 PIIINP sub analysis

Two studies (77 participants) assessed the change in fibrosis using a morphometric estimate of the portion of myocardium occupied by fibrous tissue (Brilla, Funck et al. 2000, Silva, Magalhães et al. 2017). Both studies reported a significant regression of myocardial fibrosis following ACE inhibitor treatment. The statistical subgroup analysis notes that there is important heterogeneity across the studies (figure 3-4)

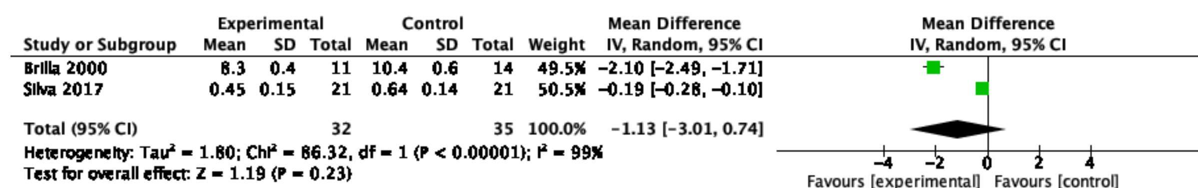

Figure 3-4 Morphometric sub analysis

Methodological quality

Figure 3-5 shows the risk of bias assessment, which includes the components of randomisation process, deviations from intended interventions, missing outcome data, selection of the reported result and overall bias. There were no missing data in any of the studies.

Table 3-3 Risk of bias assessment of included studies.

Study ID	Randomization process	Deviations from intended interventions	Missing outcome data	Measurement of the outcome	Selection of the reported result
(Brilla, Funck et al. 2000)	Low	low	Low	Low	low
(Ciulla, Paliotti et al. 2009)	Low	High	Low	Low	Low
(Hayashi, Tsutamoto et al. 2003)	Some concerns	High	Low	High	Low
(Modena, Aveta et al. 2001)	Some concerns	High	Low	Low	Low
Silva, Magalhães et al. 2017)	Low	Low	Low	Low	Low
(Zile, O'Meara et al. 2019)	Low	Low	Low	Low	Low

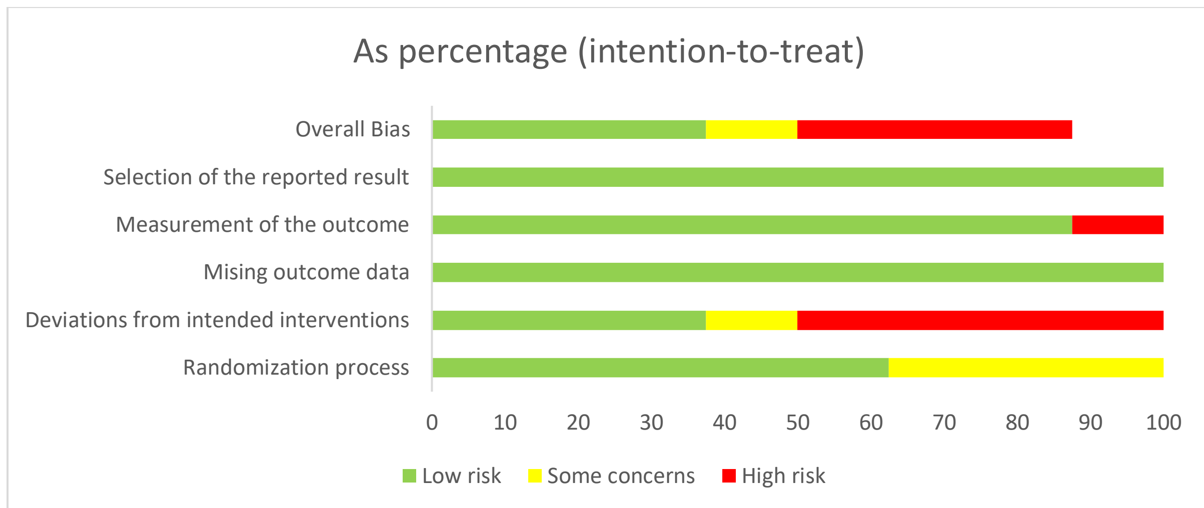

Figure 3-5 Risk of bias assessment

Discussion

We have conducted a systematic review of the literature to determine whether there is evidence from randomized controlled trials that ACE-inhibitors compared to other inhibitors of the RAAS or placebo, reduce the composite of biomarkers of fibrosis, histological evidence of fibrosis and imaging evidence of fibrosis.

There are three main findings from this systematic review. ACEi, when compared to placebo and non-RAAS inhibitors show a significant reduction in the measures of fibrosis. ACEi, compared to other RAAS inhibitors as monotherapy regress myocardial fibrosis progression. Lastly, ACE in combination with other RAAS inhibitors supress myocardial fibrosis more than with ACE inhibitors alone.

RAAS inhibition is important to fibrosis reduction. There is a role of ACEi in the regression of myocardial fibrosis, with a further improved reduction in combination with other RAAS inhibitors.

ACEi is a driver of reduction in fibrosis. Clinically, the reviewed studies show that ACE inhibitors regress myocardial fibrosis progression, improved cardiac function, and overall improved patient outcomes. Additionally, ACE inhibition has been studied to reduce the progression of fibrosis in other organs, further improving organ function and patient outcomes. These studies include, but are not limited to a reduction in fibrosis in; tubulointerstitial fibrosis (Sharma, Eltayeb et al. 1999), improved forced vital capacity in interstitial pulmonary fibrosis (Kreuter, Lederer et al. 2019), and lymphoid fibrosis in people living with HIV (Cockerham, Yukl et al. 2017).

ACE inhibition up-regulates Ac-SDKP levels is through the inhibition of hydrolysis of Ac-SDKP by the N domain of sACE. ACE inhibitors that are currently in use inhibit both the C and N domain of sACE. The studies do not report the effects of ACE inhibition in normotensive patients as ACE inhibition impairment of the C domain of sACE is the mainstay of maintaining blood pressure homeostasis in hypertensive patients. This speaks to the need of *in vitro* work to develop suitable N-selective inhibitors to carry activity without interrupting blood pressure homeostasis in normotensive patients and use of Ac-SDKP analogues resistance to ACE hydrolysis(Junot, Gonzales et al. 2001, Sharma, Douglas et al. 2012, Ramasamy 2020).

No studies could be found on the effect of ACE inhibitors on the pericardium, hence the use of a proximal model of fibrosis, the myocardium. RAAS inhibition reduces myocardial fibrosis. The chosen model of fibrosis gives a targeted to look at the pericardium to determine whether the work can be replicated on its site.

Ventricular hypertrophy can be associated with myocardial fibrosis, presenting a limitation in the search strategy. We relied on the use of medical subject headings (MeSH) to index studies in which myocardial fibrosis was a complication of ventricular hypertrophy during full text review to reduce the potential impact of weakness in the search strategy.

This work further supports work to be conducted to determine whether ACE inhibitors can be used as potential antifibrotics in TBP. Additionally, the studies show a common theme of slowed disease progression. Chronic ACE inhibition is associated with significantly higher Ac-SDKP levels(Azizi, Ezan et al. 1997). This suggests a regulates fibrotic response which manifests as slowed disease progression phenotypically. This could be of importance in the management of TBP in the prevention of pericardial fibrosis and its deleterious effects.

The main limitation of this systematic review was the heterogeneity of the studies. Heterogeneity due to the outcome measures proved difficult to conduct meta-analyses analysing the class effect of ACE inhibition, however this has been shown

in previous studies which focused on AC-SDKP as a single outcome(Mnguni, Engel et al. 2015).

The included studies provide evidence of ACE inhibition slowing the progression of myocardial fibrosis. this opens the way continued work to determine whether ACEi can safely attenuate the fibrotic response in TBP.

4. Investigating the presence of an autoimmune component to the pathophysiology of TBP

Background

Anti-cardiac antibodies have been detected in the serum and pericardial fluid of patients with pericardial disease, suggesting increased immunological reactivity in the pericardium (Karatolios, Pankuweit et al. 2016). Further, there is an association of higher anti-cardiac antibody levels with greater myocardial damage and pericardial effusion recurrence.

Patients with TB test positive for anti-citrullinated peptide antibodies (ACPA) antibodies (Lima, Oliveira et al. 2013). The positive ACPA results are caused by the TB, and not the anti-TB treatment, which can induce anti-histone and antinuclear antibodies.

This evidence suggests that there may be an autoimmune component to TBP that could affect patient outcomes.

Protein citrullination is a post-translational modification that occurs when the enzyme, protein arginine deiminase enzyme (PAD2) catalyses the calcium-dependent hydrolytic conversion of peptidyl arginine to peptidyl citrulline (ROGERS and SIMMONDS 1958). The immune system mounts an antibody response to the citrullinated proteins, resulting in autoimmunity. These autoantibody responses have been documented in diseases such as rheumatoid arthritis (RA), Alzheimer’s disease, and cancer (Schellekens, de Jong et al. 1998, Ishigami, Ohsawa et al. 2005, Chang and Fang 2010). Identification of citrullination serves to determine whether there has been an autoimmune component at play, and could offer a predictive measure for constriction.

An unchecked auto-antibody component driving constriction might explain the persistent inflammatory and fibrotic events in the pericardium despite the absence of a heavy mycobacterial load. This has resulted in interest to investigate auto-antibody formation in patients with TB pericarditis through the study of protein citrullination.

Circulating autoantibodies are a characteristic feature of autoimmune disorders. Many of them are disease-specific with an appearance early on in the disease process, often before the appearance of clinical symptoms. Identifying proteins that could play a role in the propagation of the pathophysiology of tuberculosis pericarditis towards constrictive pericarditis could thus be predictive of efficient pharmacological interventions.

Study objectives

The present study aimed to answer whether citrullinated autoantibodies are present in TBP patients, which could suggest an additional pathway for the immunopathogenesis of tuberculous pericarditis, and could serve as a diagnostic indicator for the progression of the pathophysiology disease. To achieve these aims, the study objectives were as follows:

To determine whether citrullinated autoantibodies are present in TBP patients by autoantibody detection using citrullinated protein microarray technology.

Methods

Sample preparation for protein array analysis

Pericardial fluid samples were obtained from 5 TBP patients and 5 non-TBP patients who were undergoing cardiac bypass surgery. The samples from 5 TBP and 5 control were pooled separately before protein array analysis. The pooled disease samples and control samples were in a 1:600 dilution in PBS.

The immunome protein microarray (Sengenics, Singapore) consists of 1631 proteins including cancer-associated antigens, transcription factors, kinases, and other proteins involved in inflammation and cell signalling.

Blocking immunome slides

The slides were removed from -20°C storage and blocked in CT100plus blocking buffer (25mM HEPES, 50mM KCL, 20% glycerol, 50mM biotin, and 0.1% Tween-20 for 1 hour at room temperature. The slides were then washed three times in 1x PBS.

Citrullinating immunome slides

The slides were coated in 1µg/ml PAD4 in citrullination buffer (25mM HEPES, 5mM $CaCl_2$, 150 mM NaCl, and 0.2% Tween-20) covered in foil, placed in a humidifying chamber, and incubated for 3 hours. The slides were then washed three times in 1x PBS.

Incubating with pooled patient serum/ plasma

The pooled samples (diluted 1:600 in PBS buffer) were added to the slides and incubated for 2 hours at 20˚C. Slides were removed from the chamber, dried, and washed three times in the wash buffer at 20°C. The slides were then incubated for 30 minutes with 10µg/ml AF647-α-human IgG antibody and AF555-α-IgA, washed three times in wash buffer at 20°C and dried by centrifugation in a slide tube at 1200 x *g* for 2 minutes at 20°C to dry.

Slide scanning

The slides were scanned on a high-resolution slide reader (Innoscan 710AL, Innopsys, Carbonne, France) using the Mapix software (Ver 8.2.2, Innopsys). The scan settings were as follows: a 635nm laser with 10mW power, gain at 15 and automatic gain control, and 10µm resolution.

Extraction of quantitative expression data

Scanned images of the microarrays were imported to Spotxel (SICASYS, v1.7.6, Heidelberg, Germany) plate reader and microarray image analysis. The scanned images were converted to GAL files. Spotxel hosts a flex-spot option that was used to ensure that every protein spot was included. This completed the first strep of

quantification. The boundaries for the protein spots were then examined to ensure that they were representative and the artefacts excluded . The raw data was then imported to R (ver 1.1.456, R core team), and data were processed using an in-house developed R script.

Data analysis pipeline

The raw values from the scans were used to normalize the data using quantile and intensity based normalizations as reported by Duarte et al (Duarte J 2013). The normalized expression values were then used to calculate a z-score for each protein. Cut-off values were as follows: a Z-score of >2 (median ± 2 standard deviations of the baseline signal), and a confidence interval value of <0.05. These thresholds were applied to filter low intensities, ensuring the identification of disease-relevant antigens with high reproducibility.

A linear model was fitted to? the data and an empirical Bayes method was then used to moderate the standard errors of the estimated log-fold changes. A false discovery rate (FDR) of p<0.05 was used to minimize the risk of a type 1 error (false positives). The threshold was set at LFC >1/-1 suggesting genes have doubles/halved. Further analysis with an FDR or p<0.01 was applied to the log fold-change analysis.

Results

Pools of pericardial samples from 5 control and 5 TBP patients were each applied to the commercially available Immunome Discovery microarray consisting of more than 1600 different human proteins from different protein families including kinases, signalling molecules, cytokines, interleukins, chemokines, and cancer antigens.

The average intensities across the groups were classified as low/high, with <2500 relative fluorescent units (RFU) indicating low activity. Expression intensity between the TBP and control samples showed a positively skewed data distribution in the TBP group, meaning the TBP samples had a higher proportion of 'high' intensity scores than the control samples (Figure 4-1).

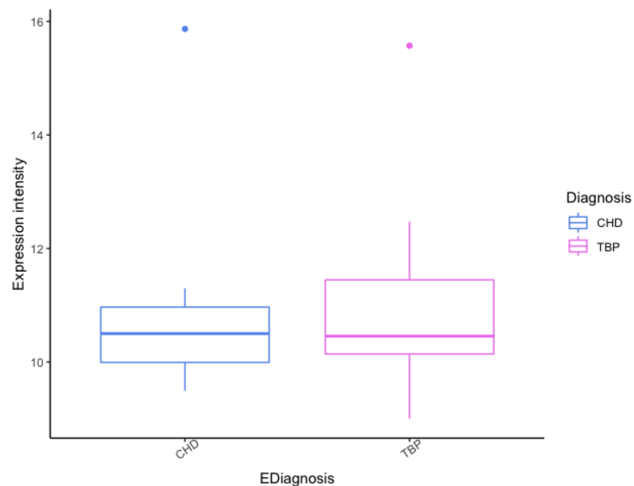

Figure 4-1 Box and Whisker plot of Intensity expressions between TBP group and control (CHD) group.

Reproducibility

A preliminary microarray was conducted on TB PCCF fluid without citrullination for autoantigen identification. The top autoantigens identified are summarized in Table 4-1 and included ALFM1, CBFB, PANK3, PIP, PSG1, RHOB1, and TPM4. These autoantigens were also identified in subsequent runs, suggesting that this method has good reproducibility.

Table 4-1Preliminary Microarray autoantigen detection

Uniprot ID	Function	Reference
Apoptosis inducing factor 1	Part of unique TB unique profile. Indicative of TB progression from latent to active.	(Maertzdorf, Repsilber et al. 2011)
Core-binding subunit beta	Proto-oncogene. Well described in AML.	(Liu, Wijmenga et al. 1996)
Pantothenate kinase 3	Essential enzyme CoA synthesis.	(Subramanian, Yun et al. 2016)
Prolactin inducible protein	Collagen crosslinking. Well described in keratoconus and diabetes.	(McKay, Priyadarsini et al. 2019)
Pregnancy specific glycoprotein	Induction and activation of TGF-β1.	(Ballesteros, Mentink-Kane et al. 2015
Rho-related GTβ1	Novel PPARγ gene target in vascular smooth muscle cells that mediates the protective effect of PPARγ (RAS antagonist)(Fang and Sigmund 2020). inhibits TGFβ/Smad3/CTGF and TGFβ/pSTAT3/pFoxO1 pathways.	(Kökény, Calvier et al. 2020)
Tropomyosin α 4	Actin-binding. Cardiac tissue specific. Cardiac hypertrophy and fibrosis. Hepatic stellate cell activation.	(Wieczorek 2018)

Identification of autoantigens

A total of 115 native proteins were recognised by antibodies in the unmodified and citrullinated arrays (Table 4-2). Of these , 9 were listed as known autoantigens in the AagAtlas dataset: src-homology 3-domain GRB2-like 1, tropomyosin 1, RalA binding protein 1, RuvB-like AAA ATPase 1, Raf-1 proto-oncogene, serine/threonine kinase, testis specific, 10, melanoma antigen family B, 2, keratin 19, and recombination signal binding protein for immunoglobulin kappa J region.

Of the 115 autoantigens identified, 55 were citrullinated and 26 of these were exclusively recognised by antibodies in the citrullination group. Of the 26 autoantigens, 19 and 7 reacted to IgA and IgG, respectively (Figure 4-2). None of the exclusive autoantigens have previously been identified as autoantigens according to the autoantigen database AagAtlas (Wang, Yang et al. 2017, Wang, Zhang et al. 2020).

Table 4-2 identification of autoantigens

DA	Modification	# of hits
IgA	Unmodified	38
	Citrullinated	31
IgG	Unmodified	22
	Citrullinated	24

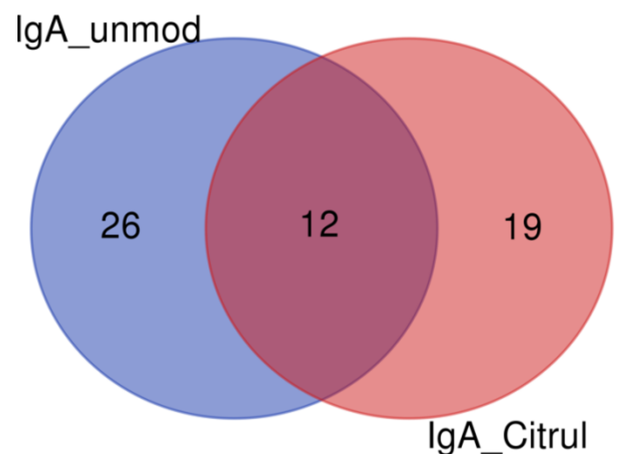

Figure 4-2 Autoantigen hits, divided by antibody response.

From the log fold change analysis, 13 autoantibodies with an LFC >1/-1 were identified from 1815 proteins. 11 of these proteins had a $p_{adjusted}$ value <0.05 (Figure 4-3). Of these 11, ASNA1, LRRFIP2, and SPANXN2 were upregulated while AURKB, KRT15, KRT19, PSMD9, RBPJ, RPA2, SPA17, and TPM1, were downregulated (Figure 4-4).

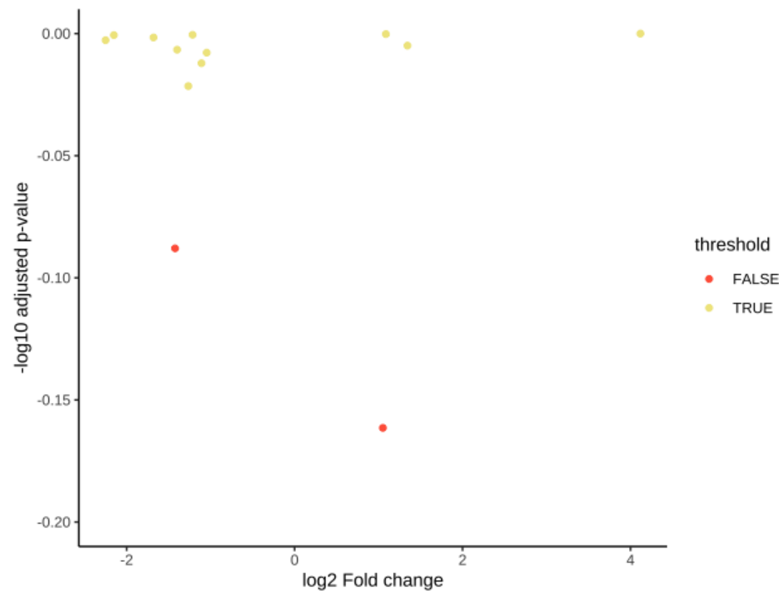

Figure 4-3 Autoantibody expression LFC >1/-1, adjusted p<0.01

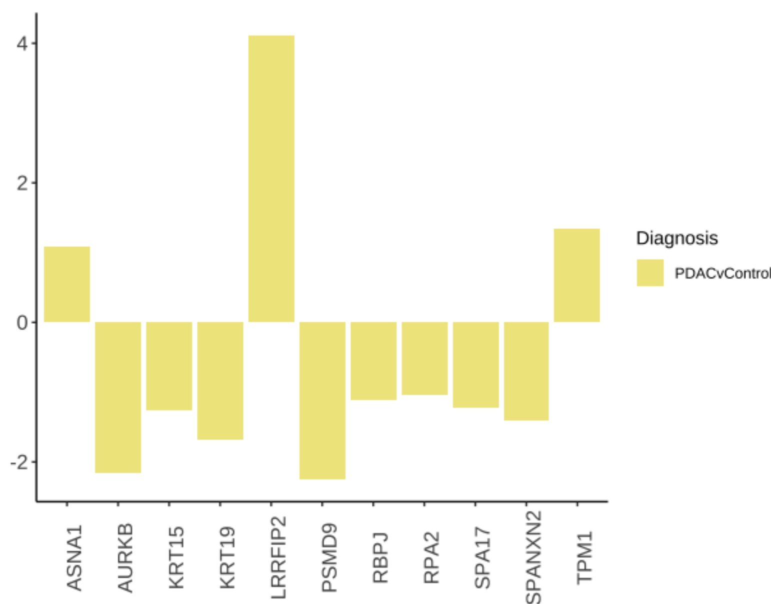

Figure 4-4 log fold changes of identified autoantibodies

On further analysis, 13 autoantibodies with LFC >1/-1 were identified from 1815 proteins. Of these, 9 autoantibodies had a $p_{adjusted}$ value <0.01 (Figure 4-5and 6). In this subset of data, ASNA1, LRRFIP2, and SPANXN2 were upregulated. The remaining 6 autoantibodies, AURKB, KRT19, PSMD9,, RPA2, SPA17, and TPM1, were downregulated (Figure 4-6).

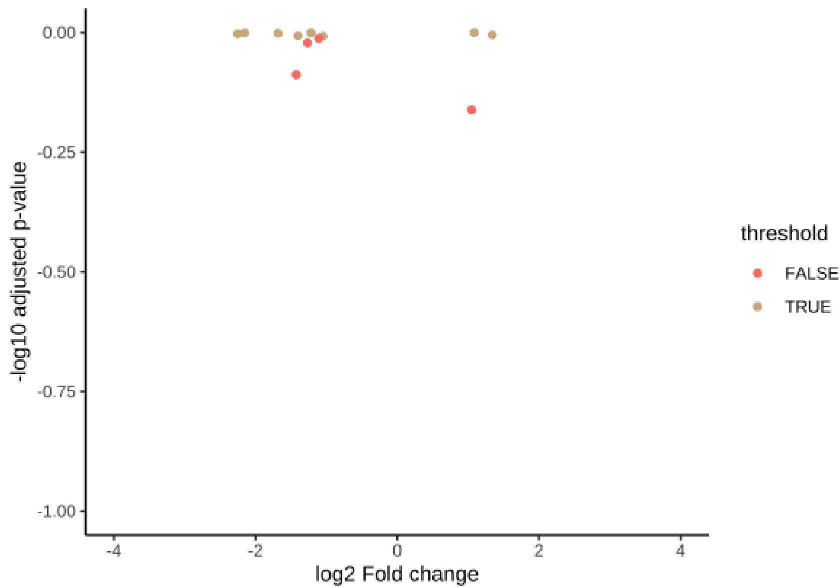

Figure 4-3 log fold changes of identified autoantibodies. LFC>1/-1, adjusted p<0.01

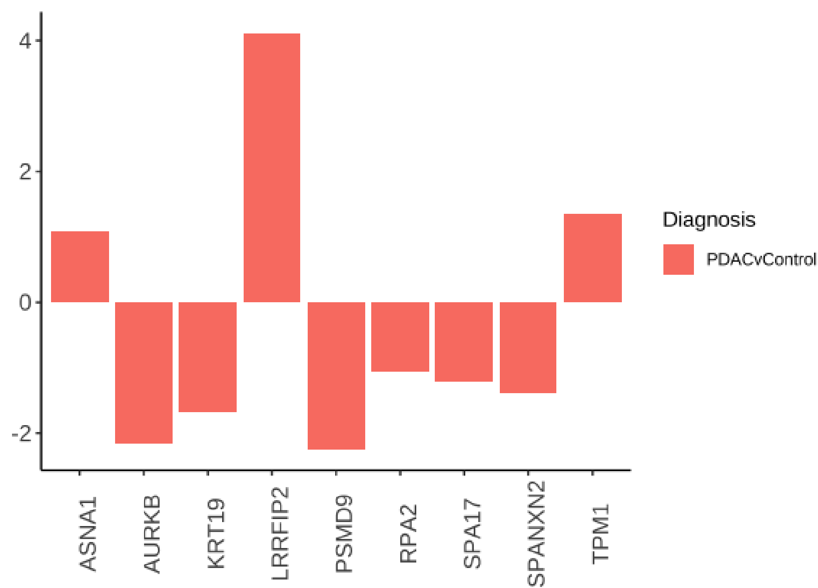

Figure 4-4 Autoantibody expression. LFC >1/-1, adjusted p<0.01

Antigens identified in pericardial fluid

In the search for potentially pathogenic TBP autoantigens, the 115 identified autoantigens were searched against the TBP proteome described in Chapter 2. Only 3 autoantigens were also identified in the LC-MS/MS analysis of the TBP proteome:

CDK regulatory subunit-associated protein 2 (CDK), human Immunoglobulin heavy constant gamma 1 (IGHG1), and human immunoglobulin heavy variable 3 (ASNA).

Discussion

This study is the first to use a high-density protein array for native autoantigen profiling in TBP. Using this novel technology, 115 potential native autoantigens were identified, of which only 9 have been previously identified as autoantigens.

Citrullination is a post-translational modification that is well studied in RA, as it is the most common systemic disease characterised by citrullination. Based on the present results, the identified autoantigens which are shared between RA and TBP are: SH3GL1, TPM1, AFF4, NAP1L3, TACC1, MAGEB1, IGHG1, RBPJ, KRT15, and SPANXN2. Of these MAGEB1, SH3G1, and KRT15 are associated with increase levels after citrullination with PAD enzymes (Poulsen, Damgaard et al. 2020). This suggests that these proteins may be targets for autoantibodies in the TBP proteome.

Of particular interest among the identified autoantigens is melanoma antigen family B2 (MAGEB2). The MAGEB2 autoantibody is documented to be a clinically relevant biomarker for paediatric systemic lupus erythematosus (SLE) disease activity and SLE glomerulonephritis disease activity (Hoftman, Tai et al. 2008).

One of the manifestations of SLE is pericarditis. A literature search of identified autoantigens in SLE was compared to those associated with TBP. This novel technique was applied in a cohort of patients with SLE for the detection of putative autoantibodies. The proteome data from the present study was run against the top 50 of the 381 autoantigens identified with significant responses in SLE (Mak, Kow et al. 2020), and to 68 novel autoantigens targeting the toll-like receptor (TLR) and SMA pathways (Lewis, McAndrew et al. 2018). Only three of the autoantigens identified in the TBP proteome could be cross-matched with the top SLE autoantigens: RNA-binding protein FUS (FUS), polyadenylate-binding protein 1 (PABPC1), and DNA repair protein RAD51 homolog 1 (RAD51). This suggests a possible antibody

response that is specific to the pericardium, differing to that associated with pericarditis, or that pericarditis occurs during SLE.

Another autoantigen identified in the present study is recombination signal binding protein for immunoglobulin kappa J region (RBPJ), which was identified as a novel autoantigen in cerebrospinal fluid (CSF) of patients with multiple sclerosis(Querol, Clark et al. 2013).

Base on the present study,

Keratin 19 (KRT19) appears to be a target for autoantibodies in both unmodified and citrullinated pools. KRT19 is a prominent autoantigen in anti-cyclic citrullinated peptide (Anti-CCP) RA (Poulsen, Damgaard et al. 2020).

Autoantibody expression

Among the identified autoantibodies, ASNA1, LRRFIP2, and SPANXN2 were upregulated.

ASNA 1 is an ATPase required for post-translational protein localisation. It is a highly conserved 40 kDa ATPase subunit of the transmembrane domain recognition complex (TRC), which delivers tail-anchored proteins into the endoplasmic reticulum membrane (Stefanovic and Hegde 2007). ASNA1 is expressed in cardiac, and skeletal muscle, epithelial cells of liver, kidney, and stomach wall (Kurdi-Haidar, Heath et al. 1998). Data is lacking on the significance of ASNA1 overexpression during disease.

LRRFIP2 positively regulates Toll-like receptor signalling (TLR)by ending to TLR4, thus activating nuclear factor kappa B signalling. LRRFIP2 downregulates the NLRP3 (NACHT, leucine-rich repeat, and pyrin domain-containing protein 3) inflammasome by reduction of caspase-1 inhibitor flightless -1 to inflammasome complex(Jin, Yu et al. 2013). The NLRP3 inflammasome is a macromolecular structure composed of three components: the sensor NLRP3, which is an adaptor or scaffolder protein, the apoptosis-associated speck-like protein containing a caspase recruitment domain

(ASC), and the effector protein, caspase-1, which cleaves pro-IL-1β and pro-IL018 into their active forms (Guo, Callaway et al. 2015).

The NLRP3 inflammasome has a potentially important role in autoimmune diseases due to its association with inappropriate autoreactive immune responses. (Zhang, Yang et al. 2021).

The NLRP3 inflammasome is upregulated in inflammatory bowel disease, and is positively correlated with the severity of disease. NLRP3 is also significantly increased in other autoimmune diseases, such as Type 1 diabetes, systemic lupus erythematosus, rheumatoid arthritis, systemic sclerosis, psoriasis, and autoimmune thyroid disease.

Of interest, NLRP3 is a central component of the pathophysiology of pericarditis. The inflammatory response initiated by the Mtb is amplified through the activation of the NLRP3 inflammasome. Additionally, the three different components of the NLRP3 inflammasome–NLRP3, ASC, and caspase-1-are all more intensely expressed in the pericardium of patients with chronic pericarditis experiencing an acute flare up (Vecchié, Del Buono et al. 2021).

The evidence for this association grew through indirect observations in which a significant reduction in pericarditis recurrences were seen in clinical cases, and animal trials in which colchicine was used as first-line treatment for pericarditis (Klein, Imazio et al. 2021). Colchicine impairs NLRP3 chromosome activation.

In patients who are colchicine resistant, anakinra, a recombinant IL-1, was highly effective in reducing pericarditis recurrence through inflammasome inhibition(Imazio, Andreis et al. 2020).

LRRFIP2 was identified as upregulated in this study, but it's relation to NLRP3 autoinduction needs to be further studied. Sometimes, inhibition of the NLRP3 inflammasome results in a paradoxical pro-inflammatory effect, indicating a complex and possible alternative autoinduction mechanisms that are yet to be explored (González-López, Martínez-Taboada et al. 2008, Karamanakos, Vergou et al. 2021).

A limitation to this work was the use of pooled plasma samples due to resource limitations, which does not allow for the resolution of individual patients-level differences in autoantigen levels. A second limitation is the use of the microarray platform which has a fixed number of antigens for identification.

5. RAS fingerprinting

Background

RAS fingerprinting is an MS-based technique that allows the mapping of RAS metabolites in healthy or diseased states. This novel technique combines sampling in the presence of a RAS-specific enzyme inhibitor cocktail with advanced LC-MS/MS to generate a cross-sectional ‘snapshot’ of enzyme activity and metabolite concentrations. This precise mapping of RAS metabolites allows us to study the complex physiology of the RAS in disease states, and its possible involvement in TBP progression to constrictive pericarditis upon RAS dysregulation. Dysregulated RAS metabolites can be correlated with disease progression and can also be potential biomarkers.

Basu *et.al.* and Pavo *et al.* used the RAS-fingerprinting method (Figure 5-1)in plasma to develop a biochemical profile of patients with heart failure (Pavo, Wurm et al. 2016, Basu, Poglitsch et al. 2017), and identified key metabolites involved in the pathogenesis of heart failure.

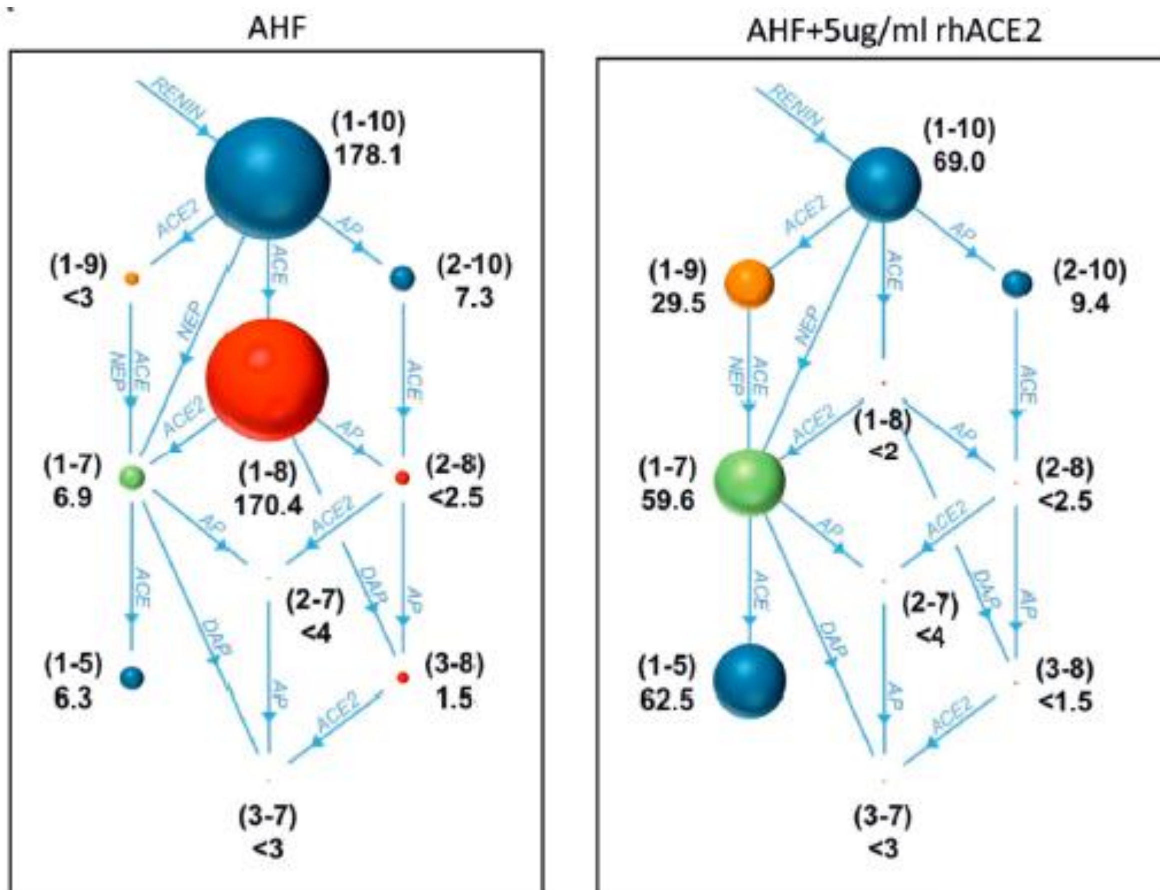

Figure 5-1 ***RAS fingerprint*** *global representation of equilibrium levels of 10 angiotensin peptides in plasma from AHF 21 patients in the absence and presences of recombinant ACE2 (rhACE2). The spheres sizes visually represent the concentrations of the metabolites (in brackets) Adapted from Basu, Poglitsch et al. 2017.*

Methods

Four PCCF samples were stored at -80ºC in a RAS angiotensin peptide inhibitor cocktail and shipped to Attoquant Diagnostics, Vienna, Austria. The PCCF samples were then incubated at 37ºC for one hour at pH 7.6, and then subjected to LC-MS/MS quantification of equilibrium angiotensin peptide levels. Stable isotope-labelled internal standards for Ang I, Ang 1-9, Ang II, Ang 2-8, Ang 3-8, Ang 1-7, Ang 1-5, Ang 2-7, Ang 3-7, and Ang 2-10 were added to the samples at concentrations of 200pg/ml each. The samples were then subjected to a C18 column solid-phase extraction (Acquity UPLC C18, Waters), prior to LC-MS/MS analysis using a reverse-phase analytical column operating in line with a XEVO TQ-S triple quadruple mass spectrometer in multiple reaction monitoring mode. The PCFF angiotensin concentrations were calculated by relating the endogenous peptide signals to that of the internal standards.

Results

The angiotensin peptide levels in pericardial fluid were measured in four unfiltered PCFF samples and in single-, double-, and triple-filtered PCFF samples using a LC-MS/MS to create a biochemical profile of RAS metabolites in pericardial fluid. The metabolites, AGT, Ang I, Ang II, Ang 1-7, and Ang 2-10 were detected in at least 4 samples. Ang I and Ang II were detected in all samples (Table 5-1).

Patient sample PCFF1 had the most strikingly different Ang I (749.8 pmol/l) metabolite as compared to the other patient samples. We suspect that this patient was on an ACE inhibitor treatment due to the high Ang I and low Ang II levels, thus inhibiting the ACE enzyme. This is supported by the presence of Ang 2-10, indicating the activation of aminopeptidase shunting Ang I metabolism towards Ang 2-10 production. The presence of Ang 1-7 suggests that ACE2 activity is shifting towards an alternative RAS axis.

PCFF2 demonstrated a mildly elevated Ang II to Ang I ratio of 1.34 compared to PCFF1 and PCFF3, which were 0.01 and 0.42, respectively. This high Ang II to Ang I ratio is also observed in PCFF4 (2.08); however, the metabolites were too close to the limit of detection to be significant.

The filtered samples underwent RAS-fingerprinting to determine whether the mandatory BSL3 filtering would influence the results. The single, double, and triple filtered samples yielded similar results to the unfiltered samples, indicating that filtering may have a negligible effect on RAS fingerprinting.

Table 5-1 RAS fingerprinting of TB PCCF. Ang concentrations in pmol/l.

	Ang I (1-10)	**Ang II (1-8)**	**Ang 1-7**	**Ang 1-9**	**Ang III (2-8)**	**Ang 3-7**	**Ang 1-5**	**Ang IV (3-8)**	**Ang 2-7**	**Ang 2-10**	**AGT (ug/ml)**
PCFF1	749.8	9.1	9.8	<3	<2	<2	<1.5	<2	<3	94.8	31.7
PCFF2	15.0	20.2	<3	<3	<2	<2	<1.5	<2	<3	<2	45.2
PCFF3	11.5	4.9	<3	<3	<2	<2	<1.5	<2	<3	<2	15.1
PCFF4	3.6	7.5	<3	<3	<2	<2	<1.5	<2	<3	<2	17.3
PCFF3 fsx1	10.6	9.1	<3	<3	<2	<2	<1.5	<2	<3	<2	n.a.
PCFF3 FSX3	7.9	5.0	<3	<3	<2	<2	<1.5	<2	<3	<2	14.6

Discussion

RAS fingerprinting was performed to further examine the role of the renin-angiotensin system during TBP. To our knowledge, this is the first time RAS fingerprinting has been performed on TB pericardial fluid. The aim of this equilibrium analysis of angiotensin metabolites was to provide a comprehensive molecular fingerprint for the

RAS, which could provide insights into the regulation of this system (Kovarik, Antlanger et al. 2015). This technique could detect some of the RAS metabolites in pericardial fluid. The angiotensin concentrations varied across the patient samples, indicating varying basal RAS activity. The patient samples all contained Ang II concentrations confirming the presence of ACE activity, and which was also detected in the ACE activity assay. The presence of Ang II in the pericardial fluid samples of patients with TB pericarditis suggests that it maybe be involved in the development of constriction due to fibrosis.

RAS fingerprinting of PCFF samples revealed lower levels of metabolites than blood, as compared to previously published results obtained from blood samples from males with hypertension, patients with heart failure, and patients with chronic renal failure undergoing haemodialysis (Kovarik, Antlanger et al. 2015, van Rooyen, Poglitsch et al. 2016, Basu, Poglitsch et al. 2017). This observation may strengthen the hypothesis of Rifai et al. that metabolites found in a proximal fluid such as pericardial fluid serve as surrogates for what is available systematically (Rifai, Gillette et al. 2006).

In the above-mentioned studies, a similar pattern of the RAS metabolites was found, with Ang I and Ang II being the most abundant observed metabolites, while the remaining metabolites were detected at low levels, or near to the-limit of detection. These findings confirm the presence of ACE activity in diseases which have fibrosis as a complication of RAS activation.

PCFF1 yielded different results from the other samples due to the high Ang I: Ang II ratio of 82 and the presence of the anti-fibrotic cardioprotective Ang 1-7 and Ang 2-10 metabolites, which is a fingerprint pattern also observed in ACEi-treated patients (Kovarik, Antlanger et al. 2015). The high Ang I: Ang II ratio occurs due to the inhibition of the production of Ang II by ACEi. The inhibition of this major angiotensin peptide allows for the activation of alternative RAS pathways, counter-regulatory to the Ang II pathway, of which Ang 1-7 is the main effector (Ferreira, Shenoy et al. 2011). The increase of Ang 1-7 caused by ACEi can be explained by the reduced signaling of Ang 1-8 and reduced degradation of Ang 1-7 (Carretero 2005). Further, ACEi has been shown to reduce fibrosis, slow disease progression, and improve organ function in patients with myocardial fibrosis, diabetic nephropathy, idiopathic pulmonary fibrosis, and Duchenne and Becker muscular dystrophy (Sharma, Eltayeb et al. 1999, Brilla, Funck et al. 2000, Brilla, Rupp et al. 2003, Marakalala, Raju et al. 2016, Silva,

Magalhães et al. 2017, Kreuter, Lederer et al. 2019). These protective effects of ACEi touch on the prospects of using domain-specific ACEi for the treatment and/or prevention of constrictive pericarditis in patients with TB pericarditis and other forms of pericarditis.

6. Conclusions, limitations and future directions

In summary, TBP is a complex disease which progresses to constrictive pericarditis. This progression has the involvement of many key modulators. In this study, proteins that are involved in the fibrotic process were identified by MS analysis needing probing for further analysis and their role in the progression of fibrosis in TBP. Time and resource constraints impacted the study in terms of sample size, and further analysis. Future studies will apply a targeted mass spectrometry approach, assay development, and inhibition studies of the identified proteins of interest to assess their diagnostic efficiency as biomarkers, and therapeutic potentials.

This study was the first of its kind to identify autoantibodies in pericardial fluid from TBP patients. The main conclusion of this proof-of-concept study lays the foundation for an additional arm of further studying the pathophysiology of disease. Other autoimmune modifications outside of citrullination can now be studied to expand our current understanding of the post-infectious inflammatory process.

RAAS and the dysregulation thereof has been implicated to be involved in the inflammatory and fibrotic processes through its pro-fibrotic components, ACE, and Ang II. The counterregulatory Ang II and inhibitor of ACE form the focus of being the potential reversing factors of the inflammatory and fibrotic process leading up to constriction. The simultaneous investigation of RAS and its inhibition forms the basis of this project. The study of the fibrotic process, the autoantibody contribution to fibrosis and the potential use of ACEi to counteract fibrosis form the basics of this project.

More work must be done on biomarker discovery in TB pericarditis to decrease the occurrence of constrictive pericarditis. This study paves the way for biomarker

discovery by applying different MS-based discovery proteomic techniques on pericardial fluid. Through MS analysis we identified proteins which have possible implications of involvement in the pathophysiology of fibrosis. However, the large range of proteomic expression and the intrinsic limitation of instrument sensitivity presented the major hurdle to this study.

This Masters project was a compilation of different methodologies from different departments in the clinical and biomedical sciences. Collaborations with the various stakeholder s within The University of Cape Town presents a strength of the work presented in which pathophysiology of disease was not approached in a monolithic manner.

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border-box; margin: 2px 0px 0px; font-family: "Trebuchet MS", Tahoma, Verdana, Arial, sans-serif; line-height: 1.3; color: rgb(235, 143, 0); font-size: 14px; padding: 0.5em 0.5em 0.5em 2.2em; border: 1px solid rgb(251, 216, 80); outline: 0px; list-style: none; background-image: url("https://prod-assets-3.rayyan.ai/assets/jquery-ui-1.9.2/ui-bg_glass_65_ffffff_1x400-f0e6cd91b837d5c5644d026e5ffeccd907953317cd5c0f689901733afda260b2.png"); background-color: rgb(255, 255, 255); border-top-left-radius: 4px; border-top-right-radius: 4px; cursor: pointer; position: relative; zoom: 1; background-position: 50% 50%; background-repeat: repeat no-repeat;" tabindex="0">Angiotensin-converting enzyme inhibitors: a potential treatment for fibrosis in tuberculous pericarditis ", from https://rayyan.ai/reviews.

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