Review

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Targeting cardiac fibrosis in heart failure with preserved ejection fraction: mirage or miracle?

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Abstract

Cardiac fibrosis is central to the pathology of heart failure, particularly heart failure with preserved ejection fraction (HFpEF). Irrespective of the underlying profibrotic condition (e.g. ageing, diabetes, hypertension), maladaptive cardiac fibrosis is defined by the transformation of resident fibroblasts to matrix-secreting myofibroblasts. Numerous profibrotic factors have been identified at the molecular level (e.g. TGFβ, IL11, AngII), which activate gene expression programs for myofibroblast activation. A number of existing HF therapies indirectly target fibrotic pathways; however, despite multiple clinical trials in HFpEF, a specific clinically effective antifibrotic therapy remains elusive. Therapeutic inhibition of TGFβ, the master-regulator of fibrosis, has unfortunately proven toxic and ineffective in clinical trials to date, and new approaches are needed. In this review, we discuss the pathophysiology and clinical implications of interstitial fibrosis in HFpEF. We provide an overview of trials targeting fibrosis in HFpEF to date and discuss the promise of potential new therapeutic approaches and targets in the context of underlying molecular mechanisms.

Keywords CMR; fibroblast; fibrosis; heart failure; HFpEF
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See the Glossary for abbreviations used in this article.

Introduction

Heart failure (HF) is a major public health problem with an estimated worldwide prevalence of over 23 million (Bui *et al*, 2011; Ponikowski *et al*, 2014)—a figure projected to rise as populations age (Conrad *et al*, 2018). Despite advances in the treatment of HF over the last decades, mortality and morbidity remain high and HF is a major contributor to the global health economic burden (Lesyuk *et al*, 2018). It is increasingly clear that cardiac fibrosis plays a role in the aetiology of all forms of HF and in particular the pathophysiology of HF with preserved ejection fraction (HFpEF) (Moreo *et al*, 2009; González *et al*, 2018).

Fibrosis is an evolutionarily conserved physiological process intended to repair, replace and reinforce severely or chronically injured tissue when tissue regenerative and homeostatic mechanisms are exhausted. Fibrosis ultimately results in the accumulation of extracellular matrix (ECM) at the site of injury and the production of a "scar". Short-term fibrotic processes can be adaptive, but persistent activation of fibrotic pathways, as occur in HFpEF, results in excess accumulation of ECM and disruption of tissue function (Rockey *et al*, 2015). Much research has been carried out in the field of cardiovascular fibrosis, identifying potential antifibrotic targets that may provide new strategies to treat HF. In this review, we summarize the processes involved in the development of HFpEF focussing on interstitial cardiac fibrosis and its contribution to HF, discuss strategies which have been implemented to date—with limited success—and highlight the new opportunities.

HFpEF

The classical definition of HF is "an inability of the heart to pump blood to the body at a rate commensurate with its needs, or to do so only at the cost of high filling pressures" (Braunwald, 1988) which presents clinically as a syndrome of exertional breathlessness, peripheral oedema and fatigue. HF can be further categorized as HF with reduced ejection fraction (HFrEF) or HFpEF (Ponikowski *et al*, 2016). In HFrEF, the systolic force generation of the heart is impaired, and consequently, the proportion of blood expelled with each contraction—the ejection fraction—is reduced. In HFpEF, routine parameters of systolic function are largely maintained but diastolic filling and relaxation are impaired (Ponikowski *et al*, 2016).

While several therapies exist for HFrEF—including beta-blockers, drugs targeting the renin–angiotensin–aldosterone system (RAAS) and sodium-glucose co-transporter 2 (SGLT2) inhibitors—no treatment has yet been shown to be effective for HFpEF despite multiple

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Glossary

Cardiac fibroblast
Cells resident within the myocardium which express mesenchymal markers and secrete ECM proteins.
Diabetic nephropathy
Kidney disease caused by the effects of hyperglycaemia on the structure and function of the glomerulus.
Dilated cardiomyopathy
Heart muscle disease, often with a genetic component, characterized by progressive dilation and dysfunction of the cardiac chambers leading to heart failure.
Extracellular matrix
A complex network of interconnected structural proteins, glycoproteins and enzymes which reside in the extracellular space and provide structural and biochemical support to surrounding cells.
Extracellular volume
Magnetic resonance imaging measurement technique which allows the quantification of diffuse collagen deposition within a tissue.
Fibrosis
The accumulation of excess extracellular matrix proteins within the extracellular space distorting the architecture and the function of the native tissue.
Heart failure with preserved ejection fraction
Clinical syndrome of heart failure where, although the routinely measured parameters of systolic function are normal, dysfunction of relaxation and passive filling results in heart failure symptoms.
Heart failure with reduced ejection fraction
Clinical syndrome of heart failure where the contractile force is reduced and the proportion of blood expelled with each contraction is below the normal range.
Heart failure
A syndrome of clinical signs and symptoms characterized by the inability of the heart to provide sufficient cardiac output to match the physiological demands of the body.
Hypertensive heart disease
Changes within the heart in response to chronic hypertension which includes cardiomyocyte hypertrophy and fibrosis which can result in heart failure.
Hypertrophic cardiomyopathy
A heart muscle condition, often inherited, resulting in ventricular hypertrophy in the absence of abnormal loading conditions.
Interstitial fibrosis
The accumulation of ECM in the absence of large-scale cell death in the interstitial and perivascular spaces.
Myocardial infarction
Myocardial damage caused by insufficient blood supply to a myocardial region leading to cell death and fibrotic scar formation
Myofibroblast
Activated fibroblasts with a highly secretory and contractile phenotype which are responsible for the majority of ECM production in pathological states.
Oxidative stress
An imbalance between the production of reactive oxygen species within a tissue and its ability to clear these with antioxidants.
Replacement fibrosis
The deposition of ECM to replace dead or damaged cells and preserve the structural integrity of a tissue.
Transverse aortic constriction
An experimental surgical animal model of pressure overload involving creation of a stenosis in the transverse aorta causing increased pressure in the left ventricular cavity, resulting in hypertrophy, fibrosis and—ultimately—heart failure.
Ventricular remodelling
The changes that occur within the ventricular myocardium in response to pressure or volume overload leading to cardiomyocyte hypertrophy and accumulation of fibrotic tissue.

randomized trials (Ponikowski *et al*, 2016; Pfeffer *et al*, 2019; Seferovic *et al*, 2019). This is of particular concern as HFpEF is common—estimated to be responsible for > 50% of HF cases (Vasan *et al*, 2018)—and is growing increasingly more so due to its association with ageing and comorbidities such as diabetes, renal dysfunction, hypertension, non-alcoholic fatty liver disease and sarcopenia (Bekfani *et al*, 2016; Dunlay *et al*, 2017; Streng *et al*, 2018).

Echocardiographic and invasive haemodynamic measurements in HFpEF have described impairment in both diastolic function (Kasner *et al*, 2011; Hummel *et al*, 2017) and in non-classical measures of systolic function, such as longitudinal contraction (Kraigher-Krainer *et al*, 2014). Characteristic changes in diastolic function include impairment of ventricular relaxation (Zile *et al*, 2004) resulting in reduced ventricular compliance and reduced efficiency of ventricular filling during diastole (Hay *et al*, 2005; Westermann *et al*, 2008). Maintenance of adequate stroke volume in this setting necessitates elevation of ventricular filling pressures particularly during exercise when diastolic filling time is limited (Westermann *et al*, 2008). Neurohormonal systems including the sympathetic and RAAS are activated which promotes salt and water retention in the kidney. Over time, the increased circulating volume and high levels of AngII and aldosterone are maladaptive, increasing ventricular stretch, oncostatic pressure in the lungs and peripheries and exerting a potent prohypertrophic and profibrotic effect within the myocardium (Díez, 2004; Brown, 2013).

HFpEF does not represent a single pathological process but is a complex disease, with many contributing pathophysiological mechanisms (Cohen *et al*, 2020) both within the cardiomyocyte, in the surrounding tissue (Fig 1) and in peripheral tissues (not discussed here). Endothelial dysfunction has repeatedly been associated with development of HFpEF and is predictive of diastolic dysfunction and subsequent HFpEF in asymptomatic patients (Yang *et al*, 2020). Healthy endothelium releases nitric oxide (NO) which is a key homeostatic mediator with effects on vascular smooth muscle cells, cardiomyocytes and fibroblasts and has been suggested to be a cornerstone of HFpEF pathophysiology (Paulus & Tschöpe, 2013). Cardiomyocyte hypertrophy (Takimoto *et al*, 2005), increased myocardial stiffness (Bishu *et al*, 2011) and cardiac fibrosis (Calderone *et al*, 1998) have all been associated with reduced NO signalling through decreased cyclic GMP production and inhibition of protein kinase G activity in various cell types (Paulus & Tschöpe, 2013).

Dysfunctional endothelial cells also express high levels of vascular adhesion molecules which promote the migration of inflammatory cells into the myocardium (Westermann *et al*, 2011). Inflammatory cell infiltration is augmented by local release of inflammatory mediators including IL-1, IL-6 and TNF-α in response

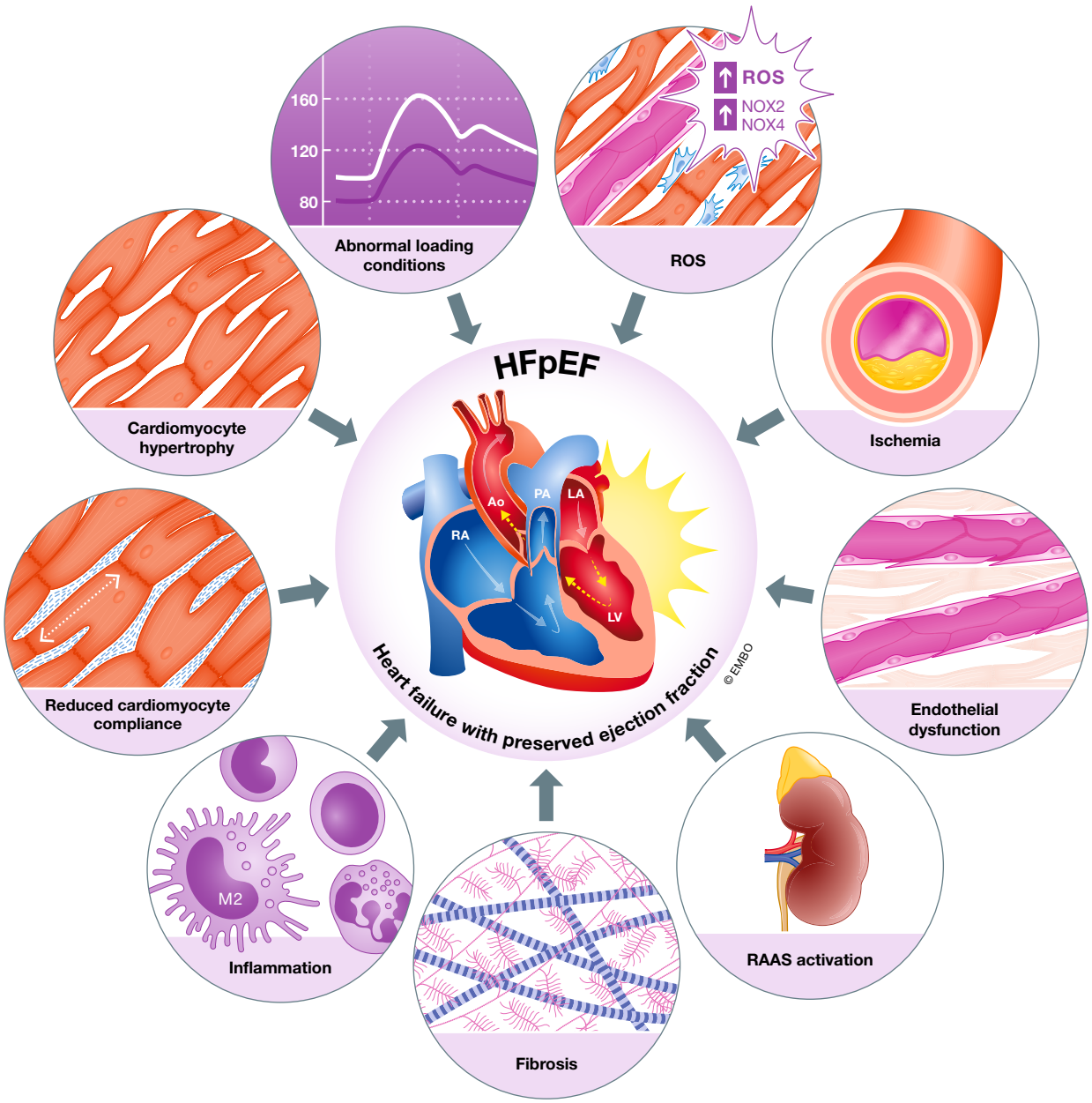

Figure 1. Illustation of the multiple interacting pathophysiological mechanisms responsible for development of HFpEF.

The development of HFpEF has multiple contributing pathophysiological mechanisms acting on the heart. The relative contribution of each of these factors varies depending on the underlying disease state; however, cardiac fibrosis is a common pathway present in almost all patients with symptomatic HFpEF.

to hypoxia or local tissue damage (Turner *et al*, 2007; Yu *et al*, 2012). Systemic inflammatory conditions including rheumatological conditions, diabetes or metabolic syndrome which are associated with HFpEF prime immune cells to initiate exaggerated inflammatory and fibrotic responses when recruited to tissues (Esposito *et al*, 2002; Umare *et al*, 2014). Inflammation within the myocardium increases oxidative stress, reduces cGMP production, damages the endothelium and impairs cardiomyocyte performance (Picchi *et al*, 2006; Waddingham *et al*, 2019). If persistent, inflammation can be associated with the emergence of profibrotic macrophages (Westermann *et al*, 2011; Peet *et al*, 2019) and infiltration of Th1 T cells (Nevers *et al*, 2017). These inflammatory cells express transforming growth factor β (TGFβ), interferon-ɣ, Galectin-3 (Gal-3), connective tissue growth factor and angiotensin-converting enzymes which activate cardiac fibroblasts (CF) thereby promoting the deposition of ECM and the occurrence of fibrosis.

At the level of cardiomyocytes, mechanical stretch, neurohormonal activation and oxidative stress lead to a hypertrophic response with increased sarcomere numbers, cardiomyocyte area, myocardial mass and impaired relaxation kinetics (Kojima *et al*,

1994; Okoshi *et al*, 2004). Post-translational modification to sarcomeric proteins such as phosphorylation of titin occurs in response inflammatory and profibrotic signals which reduces the compliance of the cardiomyocyte during relaxation (Fukuda *et al*, 2005; Krüger *et al*, 2009). Oxidative stress within the heart is elevated in HFpEF (Vitiello *et al*, 2014) particularly in conditions such as obesity, hypertension and diabetes which can cause mitochondrial dysfunction (Sverdlov *et al*, 2016; Sorop *et al*, 2018), uncouple the electron transport chain (Boudina *et al*, 2007), upregulate reactive oxygen species (ROS)-producing enzymes (Ide *et al*, 2000; Moris *et al*, 2017) and reduce antioxidant activity (Ballal *et al*, 2010). Oxidative stress impacts NO signalling, the phosphorylation state of sarcomeric proteins, calcium handling and hypertrophy within the cardiomyocyte. This results in increased myocardial stiffness, impaired energetic metabolism and a profibrotic, pro-inflammatory secretome which contributes to and perpetuates the haemodynamic changes of HFpEF.

Fibrosis in HFpEF

Among the multiple factors contributing to the development of HFpEF, fibrosis is a common pathway which exists regardless of aetiology. In patients with symptomatic HFpEF, extracellular fibrotic burden is more strongly correlated with diastolic dysfunction than is cardiomyocyte stiffness (Zile *et al*, 2015) and fibrosis is correlated with increased arrhythmias (Cho *et al*, 2018) hospitalization and mortality in HFpEF (Kanagala *et al*, 2019) making it an attractive therapeutic target. There is a prolonged asymptomatic phase prior to the development of HFpEF in which significant structural and haemodynamic changes accumulate within the heart but without limiting symptoms (Abhayaratna *et al*, 2006; Kosmala & Marwick, 2020). Although reducing cardiomyocyte stiffness, endothelial dysfunction and oxidative stress may provide beneficial effects in the early stages of disease, reversing fibrotic changes and positively remodelling the myocardium is crucial to improve cardiac function and ameliorate symptoms late in the disease as symptoms are beginning to develop (Kim *et al*, 2018).

Fibrotic tissue is predominantly composed of fibrillar collagens such as collagen I and collagen III which strongly influence the biomechanical properties of the ECM. Fibrillar collagens have high tensile strength providing structural support to the myocardium, however when present in excess, reduces myocardial compliance (de Souza, 2002). Collagen subtypes have differing elastic properties, and therefore, the ratio between collagen subtypes in addition to increased quantity is important for the physiological effects seen in the fibrotic heart. Collagen I accounts for 85–90% of collagen within the healthy heart with collagen III making up 5–10% and smaller contributions from other collagen subtypes (Weber, 1989; de Souza, 2002). Collagen I is less compliant when exposed to tension compared to collagen III which has more elastic properties (Collier *et al*, 2012; Asgari *et al*, 2017). An increased ratio of type I vs. type III collagens is seen in both animal and human models of pressure overload (Kasner *et al*, 2011; López *et al*, 2014; Echegaray *et al*, 2017) and is correlated with worsening diastolic function and increased symptoms (Kasner *et al*, 2011).

Fibrosis histology

Fibrotic changes in the heart can be broadly categorized into distinct but not mutually exclusive categories of (i) replacement or (ii) reactive/interstitial fibrosis (Fig 2). Replacement fibrosis is classically associated with myocardial infarction (MI) where cardiomyocyte cell death and muscle loss are replaced by ECM proteins to maintain the structural integrity of the heart wall. This is a crucial process to reinforce areas of the myocardium weakened by cardiomyocyte loss and prevent myocardial rupture. The resulting area of fibrotic scar is non-contractile, non-elastic tissue that does not contribute to force generation. Thus the size, composition and physical properties of the fibrotic scar have major implications for the development of HF.

So-called "reactive fibrosis" is an alternative form of cardiac fibrosis which occurs in the absence of large-scale cardiomyocyte death and will be the focus of the remainder of this review. There are two major histologically distinct forms of reactive fibrosis—interstitial and perivascular—which often coexist. Interstitial fibrosis involves the deposition of collagen-rich ECM in the interstitial space between cells and is most commonly associated with chronic stressors that include abnormal loading conditions (e.g. hypertension, post-MI or valve pathology) (Brilla *et al*, 2000; Treibel *et al*, 2018a) or profibrotic systemic conditions (Shimizu *et al*, 1993; Eschalier *et al*, 2014; Kobayashi *et al*, 2017). Perivascular fibrotic tissue is rich in inflammatory cell infiltrate and is more prominent in conditions where endothelial damage predominates such as hypertensive heart disease (HHD) or diabetes (Hinglais *et al*, 1994; López *et al*, 2006). ECM production in perivascular fibrosis may have a greater role for endothelial to mesenchymal transition (Endo-MT; a debated process) (Zeisberg *et al*, 2007; Okayama *et al*, 2012), fibroblastic differentiation of pericytes (Kramann *et al*, 2015) and infiltration of inflammatory cells (Hinglais *et al*, 1994; Hara *et al*, 2002; Nevers *et al*, 2017). Perivascular fibrosis is also associated with abnormalities in coronary blood flow (Dai *et al*, 2012), and increased diffusion distance from the endothelium to cardiomyocytes reduces the diffusion of oxygen, fatty acids, glucose and signalling molecules such as NO (Nevers *et al*, 2017). However, differentiating the effects of interstitial and perivascular fibrosis is challenging as these processes typically coexist and for technical reasons are normally grouped together in analysis.

In human disease, myocardial interstitial fibrosis typically builds up over many years before manifesting clinically, leading to the important question of its reversibility. Fortunately, fibrosis does not exist as an inert, metabolically inactive tissue but rather undergoes continual remodelling controlled by the activity of fibroblasts, immune cells and proteolytic enzymes. The removal of pressure overload in animal studies results in positive myocardial remodelling with reduced interstitial collagen (Walther *et al*, 2001; Szardien *et al*, 2012). Similarly, human studies in patients with aortic stenosis have demonstrated a gradual reduction in interstitial cardiac fibrosis following aortic valve replacement (Villari *et al*, 1995; Treibel *et al*, 2018b). This positive remodelling has also been replicated using RAAS pathway inhibitors in hypertensive heart disease with associated improvement in cardiac haemodynamics (Brilla *et al*, 2000; Díez *et al*, 2002). Replacement fibrosis, unlike diffuse fibrosis, was not shown to resolve following treatment of aortic stenosis (Treibel *et al*, 2018b) which may be partly explained by increased collagen cross-linking within areas of replacement fibrosis which render the tissue resistant to collagenase mediated degradation (Frangogiannis, 2019). Interstitial fibrosis tends to be less heavily cross-linked than replacement fibrosis but increased

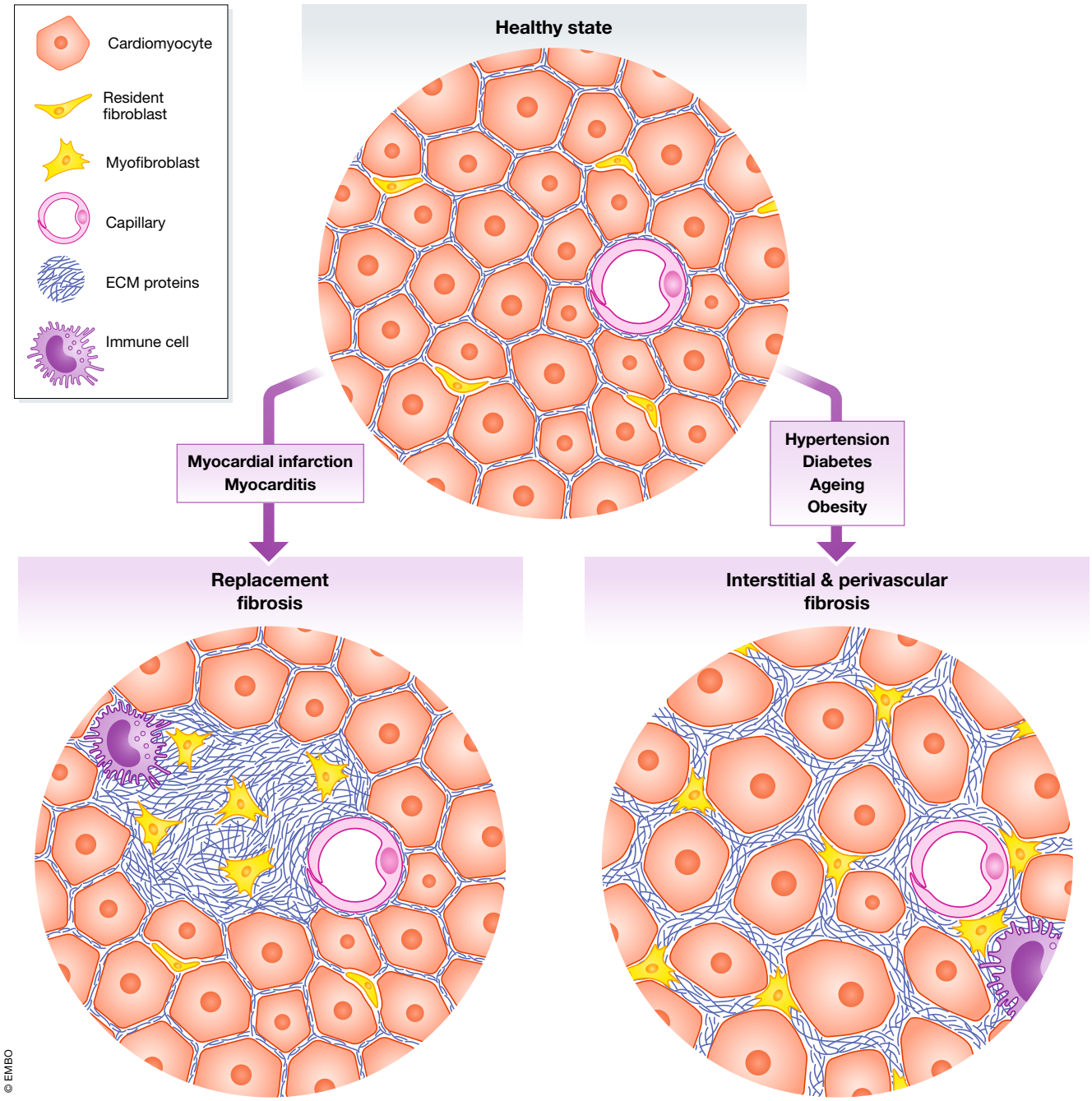

Figure 2. Histological differences between replacement fibrosis and interstitial/perivascular fibrosis.

Illustration of replacement vs. interstitial/perivascular fibrosis showing the differential spatial accumulation of ECM in the two forms of cardiac fibrosis along with altered cellular architecture, cardiomyocyte hypertrophy, inflammatory cell infiltration and activation of myofibroblasts.

cross-linking of interstitial fibrosis is associated with diabetes (Liu *et al*, 2003) and hypertension (Norton *et al*, 1997; Badenhorst *et al*, 2003; López *et al*, 2016). High levels of collagen cross-linking have been suggested as an explanation for lack of efficacy in trials of anti-fibrotics in HFpEF (Ravassa *et al*, 2018). Therefore, early identification and treatment of patients with HFpEF and cardiac fibrosis may be important for achieving optimal outcomes.

Monitoring of cardiac fibrosis in clinic

Given the heterogeneous pathophysiology of HFpEF, the ability to detect and monitor changes in fibrosis over time is optimal for identifying patients most likely to benefit from antifibrotic interventions and define the effectiveness of these treatments in clinical trials. The gold standard measure of cardiac fibrosis is histological analysis of endomyocardial biopsy (EMB) samples stained for collagen using

picrosirius red under polarized light (Whittaker *et al*, 1994). However, a number of limitations make this method impractical for routine use. Primarily, EMB samples only a small fraction of the myocardium which can lead to sampling error depending on the site of the biopsy and the site of pathology. EMB is an invasive procedure and associated with procedural risks (From *et al*, 2011). The safest and most accessible site for EMB is the right side of the interventricular septum which may not correlate with left ventricular pathology.

Surrogate markers have been developed to non-invasively quantify myocardial fibrosis and overcome some of the limitations of EMB. During ECM synthesis, collagen is released as a promolecule requiring cleavage of the amino and carboxyl-terminals by collagen peptidase to form mature collagen fibrils. These cleaved terminal peptides can be measured in serum to give an indication of the quantity of collagen formation. To date, the carboxyl-terminal of procollagen I (PICP) has shown the most promise in HFpEF. PICP is associated with raised collagen content on EMB, diastolic dysfunction and prognosis in HFpEF (Querejeta *et al*, 2000; López *et al*, 2015a,b). Alternative markers of collagen synthesis including the amino-terminal of procollagen I (PINP) and III (PIIINP) have been detected in serum and are elevated in hypertensive patients (Díez *et al*, 1995) and those with hypertrophic cardiomyopathy (HCM) (Lombardi *et al*, 2003). However PINP and PIIINP have not been convincingly associated with histological measures of cardiac collagen in HFpEF (López *et al*, 2015a,b). Similarly, the C-terminal telopeptide produced during the degradation of collagen I (CITP) can be measured in serum to provide a surrogate measure of collagen degradation which has shown an association with HFpEF symptoms (Martos *et al*, 2009). However, the extent to which these biomarkers, when measured peripherally, are representative of changes in myocardial collagen content is unclear. Studies sampling directly from the coronary sinus (CS) have yielded conflicting results regarding the cardiac contribution to the circulating levels of terminal peptides. PICP is elevated in CS samples from HHD patients (Querejeta *et al*, 2004); however, CS measurement of PINP, PIIINP and CITP do not show association with the burden of fibrosis measured by histology or cardiac magnetic resonance imaging (CMR) (Kupari *et al*, 2013; Nagao *et al*, 2018). Peripheral measurements of collagen biomarkers are more likely to represent a systemic profibrotic or inflammatory state than to specifically reflect the level of cardiac fibrosis. In spite of this, given the multisystemic nature of HFpEF, this does not preclude these markers from having role in prognosticating disease or guiding therapy (Krum *et al*, 2011) but caution must be exercised when drawing conclusions about cardiac collagen content from use of these biomarkers, and currently, they remain a research tool.

Advances in CMR have made the non-invasive quantification and localization of fibrosis within the myocardium possible, and CMR compares favourably with histological measures (Diao *et al*, 2016). CMR has many potential advantages compared to invasive histology based assessment; sampling errors seen with EMB are reduced as the entire myocardium is imaged, CMR is non-invasive and low risk allowing serial scans in the same patient to track progression or resolution of fibrosis over time, and structural and functional information can be collected as part of the same study.

Late gadolinium contrast enhancement (LGE) imaging has been used extensively to identify focal areas where volume of distribution is increased and contrast washout delayed which correlates with fibrotic areas histologically (Schelbert *et al*, 2010). Although LGE is primarily detects areas of replacement fibrosis, this still correlates with cardiac function in HFpEF across aetiologies including HHD (Rudolph *et al*, 2009; Krittayaphong *et al*, 2010), aortic stenosis (Nigri *et al*, 2009; Everett *et al*, 2020), diabetic cardiomyopathy (Kwong *et al*, 2008) and HCM (Bruder *et al*, 2010; Moravsky *et al*, 2013).

More recently, CMR measurement of the interstitial component of cardiac fibrosis has become possible due to the advent of T1 mapping (Löffler *et al*, 2019). T1 mapping uses precontrast and post-contrast magnetic resonance measurements from the myocardium to provide a quantitative assessment of the extracellular volume (ECV) within an area of myocardium. ECV is highly correlated with collagen content measured histologically (Diao *et al*, 2016; Duca *et al*, 2016), functional measures of diastolic function (Rommel *et al*, 2016) and prognosis in HFpEF (Mascherbauer *et al*, 2013; Schelbert *et al*, 2017). Clinical trials of antifibrotic HFpEF therapies are starting to use CMR-derived ECV measures in patient selection and as an outcome measure (Lewis *et al*, 2019). This may begin to address some of the long-standing issues with patient selection for HFpEF clinical trials (Kelly *et al*, 2015) ensuring these therapies are targeted at the group of patients most likely to benefit.

Cellular and molecular mechanisms of fibrosis

Myofibroblasts—the cellular driver of fibrosis

A central event in the development of fibrotic changes within the heart is the accumulation of activated myofibroblasts at the site of injury (Moore-Morris *et al*, 2014; Rockey *et al*, 2015; Kanisicak *et al*, 2016). Myofibroblasts exhibit two cardinal features: firstly, they secrete large amounts of ECM components, and secondly, via the expression of smooth muscle actin (SMA, otherwise known as ACTA2), they are contractile (Wynn, 2008; Rosenbloom *et al*, 2017). Together, these features result in the expansion of ECM, increased tissue stiffness and ventricular remodelling typical of cardiac fibrosis and HF.

The cellular origin of myofibroblasts *in vivo* has been debated extensively (Di Carlo & Peduto, 2018) with multiple candidates having been identified in the literature including epicardial or endothelial cells undergoing mesenchymal transition or migration of hematopoietic cells or pericytes into the interstitium (van Amerongen *et al*, 2008; Krenning *et al*, 2010; Widyantoro *et al*, 2010). Lineage tracking studies of myofibroblasts using periostin as a marker of myofibroblast activation have found that resident cardiac fibroblasts (CF) in the myocardium are the most significant contributor to the myofibroblasts population in cardiac injury with minimal input from extracardiac sources or endothelial structures (Acharya *et al*, 2012; Moore-Morris *et al*, 2014; Kanisicak *et al*, 2016).

Recent cell sorting experiments suggest that resident CF make up approximately 10% of the total cell number within the heart which is significantly less than previous estimates when fibroblasts were considered to be the most abundant cell within the heart (Banerjee *et al*, 2007; Pinto *et al*, 2016; Tallquist & Molkentin, 2017). This lack of consensus on the origin, definition and relative proportion of ECM-producing cells within the heart is in part due to the extensive heterogeneity among this cell type. Significant variation in synthetic

function, morphology and gene expression exists among CF depending upon their origin, anatomical site within the heart and state of activation. A variety of cell surface and intracellular markers has been used to identify these cells including fibroblast-specific protein (FSP1), DDR2, Sca-1, Thy-1, fibronectin and vimentin (Hudon-David *et al*, 2007; Ivey & Tallquist, 2016). However, no single marker is sufficiently comprehensive or specific (Kong *et al*, 2013), and as a result, combinations of cell markers have been used to define CF, although likely incompletely.

Two markers which appear to be relatively more specific to CF are the platelet-derived growth factor receptor α (PDGFRα) and the transcription factor Tcf21, both of which are expressed in the majority of myofibroblast-forming cells (Smith *et al*, 2011; Acharya *et al*, 2012; Pinto *et al*, 2016). PDGFR-α and Tcf21 are both expressed in the epicardial layer of the developing heart and are necessary for epithelial to mesenchymal transition of epicardial cells. Genetic knockout of Tcf21 leads to a paucity of CF within myocardium, and lineage tracking studies suggest an epicardial origin for a majority of resident CF (Smith *et al*, 2011; Acharya *et al*, 2012). An endothelial origin has been identified for a smaller proportion of resident CF using the endothelial marker Tie2 (Moore-Morris *et al*, 2014) which comprise approximately 10% of the left ventricular fibroblasts and have indistinguishable behaviour in response to cardiac injury (Acharya *et al*, 2012; Tallquist & Molkentin, 2017).

Although activation of myofibroblasts is of central importance in most profibrotic settings, recent evidence—particularly in the setting of metabolic disease, has suggested that increased ECM synthesis may occur in the absence of myofibroblast activation. High glucose-containing media increases collagen production from CF (Zhang *et al*, 2007; Gu *et al*, 2017) without myofibroblast activation. Animal studies in diabetic mice have indicated that ECM deposition is upregulated independently of myofibroblast activation and α-SMA expression and is not dependent on TGFβ stimulation (Alex *et al*, 2018). Furthermore, fibroblasts isolated from the atria of humans with type 2 diabetes have increased expression of collagen I in the absence of TGFβ stimulation (Sedgwick *et al*, 2014) indicating that CF from diabetic individuals may possess an inherently profibrotic phenotype.

Molecular processes in fibrosis

The molecular processes involved in fibrosis are expansive, complex and interacting, and a comprehensive description is beyond the scope of this review. However, certain groups of factors are frequently implicated in fibrosis and have consequently been explored as potential therapeutic targets. Chief among these is the TGFβ family of proteins, which are potent drivers of fibroblast-to-myofibroblast transition and powerful stimuli for ECM synthesis (Meng *et al*, 2016).

More recently, a search for factors acting downstream of TGFβ1 led to the re-evaluation of interleukin-11 (IL11) as a profibrotic molecule (Schafer *et al*, 2017). Similarly, Gal-3 (Shen *et al*, 2018) and processes resulting in oxidative stress have emerged as new antifibrotic targets (Somanna *et al*, 2016). A new and alternative paradigm has been to target the fibroblast itself, aiming to deplete myofibroblast populations (Aghajanian *et al*, 2019). Below, we describe attempts to produce antifibrotic therapies against the more established targets of the TGFβ family and vasoactive peptides before reviewing more novel targeting of IL11, Gal-3, oxidative stress or the activated myofibroblast itself.

Established targets

TGFβ inhibitors—effective but toxic

The TGFβ family of proteins are the most well established and potent activators of profibrotic effects in cells, have been implicated in virtually all forms of fibrosis and have been described as the "master regulators" of fibrosis (Meng *et al*, 2016). TGFβ is a ubiquitously expressed protein with an expansive range of biological effects including enhanced ECM synthesis, cell differentiation, apoptosis, angiogenesis and immune cell function depending on the site of action. Myocardial TGFβ expression is consistently upregulated in the heart of patients with HFpEF irrespective of aetiology including HCM (Li *et al*, 1997), HHD (Almendral *et al*, 2010) and aortic stenosis (Hein *et al*, 2003). There are three isoforms of TGFβ of which TGFβ1 is the most well studied in the context of fibrosis. TGFβ is secreted as an inactive peptide due to the presence of the bound latency-associated peptide (LAP) which prevents it accessing its receptor (Taipale *et al*, 1994) and is further sequestered into the structure of the ECM by latency TGFβ binding proteins. Dissociation of LAP from TGFβ is an important step in the profibrotic response which occurs in response to tissue damage, inflammation or profibrotic signals and is mediated by a variety of factors which are upregulated in patients with HFpEF. These factors include MMP-2, MMP-9, ADAMTS16 and plasmin which have protease activity that cleaves LAP from the TGFβ molecule (Khalil *et al*, 1996; Yu & Stamenkovic, 2000; Wang *et al*, 2006a; Yao *et al*, 2020). Thrombospondins (Reed *et al*, 1995), ROS (Barcellos-Hoff & Dix, 1996) and specific integrins (Munger *et al*, 1999; Wipff *et al*, 2007) induce a conformational change in the LAP which exposes the receptor binding site on the TGFβ molecule. Constituently, active forms of TGFβ, resistant to LAP inactivation, have been used in both large and small animal models to stimulate atrial and/or ventricular cardiac fibrosis resulting in HF, arrhythmias and reduced survival (Nakajima *et al*, 2000; Verheule *et al*, 2004; Accornero *et al*, 2015; Polejaeva *et al*, 2016).

Cellular signalling of TGFβ in fibrosis is mediated by the membrane-bound TGFβRII and can involve the canonical, SMAD-dependent pathway or non-canonical, SMAD-independent pathway. In the SMAD-dependent pathway, TGFβ binding induces the formation of a heterotetrameric complex between with TGFβRII and TGFβRI (also known as ALK-5) molecules. Formation of this complex activates the phosphorylation activity of the receptor and activates SMAD2 and SMAD3 (Wells *et al*, 1999), which dissociate from the receptor and complex with SMAD4 in the cytoplasm (Derynck & Zhang, 2003). This SMAD complex translocates to the nucleus where it binds to SMAD binding elements in the genome to act as a transcription factor independently (Dennler *et al*, 1998; Martin-Malpartida *et al*, 2017) or in combination with multiple other transcription factors (Zhang *et al*, 1998; Mullen *et al*, 2011). Within the fibroblast, activation of the canonical pathway results in myofibroblast differentiation (Khalil *et al*, 2017) and upregulation of multiple profibrotic genes including collagen, smooth muscle actin and periostin, along with IL11 (Schafer *et al*, 2017). Non-canonical signalling mediates similar profibrotic effects in fibroblasts (Chen

et al, 2005; Dolivo et al, 2019), and significant cross-talk exists between these pathways (Engel et al, 1999; Funaba et al, 2002). The mitogen-activated protein kinase (MAPK) pathways—including ERK (Lee et al, 2007), p38 (Molkentin et al, 2017) and JNK (Yoshida et al, 2005)—are chief mediators of this non-canonical response. Blocking this signalling using transgenic mice or specific inhibitors of the MAPK pathways reduces myofibroblast formation and ECM production (Gao et al, 2013; Xu et al, 2017).

TGFβ signalling within the cardiomyocyte also has effects on cardiac remodelling particularly via the non-canonical p38 pathway (Gao et al, 2013; Xu et al, 2017) which influences release of profibrotic mediators in response to stress (Koitabashi et al, 2011) and upregulates genes related to cardiomyocyte hypertrophy (Matsumoto-Ida et al, 2006). The canonical TGFβ signalling pathway within cardiomyocytes provides important survival signals and maintains contractility during cell stress (Wang et al, 2005; Umbarkar et al, 2019) which may underlie toxicity when inhibiting this pathway.

In vivo, transgenic mice with fibroblast-specific disruption of TGFβR1/2 or SMAD3 protect against cardiac fibrosis and improve diastolic function in response to transverse aortic constriction (TAC) (Khalil et al, 2017). Similarly, mice with a single functional allele of the TGFβ gene are relatively resistant to age-related cardiac fibrosis and have an increased lifespan compared to wild-type mice (Brooks & Conrad, 2000) suggesting a potential therapeutic target. These findings have been replicated in preclinical studies over the last two decades employing either neutralizing monoclonal antibodies (mAb) against TGFβ (Kuwahara et al, 2002; Teekakirikul et al, 2010) or small molecule kinase inhibitors targeting TGFβRI (Kuwahara et al, 2002; Derangeon et al, 2017) which have markedly reduced cardiac fibrosis and improved LV compliance in rodent models of HFpEF. However, the multifunctional role of TGFβ provides a significant challenge—repeatedly encountered with anti-TGFβ therapy in both animal and human studies—of on-target toxicities that are dose-limiting thus hindering treatment efficacy.

The problems encountered with anti-TGFβ therapies are highlighted in *Tgfb1* knockout (KO) mice and humans. Mice have high embryonic lethality and those that survive to birth die between 3 and 5 week of age due to an excessive and widespread inflammatory response resulting in multiorgan failure (Shull et al, 1992; Kulkarni et al, 1993). In humans, biallelic loss of function mutations of TGFB1 gene results in severe colitis and neurological deficits with frequent seizures and cerebral atrophy (Kotlarz et al, 2018). Cardiovascular toxicity is common in trials targeting TGFβ which may relate, in part, to the protective role played by TGFβ in the cardiomyocyte. Umbarkar et al, using a tamoxifen inducible cardiomyocyte-specific KO of *Smad4*, found that these mice developed LV dilation and HF following tamoxifen treatment (Umbarkar et al, 2019). Similar effects have been seen with global inhibition of the TGFβ pathway using mAbs (Frantz et al, 2008; Koitabashi et al, 2011), kinase inhibitors (Engebretsen et al, 2014) or global *Smad3* KO animals (Divakaran et al, 2009). Although surviving animals have reduced cardiac fibrosis in response to MI or TAC, in the acute phase there are increased rates of LV dilation and increased mortality (Divakaran et al, 2009).

Human studies have been undertaken using fresolimumab—a mAb which neutralizes all three isoforms of TGFβ; however, this has shown minimal benefit in renal fibrosis or scleroderma and was associated with the development of keratoacanthomas in the skin (Lacouture et al, 2015; Rice et al, 2015; Vincenti et al, 2017). Additionally, other pan-TGFβ neutralizing antibodies have been associated with fatal haemorrhage in both mice and non-human primates and have again demonstrated significant cardiovascular toxicity with inflammatory infiltration of the coronary vessels, aorta and heart valves (Mitra et al, 2020). Selective targeting of the TGFβ1 isoform has not improved the toxicity profile with dose-related effects seen on epithelial hyperplasia, enteropathy and renal tubular inflammation in non-human primate studies (Brennan et al, 2018). When translated to humans, lower doses were tolerated in trials of diabetic nephropathy; however, this was at the expense of efficacy as progression of renal dysfunction was not reduced (Voelker et al, 2017). Small molecule inhibitors targeting the TGFβRI have produced similar toxicity concerns including degenerative changes of heart valves and aorta and mucosal inflammation in the intestine (Anderton et al, 2011; Stauber et al, 2014). Recently, a therapeutic window has been identified for the use of the TGFβRI inhibitor, galunisertib, in the treatment of myelodysplasia; however, this requires a stringent intermittent dosing schedule to avoid cardiac toxicity (Kovacs et al, 2015; Santini et al, 2019) and efficacy as an antifibrotic has not been tested.

In summary, TGFβ signalling is strongly and irrefutably profibrotic *in vitro* and *in vivo* and targeting this pathway directly or indirectly has potent antifibrotic effects. However, the toxicity profile associated is consistently too high and is sufficiently dose-limiting to render the treatment ineffective for human disease, to date.

Renin/angiotensin/aldosterone system inhibitors—mainstay therapy in heart failure

Targeting the RAAS has been a pillar of HF treatment for over 30 years and is perhaps the seminal success of modern day disease-modifying therapy in HFrEF (Swedberg, 1987; Pfeffer et al, 1992; Pitt et al, 1999). Classically, angiotensin II (AngII) is a profibrotic circulating factor produced by the serial actions of renin- and angiotensin-converting enzymes (ACE) on angiotensinogen. In CF, AngII treatment stimulates myofibroblast formation and ECM production (Brilla et al, 1994; Siddesha et al, 2013). AngII signalling in the heart is mediated through G protein-coupled receptors (GPCR) designated angiotensin receptor type 1 (AT1R) and type 2 (AT2R). The intracellular effect of AngII involves Gq-mediated activation of phospholipase C, β-arrestin signalling and transactivation of other membrane-bound growth factor receptors. AT1R-mediated β-arrestin signalling (McDonald et al, 2000; Rakesh et al, 2010) and transactivation of PDGFR and epithelial growth factor receptor (EGFR) (Mondorf et al, 2000; Schellings et al, 2006) have all been shown to activate MAPK pathways in CF (Schorb et al, 1995). AT1R stimulation has also been shown to directly augment the SMAD-dependent pathways of TGFβ signalling (Wang et al, 2006b) and in concert with endothelin-1 (ET-1) (Fujisaki et al, 1995) and largely indirectly—induces hypertrophic changes in cardiomyocytes (Gray et al, 1998). In rodents, AngII infusion is a well established *in vivo* model for stimulating cardiac fibrosis and has been used in many 100s of publications (Sun et al, 1997). This profibrotic of effect AngII is maintained even at subpressor doses which despite not increasing blood pressure results in fibrosis accumulation and diastolic dysfunction (Regan et al, 2015).

AT1R antagonists or ACE inhibitors (ACE-I) have in multiple settings reduced ECM production, cardiac hypertrophy and HF in both cell culture and animal models (Pahor *et al*, 1991; Brilla *et al*, 1994; Ham *et al*, 2018). In short-term human studies of HHD, following 6 months of ACE-I treatment the myocardial collagen content on EMB and echocardiographic features of diastolic function were reduced compared to treatment with antihypertensive alone (Brilla *et al*, 2000). However, multiple clinical trials in HFpEF have failed to demonstrate a mortality benefit or reduction in hospitalization with AngII inhibition, suggesting that this approach is insufficient to block the activity of the multiple profibrotic pathways which are active in HFpEF and suggests a high degree of redundancy within this system (Yusuf *et al*, 2003; Cleland *et al*, 2006; Martin *et al*, 2018).

Mineralocorticoid receptor antagonists—old drugs but effective

Aldosterone binds to the mineralocorticoid receptor (MR) in the cytoplasm and translocates to the nucleus where it complexes with a variety of co-activators and is responsible for upregulating profibrotic genes. Additionally, aldosterone has multiple non-transcriptional dependent effects which occur more rapidly and can occur independently of the MR (Mihailidou *et al*, 2004; Markos *et al*, 2005). In particular, AngII and aldosterone work synergistically to produce a potent profibrotic effect (Lemarié *et al*, 2009). Aldosterone augments AngII signalling by upregulation of MAPK pathways in both cardiomyocytes (Tsai *et al*, 2013; Somanna *et al*, 2015) and CF (Stockand & Meszaros, 2003; Lemarié *et al*, 2009) in a process dependent on G protein-coupled receptor kinases (Cannavo *et al*, 2016). Aldosterone has been implicated in multiple other processes linked to HFpEF including production of ROS (Hayashi *et al*, 2008) and development of a pro-inflammatory infiltrate within the heart during pressure overload by promoting differentiation of profibrotic "M2" macrophages (Rickard *et al*, 2009) and infiltration of profibrotic T cells (Li *et al*, 2017).

In vivo studies in HFpEF models have been promising with inhibition of this pathway using either cardiomyocyte-specific KO of the MR (Lother *et al*, 2011; Rickard *et al*, 2012) or specific MR antagonists (MRA) (Nishioka *et al*, 2007; Leader *et al*, 2019) demonstrating reduced ECM production and improved LV function. Mechanistic studies in humans with MRA have mirrored these results showing a reduction in myocardial collagen accumulation histologically or using ECV on CMR (Table 1; Kosmala *et al*, 2011; McDiarmid *et al*, 2020).

However—as with ACE-I—rodent and intermediate phenotype studies have so far failed to translate to improved outcomes in large randomized controlled trials (RCT) of MRA in HFpEF (Edelmann *et al*, 2013; Pitt *et al*, 2014). A notable caveat is that post hoc analysis of the TOPCAT trial of MRA in HFpEF suggested that hospitalization and symptoms may be improved in subgroups of the population (Girerd *et al*, 2016) and that markers of collagen turnover, PICP and PIIINP are reduced (Kosmala *et al*, 2011; Ravassa *et al*, 2018; Xiang *et al*, 2019). Despite this, in the absence of positive prospective RCT data in HFpEF, conclusive evidence for a clinically meaningful cardiac antifibrotic effect of MRA remains elusive.

Neprilysin inhibitors—the "new" old

The natriuretic peptides (NP), atrial natriuretic peptide and brain natriuretic peptide (BNP) are released by cardiomyocytes in response to stress including mechanical stretch or stimulation by profibrotic factors (Liang & Gardner, 1999; Pikkarainen *et al*, 2003). The effects of NP provide endogenous antifibrotic, vasodilatory and natriuretic effects which counters many of the deleterious effects of the RAAS (Kerkelä *et al*, 2015). Natriuretic peptide receptors A (NPRA), B (NPRB) and C (NPRC) are guanylyl cyclase-coupled receptors, with NPRA being the most relevant in cardiovascular disease (Kerkelä *et al*, 2015). NP binding increases intracellular cGMP and decreases cAMP and IP_3 which counters the profibrotic and hypertrophic signalling in fibroblasts and cardiomyocytes (Fujisaki *et al*, 1995). This protective role is highlighted by the increased fibrosis and LV hypertrophy which occurs animals with KO of BNP gene (*Nppb*) or NPRA gene (*Npr1*) in response to AngII infusion or TAC (Tamura *et al*, 2000; Patel *et al*, 2005; Parthasarathy *et al*, 2013).

Exogenous administration of recombinant human BNP has been trialled in patients with acute HF; however, no significant survival or rehospitalization benefit was demonstrated in these trials (O'Connor *et al*, 2011). The half-life of circulating NPs is under 20 mins as the molecules are readily degraded by the widely expressed membrane-bound peptidase, neprilysin (Charles *et al*, 1996). Consequently, NPs can be used only as a continuous infusion, unsuitable for use in chronic HF. Inhibitors of the neprilysin peptidase have instead been employed to prolong the half-life of endogenously produced NPs and have shown promise. Treatment with the combination of angiotensin receptor blockers (ARB) and neprilysin inhibitors in diabetic mice results in reduced interstitial fibrosis and cardiomyocyte hypertrophy (Suematsu *et al*, 2016). Further *in vitro* experiments have shown that, in contrast to angiotensin receptor inhibition which primarily reduces fibroblast proliferation, the NP system more potently inhibits myofibroblasts activation in response to profibrotic stimuli therefore providing a complimentary antifibrotic effects on the CF (Burke *et al*, 2019).

Clinical trials of the ARB—neprilysin inhibitor (ARNI) combination of valsartan and sacubitril, have demonstrated significant efficacy in reducing symptoms, hospitalization and mortality in HFrEF in addition to standard therapy, including RAAS inhibition (McMurray *et al*, 2014). Subgroup analysis showed that an effect on fibrosis may be responsible, in part, for the improvement in outcomes: MMP-2 and MMP-9 levels were lower in treated patients compared to controls, and PINP was also reduced in the treatment group (Zile *et al*, 2019). However disappointingly, a recent RCT in HFpEF failed to meet its primary endpoints of reducing hospitalization for HF or death from cardiovascular causes (Solomon *et al*, 2019). Exploratory subgroup analysis of this trial has yielded some interesting results in particular a significant improvement in the primary outcome in women (Solomon *et al*, 2019) which is particularly intriguing given the high burden of HFpEF in women (Vasan *et al*, 2018), and this finding may stimulate further investigation into sex-specific differences in the development of cardiac fibrosis.

New directions

Given the issues that have emerged with established fibrosis targets, including the lack of clinical benefit in multiple large clinical trials in HFpEF and the toxicities associated with TGFβ therapy, once the front runner, there is a need to identify alternative approaches and targets for fibrosis. This may include augmenting the effects of

Table 1. Clinical trials of drugs where mode of action includes the potential to target cardiac fibrosis that is shown here as an endpoint outcome.

Treatment	Duration	Population	Measure of fibrosis	*N* Rx vs. placebo	Year	Fibrosis-related outcome	PMID/NCT
Mineralocorticoid receptor antagonists							
Spironolactone	6 months	HFrEF	PINP/PIIINP	81 vs. 70	2000	Reduced PINP/PIIINP	11094035
Spironolactone	12 months	HFrEF—DCM	PICP CVF on EMB	13 vs. 0	2005	Reduced PICP/CVF	16275882
Spironolactone	3 months	IHD	PIIINP	98 vs. 98	2007	Reduced PIIINP	17921831
Eplerenone	6 months	HFpEF	PINP	22 vs. 22	2011	Reduced PINP Improved diastolic function	21807324
Spironolactone	6 months	HFpEF—obesity	PICP/PIIINP	58 vs. 55	2013	Reduced PICP/PIIINP Improved diastolic function	23343682
Spironolactone	6 months	HFpEF—female	PIIINP	24 vs. 24	2014	Reduced PIIINP Improved diastolic function	24905296
Spironolactone	Variable	HFpEF	PICP	167 vs. 161	2015	Reduced PICP Improved diastolic function	26459931
Canrenone	6 months	HFpEF	PIIINP	197 vs. 197	2017	Negative	28855452
Spironolactone	12 months	HCM	PINP/PIIINP LGE on CMR	26 vs. 27	2018	Negative	29604289
Spironolactone	12 months	HFpEF	PICP	190 vs. 180	2018	Reduced PCIP levels Improved diastolic function	29709099
Spironolactone	6 months	HFpEF	ECV on CMR	19 vs. 21	2019	Negative	31852424
Spironolactone	24 months	HCM	LGE on CMR	130 vs. 130	Ongoing	–	NCT02948998
Angiotensin inhibition							
Lisinopril	6 months	HHD	CVF on EMB	18 vs. 17	2000	Reduced CVF Improved diastolic function	10993857
Losartan	12 months	HHD	CVF on EMB	19 vs. 0	2002	Reduced CVF & improved diastolic function in severe fibrosis	12034658
Losartan	6 months	HFpEF—ESRF	PICP	13 vs. 13	2005	Reduced PICP	16471172
Enalapril	6 months	HFpEF—ESRF	PICP	13 vs. 13	2005	Negative	16471172
Irbesartan	12 months	HHD	PICP	56 vs. 58	2007	No difference PICP Improved diastolic function	17762662
Irbesartan	6 months	HFpEF	PIIINP	149 vs. 164	2011	Negative	21750125
Candesartan	3-4 months	HFrEF—Anthracycline	ECV on CMR	38 vs. 32	2018	Reduced ECV	29106497
Ramipril	36 months	ARVC	MMP, TIMP	60 vs. 60	Ongoing	–	29574980
Valsartan	24 months	HCM	ECV on CMR	75 vs. 75	Ongoing	–	28454798
Vasodilators							
Sildenafil	6 months	HFpEF	PIIINP	113 vs. 103	2014	Negative	23478662
Isosorbide dinitrate	6 months	HFpEF	ECV on CMR	13 vs. 16	2017	Negative	28219917
Isosorbide dinitrate + Hydralazine	6 months	HFpEF	ECV on CMR	15 vs. 16	2017	Negative	28219917
Neprilysin inhibitors							
Sacubitril-Valsartan	9 months	HFpEF	PIIINP/MMP2	149 vs. 152	2016	Negative	26754625

Table 1 (continued)

Treatment	Duration	Population	Measure of fibrosis	*N* Rx vs. placebo	Year	Fibrosis-related outcome	PMID/NCT
Diuretics							
Torasemide	8 months	HFrEF	CVF on EMB PICP	19 vs. 17	2004	Reduced in CVF Reduced PICP	15172408
Torasemide	8 months	HFpEF	PICP	77 vs. 78	2011	Negative	21906812
Torasemide	9 months	HFpEF	PICP	17 vs. 18	2017	Negative	28891228
Antihyperglycaemic							
Empagliflozin	6 months	T2DM	ECV on CMR	35 vs. 0	2019	Negative	31653956
Dapagliflozin	12 months	T2DM	ECV on CMR	30 vs. 30	Ongoing	–	NCT03782259
Metformin	24 months	HFpEF (metabolic syndrome)	TIMP1	27 vs. 27	Ongoing	–	24515256
Statins							
Rosuvastatin	6 months	HFrEF	PINP/PIIINP	32 vs. 37	2011	Negative	20085851
Atorvastatin	6 months	HFrEF	PIIINP	28 vs. 28	2012	Reduction in PIIINP levels	22154198
Other							
Vitamin D3	6 weeks	HFrEF	PINP/PIIINP	50 vs. 51	2013	Negative	23895820
Mirabegron	12 months	HFpEF	ECV on CMR	148 vs. 148	Ongoing	–	29932311
Supervised exercise program	4 months	Mixed	ECV on CMR	60 vs. 30	Ongoing	–	NCT03084679
Pirfenidone	12 months	HFpEF	ECV on CMR	65 vs. 65	Ongoing	–	31069575
Stem cell therapy							
Allogenic Mesenchymal Stromal Cells	12 months	HFrEF—Anthracycline	ECV on CMR	21 vs. 15	Ongoing	–	29910056

ARVC, arrhythmogenic right ventricular cardiomyopathy; CVF, collagen volume fraction; LGE, late gadolinium enhancement; NCT, ClinicalTrials.gov registry number; PMID, PubMed identification number; TIMP, tissue inhibitor of metalloproteases.

currently used treatments, repurposing of drugs which have been proven to be effective in other fibrotic diseases and novel approaches and targets which may provide a much needed alternative antifibrotic treatment.

Alternative angiotensin inhibitors

Traditional RAAS inhibition has limited effect in treating cardiac fibrosis in HFpEF; hence, the role of ACE-independent AngII production has been explored as an alternative strategy. Chymases are proteolytic enzymes released predominantly by neutrophils but also by cardiomyocytes and fibroblasts (Urata *et al*, 1990). Chymases activate angiotensinogen via an alternative pathway independent of ACE bypassing the effect of ACE-I (Prosser *et al*, 2009; Ahmad *et al*, 2016; Froogh *et al*, 2017). Furthermore, there is evidence that this alternative pathway for AngII production can occur intracellularly without the need for the membrane-bound receptor thereby also evading the action of ARBs (Ferrario *et al*, 2005; Baker & Kumar, 2006). This *intracrine* pathway may be especially important in diabetes (Singh *et al*, 2008) and in hypertension where chymase-dependent intracellular AngII synthesis is upregulated (Tadevosyan *et al*, 2017).

In animal models, chymase inhibitors reduce TGFβ expression, ECM matrix deposition and improve diastolic parameters in the setting of myocarditis or tachycardia-mediated fibrosis (Matsumoto *et al*, 2003; Palaniyandi *et al*, 2007; Wei *et al*, 2010) despite having no significant effect on blood pressure in rodents or humans (Kirimura *et al*, 2005; Kanefendt *et al*, 2019). Early clinical trials of oral chymase inhibitors are ongoing, they appear safe in phase I studies (Kanefendt *et al*, 2019), and this could represent a promising future additive therapy to target the angiotensin pathway.

Small molecule inhibitors of generic fibrosis pathways

Treatment of idiopathic pulmonary fibrosis (IPF) has been transformed by the use of the small molecule inhibitors pirfenidone and nintedanib (King *et al*, 2014; Flaherty *et al*, 2019), and tranilast has been used in the treatment of asthma and keloid scars for over 30 years in Japan (Darakhshan & Pour, 2015). All three drugs also have antifibrotic effects outside the lung in animals (Seniutkin *et al*, 2018; Susutlertpanya *et al*, 2019), and repurposing these drugs for the treatment of cardiac fibrosis is being actively explored.

Pirfenidone and tranilast, through unclear mechanisms (Aimo *et al*, 2020), both reduce the expression and secretion of TGFβ in CF *in vitro* and subsequently reduce myofibroblast transformation in response to profibrotic stimulation (Martin *et al*, 2005; Shi *et al*, 2011). *In vivo* studies in mice with TAC, AngII infusion, diabetes and doxorubicin induced models of cardiomyopathy demonstrated a protective effect of pirfenidone on myocardial collagen accumulation and LV function without the early increased mortality which is seen in the acute phase with *Tgfb1* KOs or TGFβRI inhibitors (Giri *et al*, 2004; Yamazaki *et al*, 2012; Wang *et al*, 2013). Similarly, tranilast reduced the expression of TGFβ in canine models of atrial fibrosis induced by tachypacing and prevented the development of atrial arrhythmias (Nakatani *et al*, 2013).

Pirfenidone and tranilast are already approved for use in humans, and toxicity profiles are well understood. Long-term pirfenidone treatment requires surveillance for hepatotoxicity; however, it is generally well tolerated and tranilast has limited toxicities (Lancaster *et al*, 2017). The PIROUETTE trial is a clinical study currently ongoing in the UK to investigate the antifibrotic effect of pirfenidone in HFpEF using CMR measures of fibrosis (Lewis *et al*, 2019). Importantly, this trial is selecting patients with HFpEF based on fibrosis burden using ECV on CMR which will provide a stratified population with proven cardiac fibrosis in contrast to the more heterogeneous populations normally component of HFpEF trials (McMurray & O'Connor, 2014).

Nintedanib, a tyrosine kinase inhibitor, has a wide range of targets including the vascular endothelial growth factor receptor, PDGF and fibroblast growth factor receptor (Hilberg *et al*, 2008). Its effects on the fibroblast prevent ECM production and myofibroblast activation (Hostettler *et al*, 2014; Rangarajan *et al*, 2016). In animal studies of pulmonary hypertension, nintedanib reduces right heart fibrosis and remodelling (Rol *et al*, 2019). However, studies specifically in HFpEF have yet to be done.

Galectin-3 inhibitors

Gal-3 is a member of the lectin family of carbohydrate binding molecules which stimulates ECM production and myofibroblast activation when applied to CF (Shen *et al*, 2018). The effects of Gal-3 increase macrophages infiltration into the myocardium (Sharma *et al*, 2004) and increase oxidative stress secondary to the upregulation of NADPH oxidase 4 (NOX4) (He *et al*, 2017) and downregulation of antioxidant molecules (Ibarrola *et al*, 2018).

Serum levels of Gal-3 are elevated in HF, and levels are correlated with ventricular dysfunction, arrhythmias and mortality (Ho *et al*, 2012; Wu *et al*, 2015). Multiple profibrotic *in vivo* models increase the expression of Gal-3 in the heart including TAC, AngII or aldosterone infusion (Calvier *et al*, 2013; Song *et al*, 2015; Frunza *et al*, 2016). The initial work on Gal-3 by Sharma *et al* (2004) demonstrated that exogenous infusion of Gal-3 into the pericardial space results in extensive deposition of myocardial collagen and LV dysfunction in rats. Interference with Gal-3 signalling by transgenic KO or specific Gal-3 inhibitors blunts the fibrotic response and prevents LV deterioration in rodent models of pressure overload, hypertension and obesity-related cardiac fibrosis (Yu *et al*, 2013; Calvier *et al*, 2015; Martínez-Martínez *et al*, 2015).

As yet, there have been no clinical trials investigating the effect of Gal-3 inhibitors in HF. However, trials in a number of non-cardiac organ systems are ongoing including pulmonary fibrosis (NCT02257177) non-alcoholic steatohepatitis (NCT02421094) and psoriasis (NCT02407041).

Oxidative stress

Oxidative stress is increased in the heart in all forms of HF, and increased ROS is associated with decompensation of HF (Hill & Singal, 1996; Mallat *et al*, 1998), activation of the MAPK pathways (Tanaka *et al*, 2001; Qin *et al*, 2003) and increased interstitial fibrosis (Cheng *et al*, 2003; Lijnen *et al*, 2006). Mice with transgenic knockout of the ROS scavenger, superoxide dismutase, rapidly develop cardiac fibrosis and die within 10 days of life (Li *et al*, 1995). In contrast, overexpression of this enzyme prevents the development of cardiac fibrosis in aged mice (Kwak *et al*, 2015). The main sources of ROS in HF are derived from mitochondria dysfunction, NADPH oxidases (NOX, particularly NOX2 and 4), nitric oxide synthase and xanthine oxidases (Ide *et al*, 2000; Moris *et al*, 2017). Inhibition of the NOX enzymes in particular has emerged as an intriguing antifibrotic target. NOX activity has been found to be upregulated in the explanted hearts of patients with end-stage HF (Heymes *et al*, 2003; Nediani *et al*, 2007). Multiple profibrotic factors including AngII (Byrne *et al*, 2003; Johar *et al*, 2006), ET-1 (Duerrschmidt *et al*, 2000) and aldosterone (Johar *et al*, 2006) infusion as well as TAC (Kai *et al*, 2006; Ago *et al*, 2010) upregulate the activity of NOX enzymes resulting in increased ROS production in mice.

NOX2 and NOX4 are the major isoforms responsible for ROS production in the heart and are expressed in cardiomyocytes, endothelial cells and fibroblasts (Lassègue *et al*, 2012; Matsushima *et al*, 2016). Transgenic global KO of *Nox2* reduces cardiac fibrosis in response to AngII or aldosterone infusion compared to wild-type animals (Byrne *et al*, 2003; Johar *et al*, 2006). However, NOX2 has an important role in the bactericidal effects of phagocytic cells, and hence, NOX2 deficiency results in granulomatous disease in both mice and humans, making it a challenging therapeutic target (O'Neill *et al*, 2015).

Nox4 KO mice are phenotypically normal displaying only moderately increased body weight and exhibit no notable immune dysfunction (Carnesecchi *et al*, 2011). At a cellular level in CF, NOX4 expression is elevated in response to TGFβ signalling (Cucoranu *et al*, 2005) and knockdown of NOX4 activity using siRNA or small molecule inhibitors reduces fibrosis and myofibroblasts differentiation in response to TGFβ or AngII stimulation (Cucoranu *et al*, 2005; Chan *et al*, 2013; Somanna *et al*, 2016). TAC-mediated myocardial fibrosis is enhanced in mice by transgenic overexpression of *Nox4* (Kuroda *et al*, 2010), and cardiomyocyte-specific KO is protective against myocardial fibrosis and left ventricular dysfunction (Kuroda *et al*, 2010; Zhao *et al*, 2015). However, conflicting results have emerged, suggesting that pleiotropic roles of NOX4 in angiogenesis and fatty acid oxidation may be adaptive in the stressed heart and that inhibition of *Nox4* may be detrimental in some contexts (Zhang *et al*, 2010; Nabeebaccus *et al*, 2017; Schnelle *et al*, 2019), and it may demonstrate on-target toxicities in other systems, such as promoting atherosclerosis (Schürmann *et al*, 2015).

Recent translational trials of the NOX1/4 inhibitor GK137831 in humans were well tolerated in diabetic nephropathy however failed to show a significant reduction in albuminuria at relatively low dose of the drug (Reutens *et al*, 2019; NCT02010242). Further studies in diabetic nephropathy (Reutens *et al*, 2019) and idiopathic pulmonary fibrosis (NCT03865927) are planned, and trials of specific NOX inhibitors in cardiac fibrosis may follow depending on the tolerability and success of these trials.

SGLT2 inhibitors

SGLT2 inhibitors (SGLT2-I) are a class of antidiabetic drugs that demonstrated unexpectedly beneficial effects in HF during the EMPA-Reg trial in diabetes management (Zinman *et al*, 2015). This has subsequently been confirmed to be a class effect in alternative SGLT2 inhibitor trials (Neal *et al*, 2017; McMurray *et al*, 2019), and much interest has since surrounded the use of these drugs in the management of fibrosis and HFpEF, with or without comorbid diabetes mellitus.

SGLT2 is a membrane transporter acting primarily in the proximal convoluted tubule of the kidney to reabsorb glucose and sodium (Kalra, 2014). Mouse studies demonstrated a reduction in cardiac collagen content and improved indices of diastolic function with SGLT2-I in both diabetic (Habibi *et al*, 2017; Ye *et al*, 2017; Li *et al*, 2019) and non-diabetic animals (Lee *et al*, 2019; Oh *et al*, 2019).

However, the mechanism responsible for these cardiovascular effects remains elusive particularly as the SGLT2 receptor is not expressed within the heart (Di Franco *et al*, 2017). A recent study in mice demonstrated that NOX4 expression was reduced following SGLT2-I treatment with an associated reduction in myocardial oxidative stress (Li *et al*, 2019) and improvement in both systolic and diastolic function in the diabetic mouse heart (Osorio *et al*, 2012; Kusaka *et al*, 2016).

There are also a number of potentially beneficial off-target effects which may play a role in the antifibrotic effects of SGLT2 inhibition. This includes the sodium hydrogen exchanger (Baartscheer *et al*, 2017) and the SGLT1 channels (Zhou *et al*, 2003; Di Franco *et al*, 2017) both of which are expressed within the heart, are involved in myocardial hypertrophy and fibrosis and are targeted by SGLT2-I. A multitude of beneficial systemic effects are also associated with SGLT2-Is including reduction in blood pressure, blood sugar and reduced renal RAAS expression in diabetic mice (Georgianos & Agarwal, 2019; Woods *et al*, 2019), and it is likely that the beneficial effects on cardiac health are multifactorial (Chin *et al*, 2019).

Clinical studies in HFpEF have demonstrated an improvement in LV mass and diastolic dysfunction following 3 months of SGLT2-I treatment (Verma *et al*, 2016). However, ECV measured using CMR was not improved after 6 months of SGLT2 inhibition (Hsu *et al*, 2019). RCTs are currently underway in patients with HFpEF (NCT03619213, NCT03057951), and further mechanistic studies will be vital to improve understanding into the mechanisms involved in these apparent antifibrotic effects.

Interleukin-11

IL11 was recently identified as a critical regulator of the TGFβ pathway and cardiac fibrosis: in the absence of IL11 activity, TGFβ cannot exert a profibrotic effect on human cardiac fibroblasts (Schafer *et al*, 2017). Cell culture experiments with human atrial fibroblasts found that stimulation with TGFβ increases the expression of IL11 mRNA by 8.4-fold on average, making IL11 the most highly upregulated gene downstream of TGFβ/SMAD signalling in CFs. Similar findings have been reported for lung fibroblasts, hepatic stellate cells and coronary artery VSMCs (Lebastchi *et al*, 2011; Schafer *et al*, 2017; Ng *et al*, 2019; Widjaja *et al*, 2019b).

IL11 is a member of the interleukin-6 (IL6) family of cytokines but has distinct properties from other family members. In the heart, the IL11 receptor (IL11RA1) is highly expressed on fibroblasts (Schafer *et al*, 2017), and following binding of IL11 to its receptor, it then binds gp130 and results in dimerization of a hexameric receptor complex. Canonical gp130 signalling—crucial for IL6 signalling—occurs via the Jak-STAT pathway and stimulates pro-inflammatory gene transcription. In contrast, IL11 exerts its main effects in human and mouse fibroblasts at the post-transcriptional level, via sustained activation of the non-canonical ERK signalling pathway, with little evidence for a role of STAT although it is mildly phosphorylated (Heinrich *et al*, 2003; Schafer *et al*, 2017). In IL11 stimulated fibroblasts, collagen, ACTA2, periostin and MMP2 are strongly upregulated at the protein level but—surprisingly—there is no detectable change in their respective mRNA expression levels. The mechanism underlying this effect is not yet clear, but there is evidence that IL11 stimulates downstream targets of ERK which activate translation, including 40S ribosomal protein S6 kinase and eukaryotic translation initiation factor 4E (Schafer *et al*, 2017). Glutamyl-prolyl-tRNA synthetase, which mediates translation of proline-rich proteins such as collagen (and IL11 itself), may also play a role in the post-transcriptional control mechanism (preprint: Wu *et al*, 2019).

In rodents, IL11 is highly expressed in the heart after MI (Obana *et al*, 2010) or pressure overload (Schafer *et al*, 2017). *In vivo,* overexpression of IL11 specifically within fibroblasts produces extensive fibrosis across multiple organs including the heart, lungs and kidney along with a HF phenotype (Schafer *et al*, 2017; Ng *et al*, 2019). In contrast, mice with germline KO of the mouse *Il11ra1* or following treatment with an IL11 neutralizing mAb exhibit resistance to cardiac fibrosis in response to pressure overload or AngII infusion (Schafer *et al*, 2017).

These data contrast with previous work that showed recombinant human IL11 (rhIL11) is protective and antifibrotic in the mouse heart (Obana *et al*, 2010, 2012). However, the use of non-species-specific rhIL11 in these earlier studies is of central importance because more recent work (preprint: Widjaja *et al*, 2019a) has shown that rhIL11 unexpectedly functions as an inhibitor of endogenous IL11 in mice and rhIL11 does not activate mouse fibroblast ERK signalling (Schafer *et al*, 2017). In contrast, administration of recombinant *mouse* IL11 is strongly profibrotic in mice *in vivo* and to mouse fibroblasts *in vitro* (Schafer *et al*, 2017). In humans, circulating levels of IL11 are elevated in patients with HF, increase with progressive worsening of HF symptoms and are correlated with cardiovascular events including HF hospitalization, stroke, MI and death (Ye *et al*, 2019).

There is an intriguing side story to IL11, relating to its effect when injected to humans. RhIL11 was developed in the 1990s as a drug (Neumega) for treating chemotherapy-associated thrombocytopenia as it was opportunistically found to increase platelet counts when injected at high doses, although IL11 has no detectable physiological role for normal platelet production (Nandurkar *et al*, 1997). Notable side effects seen in patients receiving rhIL11 include cardiac arrhythmia including AF, pulmonary congestion, dilutional anaemia and raised brain natriuretic peptide (Smith, 2000; Bhatia *et al*, 2007; Liu *et al*, 2019). In a recent study of leukaemic patients receiving rhIL11 therapy, all patients exhibited increased BNP levels, 80% reached BNP levels consistent with a diagnosis of HF and 16% developed a clinical HF syndrome (Smith, 2000; Bhatia *et al*, 2007; Liu *et al*, 2019).

Targeting the fibroblast

As myofibroblasts play a central role in the development of fibrosis, the ability to selectively target-specific populations of fibroblasts is an intriguing potential method to treat fibrotic diseases. The developing oncology treatment, chimeric antigen receptor T-cell (CAR-T cell) therapy, uses re-engineered cytotoxic T cells to target specifically selected surface markers to deplete a defined cell population (June *et al*, 2018). CAR-T therapy has been used effectively in the clinic to treat B-cell leukaemias and lymphomas resistant to standard therapy by targeting CD19-positive cells (Porter *et al*, 2011; Schuster *et al*, 2017; Park *et al*, 2018). In a recent study, Aghajanian

et al (2019) repurposed this technology to selectively target fibroblasts. Using RNA sequencing data from the tissue of heart transplant donors and recipients, they identified a surface marker minimally expressed in the normal heart or extracardiac tissue but significantly upregulated in CF of humans with HCM and DCM (Tillmanns *et al*, 2015; Nagaraju *et al*, 2019). The marker, fibroblast activation protein (FAP), has previously been shown to be present on activated fibroblasts within malignant tumours (Cortez *et al*, 2014; Kilvaer *et al*, 2015) and is present in activated CF in mice following AngII/phenylephrine infusion. Selective elimination of FAP-positive cells using this treatment reduced cardiac fibrosis in mice within the 8-week treatment period and improved cardiac function (Aghajanian *et al*, 2019). Although still at a very early stage of preclinical development, the potential to deplete the activated fibroblast population may be a powerful tool to treat fibrosis within the myocardium and elsewhere.

Another potential method of targeting the CF is to use defined transcription factors to promote fibroblast transdifferentiation towards a cardiomyocyte phenotype, thereby reducing ECM production and potentially augmenting cardiac contraction (Ieda *et al*, 2010). Delivery of these factors using retroviruses or adeno-associated viral vectors has been trialled successfully in a number of mouse models of HF following MI. Direct injection of the vector into the peri-infarct area induces the expression of transcription factors GATA4, MEF2C and Tbx5 within fibroblasts and reprograms these cells towards a cardiomyocyte phenotype (Qian *et al*, 2012; Song *et al*, 2012). The result is depletion of the fibroblast population, reduced collagen accumulation and improved cardiac function (Qian *et al*, 2012; Yoo *et al*, 2018). This approach remains very much in its infancy and safety concerns remain regarding the use and specificity of viral vectors as well as the arrhythmogenic potential of generating new cardiomyocytes. However, this work again highlights the diversity of the novel tools being explored to treat cardiac fibrosis.

Pending issues

(i) Identify non-redundant mediators of cardiac fibrosis which can be therapeutically targeted with an acceptable safety profile.
(ii) Develop therapies to deplete matrix-secreting cardiac myofibroblasts that do not adversely affect homeostatic functions of resident cardiac fibroblasts.
(iii) Large animal and first-in-man studies for preclinical targets including IL11, gal-3 or NOX inhibition, among others.
(iv) Dissect the interplay of fibrosis and inflammation in the heart to prioritize nodal points of disease pathogenesis and cross-talk.
(v) Identify cross-tissue mediators of fibro-inflammation to enable treatment of the HFpEF, multiorgan syndrome rather than cardiac-specific pathology.

Conclusion

Cardiac fibrosis is central to the pathogenesis of HF, particularly HFpEF, and addressing the lack of available treatments for HFpEF is a priority given the rising demographics of obese, diabetic, hypertensive and ageing populations around the world. The cellular and molecular processes which lead to fibrosis are intricate and overlapping. Some of the pathways and treatment strategies discussed in this review have been understood and used for many decades but none specifically target cardiac fibrosis. It is an exciting time in the field of cardiac fibrosis as several emerging targets and approaches show promise and could be developed to treat, and perhaps even reverse cardiac fibrosis, but this can only be assessed through clinical trials. Given redundancies, it is possible that combination therapies that target multiple components of the fibrotic pathway will be more effective than any single therapy but polypharmacy comes with polytoxicity and this is particularly troublesome in elderly patients. It is important to remember also that HFpEF is a multisystem disorder, and therapies that alleviate skeletal muscle, renal and metabolic dysfunction as well as cardiac dysfunction are likely to have greatest clinical efficacy.

Ultimately, fibrosis depends on activation of the fibroblast and its transformation into a matrix-secreting and pro-inflammatory myofibroblast. This central pathology is a point of convergence for all upstream stimuli: from mechanical stretch to endocrine or paracrine factors. We end by suggesting that targeting non-redundant pathways for myofibroblast activation represents the most promising means of treating fibrosis. While the days of attempting to target TGFβ activation, either directly or indirectly, are likely limited due to dose-limiting, on-target toxicities—new opportunities are available in the form of cell therapy and novel targets, which should be explored.

Conflict of interest

MS and BC have no conflicts of interest to disclose. SAC is a co-inventor on a number of patent applications relating to the role of IL11 in human diseases that include the published patents: WO2017103108, WO2017103108 A2, WO 2018/109174 A2, WO 2018/109170 A2. SAC is also a co-founder, director and shareholder of Enleofen Bio PTE LTD, a Singapore-based biotechnology.

For more information

(i) https://www.escardio.org/Sub-specialty-communities/Heart-Failure-Association-of-the-ESC-(HFA)
(ii) https://www.bsh.org.uk/
(iii) https://www.heart.org/en/health-topics/heart-failure
(iv) https://clinicaltrials.gov

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