INTRODUCTION AND AIM OF THIS THESIS
“Science, my lad, is made up of mistakes, but they are mistakes which it is useful to make, because they lead little by little to the truth”. Jules Verne, Journey to the Center of the Earth, 1864

ORGAN FIBROSIS AT A GLANCE

The evolutionary conserved ability to repair tissues is essential for the survival of an organism [1]. As a fundamental step of the wound healing process, scar formation represents a reaction that aims to limit organ damage and maintain architectural integrity of the remnant tissue [2]. However, when normal wound healing fails, usually under sustained or repetitive injury, scarring becomes a pathological condition, known as fibrosis, that leads to the excessive accumulation of extracellular matrix (ECM) components at the site of injury (Figure 1). Fibrosis disrupts the normal tissue architecture and ultimately results in a loss of organ function [3,4]. Fibrotic diseases account for 45% of deaths worldwide [5]. Fibrosis is a dynamic, highly integrated and immensely complex process that is triggered and orchestrated by a cornucopia of different cells and signalling molecules [6,7]. It can affect virtually any human organ, including the lung, skin, heart, kidney, liver and intestine. Considering that different types of tissues and organs share common mechanisms of fibrosis and a large variety of pathophysiologically distinct diseases converge into this single process, fibrosis represents an attractive therapeutic target [8,9]. Therefore, understanding the mechanism of fibrogenesis is crucial for the development of therapeutic options that allow us to prevent, stop or revert the progression of fibrosis and improve organ function in numerous diseases.

Injury and inflammation

Mechanical trauma, ischemic injury, infections, toxins, or autoimmune reactions, can all cause tissue damage. Irrespective of the initiating insult and affected organ, tissue injury induces the progressive loss of parenchyma (i.e., hepatocytes in the liver, nephrons in kidney) and activation of local inflammatory processes [10]. Upon injury, damaged epithelial and endothelial cells release various danger signals – damage-associated molecular patterns (DAMPs) – and chemokines that recruit inflammatory cells to the site of injury [11,12]. As a result, lymphocytes, monocytes, macrophages and dendritic cells migrate to the area to facilitate tissue repair by removing pathogens and/or apoptotic cells. Therefore, acute inflammation, as a response to a disrupted epithelial surface, is a physiological part of normal wound healing [13]. However, persistent inflammation is a powerful stimulus that triggers fibrosis [14]. Once inflammatory cells have infiltrated the wound, they become activated and start to produce a large variety of cytokines and chemokines, including interleukins IL-1β, IL-6 and tumor necrosis factor TNFα, as well as other profibrotic molecules, such as reactive oxygen species (ROS) [15,16]. These factors, in turn, promote fibrogenesis by triggering the proliferation and activation of fibroblasts that continuously sustain ECM deposition.

Myofibroblasts and tissue remodelling

Activation of fibroblasts is a central driving force of fibrogenesis and tissue remodelling [17,18]. In healthy tissues, fibroblasts are primarily involved in homeostatic ECM turnover [19]. During both physiological and pathological wound healing, fibroblasts differentiate into myofibroblasts that produce large amounts of ECM components, including fibrillar collagens (mainly collagen type I and III), cellular fibronectin, laminin, elastin, glycoproteins and proteoglycans [7,20]. A key feature of myofibroblasts is their contractile function, which is aided by the expression of α-smooth muscle actin (α-SMA) [21,22]. Myofibroblasts not only deposit matrix, but also directly contribute to tissue damage by producing an array of inflammatory cytokines and growth factors, and generating ROS [23,24]. The latter can further amplify inflammation and fibrosis [25,26].

Interestingly, the myofibroblast is not a specific cell type, but rather it represents a functional cell fate that develops in response to injury [4,27]. The cellular origin of myofibroblasts has been a major focus of fibrosis research over the past 20 years, yet, it remains a matter of an ongoing debate. The main hurdle for elucidating the origin of myofibroblasts is the lack of specific cell markers, and inter-organ and inter-species differences in existing markers [24]. Figure 2 illustrates possible cellular...
progenitors of myofibroblasts. Until very recently, it was widely accepted that injured epithelial cells served as the primary source of myofibroblasts [28,29]. In experimental models of organ fibrosis, fully mature epithelial and endothelial cells acquire a mesenchymal-like phenotype and function [30,31]. This phenomenon of sequential loss of epithelial/endothelial markers and de novo acquisition of mesenchymal markers is termed epithelial-to-mesenchymal (EMT) or endothelial-to-mesenchymal transition (EndoMT). However, state-of-the-art lineage tracing studies have provided strong evidence that mesenchymal cells – resident interstitial fibroblasts and pericytes – are the major, if not the only, source of ECM-producing myofibroblasts in multiple organs [32–40]. The exact definition of fibroblasts is somewhat elusive: while it is well recognized that fibroblasts are a heterogeneous cell population that can differ even within an organ, some argue that historically defined fibroblast is actually not a cell type, but a general term for heterogeneous subsets of mesenchymal cells [8,40,41]. Other potential myofibroblast progenitors include circulating bone marrow-derived fibrocytes that migrate to the area of tissue injury, and resident mesenchymal stem cells (MSCs) that can be found in perivascular niches in human tissues [42–44].

Fibrosis is not just defined by the matrix and cells that produce matrix, but also by the core molecular pathways that drive fibrogenesis. These pathways involve a large variety of growth factors – proteins that stimulate cell growth and proliferation – that are secreted by epithelial/endothelial cells, immune cells and myofibroblast progenitors, and are able to orchestrate cellular responses [55]. Among these growth factors, transforming growth factor beta (TGFβ) dominates the pathobiology of organ fibrosis and is therefore considered as the central profibrotic mediator [56,57]. TGFβ strongly promotes the production of ECM components, including collagen I and III, and fibronectin, and drives differentiation of myofibroblast progenitors [58]. Sustained TGFβ activation contributes to deregulated matrix turnover by inhibiting the synthesis of MMPs and increasing the synthesis of TIMPs and plasminogen activator inhibitor 1 (PAI-1). On the other hand, TGFβ exerts immunoregulatory properties and acts as a negative regulator of pro-inflammatory responses in various organs, reflecting its pleiotropic nature [59]. Multifunctionality of TGFβ is further demonstrated by its critical involvement in the regulation of embryogenesis, carcinogenesis, cell proliferation and migration, among others [10].

**Figure 2. Proposed origin of myofibroblasts in fibrosis.** Activated myofibroblasts can originate from mesenchymal progenitors, such as tissue-resident fibroblasts and pericytes, that undergo differentiation directly under the influence of the profibrotic environment. Mesenchymal cells derived from circulating fibrocytes or bone marrow-derived stem cells contribute to the pool of activated myofibroblasts. Epithelial and endothelial cells also can become myofibroblasts once they acquire a mesenchymal-like phenotype through epithelial-to-mesenchymal (EMT) or endothelial-to-mesenchymal transition (EndoMT), respectively. Damaged epithelial and endothelial cells release various danger signals and proinflammatory mediators that attract immune cells to the site of injury. Sustained inflammatory environment promotes differentiation of progenitor cells into myofibroblasts that produce excessive amounts of ECM.

Not only myofibroblasts, but also deposited ECM actively contributes to fibrogenesis. As fibrosis progresses, ECM proteins, particularly fibrillar collagens, undergo extensive enzymatic cross-linking by collagen oxidases, including lysyl oxidase-like (LOXL) family members and transglutaminases [20]. This biochemical modification increases rigidity of the fibrotic matrix, rendering it resistant to proteolysis and degradation [45]. Successful matrix degradation is a critical component of the resolution phase of normal wound healing, whereas organ fibrosis is characterized by an aberrant ECM turnover [46,47]. In the latter case, an imbalance in the expression and activity of ECM remodelling enzymes, such as matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs), favours pathological deposition of ECM. MMPs degrade various matrix components, cell surface receptors, cytokines and growth factors [48]. For instance, collagenases MMP-1, MMP13 and MMP-14 as well as gelatinases MMP-2 and MMP-9 are able to process intact or denatured collagen fibrils, respectively [49]. Generally, increased levels of MMPs are associated with fibrosis progression, and have been implicated in other diseases, such as cancer, atherosclerosis and osteoarthritis; however, their multifunctional nature makes it challenging to precisely define the role of MMPs in these disease states [49–51].

Failure to degrade accumulated ECM leads to increased tissue stiffness, which in turn stimulates fibrogenesis by activating mesenchymal cells and enhancing a variety of fibrosis-associated signalling pathways via a positive feedback loop [29,47,52]. In other words, excessive ECM deposition and increased tissue stiffness, even in the absence of ongoing injury or inflammation, are capable of sustaining progressive fibrosis via a vicious circle [53]. Similarly, Herrera et al. [54] suggest that at some point during the course of fibrosis progression, mesenchymal cells acquire “autonomy”, so that they no longer respond to ECM cues that would normally terminate the fibrotic response. Therefore, at a certain, as of yet undefined, stage in the fibrotic process, disease progression becomes irreversible and self-sustainable.

**Molecular pathways in fibrogenesis**

Fibrosis is not just defined by the matrix and cells that produce matrix, but also by the core molecular pathways that drive fibrogenesis. These pathways involve a large variety of growth factors – proteins that stimulate cell growth and proliferation – that are secreted by epithelial/endothelial cells, immune cells and myofibroblast progenitors, and are able to orchestrate cellular responses [55]. Among these growth factors, transforming growth factor beta (TGFβ) dominates the pathobiology of organ fibrosis and is therefore considered as the central profibrotic mediator [56,57]. TGFβ strongly promotes the production of ECM components, including collagen I and III, and fibronectin, and drives differentiation of myofibroblast progenitors [58]. Sustained TGFβ activation contributes to deregulated matrix turnover by inhibiting the synthesis of MMPs and increasing the synthesis of TIMPs and plasminogen activator inhibitor 1 (PAI-1). On the other hand, TGFβ exerts immunoregulatory properties and acts as a negative regulator of pro-inflammatory responses in various organs, reflecting its pleiotropic nature [59]. Multifunctionality of TGFβ is further demonstrated by its critical involvement in the regulation of embryogenesis, carcinogenesis, cell proliferation and migration, among others [10].
TGFβ exists as an inert complex covalently bound to the ECM, and is released/activated upon injury via interaction with integrins – mechanosensing cell-surface receptors that mediate cell-adhesion [60]. This mechanism is directly correlated to the contraction of myofibroblasts and stiffness of the ECM, representing a reservoir of latent TGFβ in the tissue [27,61]. While controlled amounts of TGFβ play an essential role in the physiological wound healing process, overexpression of TGFβ in fibrotic tissues is detrimental, with matrix stiffness acting as an amplifier of TGFβ activity [18,62,63]. TGFβ mediates signalling through two types of transmembrane serine/threonine kinases, TGFβ type I (TGFBRI, ALK5) and type II (TGFBRII) receptors [64,65]. Interaction of TGFβ with its receptors elicits a multitude of cellular responses via both SMAD and non-SMAD-dependent signalling pathways [56,66]. In the canonical SMAD-dependent pathway, binding of TGFβ to TGFBRII induces the recruitment and phosphorylation of TGFBRI, activating its kinase activity [67]. Signal transduction is propagated through phosphorylation of receptor-regulated SMAD proteins, namely SMAD2 and SMAD3, which then translocate to the nucleus and regulate the transcription of the target genes [66,68]. Alternatively, TGFβ modulates downstream cellular responses via non-SMAD pathways [64,65], such as mitogen-activated protein kinases (MAPK) signalling cascades involving p38, extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK). TGFβ also activates Rho-like GTPases, phosphatidylinositol-3-kinase PI3K/Akt and nuclear factor-xB (NF-xB) pathways. Although non-SMAD pathways have been reported to be involved in TGFβ-induced fibrosis, TGFβ mainly drives fibrosis via SMAD signalling [57].

Other important soluble profibrogenic mediators include platelet-derived growth factor (PDGF), connective tissue growth factor (CTGF), vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF) and epidermal growth factor (EGF). These mediators function as ligands for the corresponding receptor tyrosine kinases (RTKs). Activation of RTKs after injury plays an important role in organ fibrosis [52,71–74]. Binding of the ligand results in RTK dimerization and autophosphorylation, with subsequent activation of the downstream signalling pathways [75]. Among the mentioned growth factors, members of the PDGF family are potent mitogens and chemo-attractants for mesenchymal cells in most organs, driving their recruitment and proliferation during fibrosis [76,77]. Similar to TGFβ, PDGF stimulates synthesis of major ECM components. Altogether, the diversity in molecular pathways driving fibrogenesis highlights the complexity of the regulation of organ fibrosis.

**ANTIFIBROTIC STRATEGIES UNDER CLINICAL DEVELOPMENT**

Decades of research significantly advanced our understanding of the central cellular and molecular mechanisms underlying the progression of organ fibrosis. This has laid the foundation for the development and clinical validation of rational mechanism-based antifibrotic strategies. Here, we outline current therapies that are under clinical development for fibrotic diseases in liver, kidney and gut, as these organs comprise the main focus of this thesis (Figure 3).

**Figure 3. Pipeline for therapeutic strategies to combat organ fibrosis.** Agents are classified based on their mode of action, and colours code for a particular organ – liver (yellow), kidney (red) and intestine (green). Therapeutics denoted by circles are experimental treatments, while stars mark those that are commercially available for indications other than fibrotic diseases of liver, kidney or gut. Data are derived from clinicaltrials.gov and literature; for more details see [14,16,77–86].

mAb, monoclonal antibody; CTGF, connective tissue growth factor; PDGF, platelet-derived growth factor; TGFβ, transforming growth factor beta; TKI, tyrosine kinase inhibitor; IL-13, interleukin-13; POE, phosphodiesterase; Nrf2, nuclear factor erythroid 2-related factor 2; CNI, calcineurin inhibitor; TNF, tumor necrosis factor; CCR2/CCR5, C-C chemokine receptor type 2/type 5; MCP, monocyte chemoattractant protein; ASK1, apoptosis signal-regulating kinase 1; NOX, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase; PPAR, peroxisome proliferator-activated receptor; FXR, farnesoid X receptor; HIF-PHI, hypoxia-inducible factor prolyl hydroxylase inhibitor; mTOR, mammalian target of rapamycin; JAK, Janus kinase; ACC, acetyl-CoA carboxylase; AT1 inhibitor, angiotensin II type 1 receptor inhibitor; LOXL2, lysyl oxidase-like 2; ERAs, endothelin-1 receptor antagonists; MMP9, matrix metalloproteinase 9.
Main antifibrotic strategies include: 1) eliminating the pathological trigger; 2) suppressing inflammation; 3) preventing myofibroblast activation; and 4) promoting ECM degradation and remodelling [78]. The most straightforward way to halt the progression of fibrosis is by removing the cause of tissue damage or inflammatory stimulus, as has been observed in individuals with chronic hepatitis B infection treated with entecavir, a highly effective oral antiviral drug [10]. However, this is not always feasible, since in many fibrotic disorders the tissue-damaging stimulus is either unknown or cannot be easily eliminated. Therefore, as illustrated in Figure 3, current clinical efforts are invested in antifibrotic strategies and agents that target downstream profibrotic pathways (e.g. inhibitors of TGFβ, PDGF, CTGF), inflammation (e.g. antibodies against IL-13; monocyte chemoattractant protein, MCP; thymic humoral factor, THF), oxidative stress (e.g. inhibitors of NADPH oxidase, NOX; activator of nuclear factor erythroid 2-related factor 2, NRF2) or pathologic ECM (e.g. targeting ECM molecules and their receptors, crosslinking enzymes). Notably, while multiple pharmacological interventions are under evaluation for the treatment of liver and kidney fibrosis, there are hardly any trials investigating antifibrotic therapies for intestinal fibrosis.

Given the prominent role of TGFβ and PDGF signalling in organ fibrosis, strategies to inhibit these pathways will be addressed in this thesis. Essentially, there are three main approaches for pathway inhibition: 1) sequestering the ligand (e.g. with antisense oligonucleotides); 2) disrupting ligand-receptor interaction (e.g. with neutralizing monoclonal antibodies against the ligand – TGFβ or PDGF – or their receptors); and 3) blocking receptor kinase activity to prevent signal transduction (e.g. with tyrosine kinase inhibitors, TKI) [87,88]. Two experimental monoclonal antibodies against TGFβ, fresolimumab and LY2382770, have reached clinical studies in patients with fibrosis-associated kidney diseases, but failed to show clear efficacy [18,89]. Evaluation of pirfenidone for liver and renal fibrosis falls under the category of “drug repurposing”, which is a pharmaceutical development strategy that oversees the reuse of existing licensed drugs for new medical indications. The indisputable benefits of drug repurposing include reduced development time and costs for the drugs, since they already have been proven safe and effective for other diseases. Pirfenidone is the first ever antifibrotic drug approved for the treatment of idiopathic pulmonary fibrosis [90]. The proposed mechanism of action of pirfenidone is inhibiting the nuclear accumulation of SMAD2/3, thus disrupting the TGFβ signalling, although the exact molecular targets remain unknown [91]. As illustrated in Figure 3, pirfenidone is not the only repurposed therapeutic agent. For instance, TKI that block the enzymatic activity of the PDGF receptor (PDGFR) represent a majority of current cancer therapies that inhibit PDGF signalling. These small molecules bind to the ATP-binding pocket of kinases, which is a conserved structure in this group of enzymes; therefore, TKI are not entirely specific and show multi-targeted effects [77]. Among the TKI, imatinib was the first to be approved for clinical use in 2001, and it is now the frontline treatment of chronic myeloid leukaemia. Imatinib primarily targets PDGFR, BCR-Abl kinase and c-kit. Second generation TKI have an even broader spectrum of action: for example, sorafenib, clinically approved for the treatment of renal and liver cancer, inhibits PDGFRs, FLT3, CSF1R and VEGFR [88].

TKI have been evaluated in various fibrotic diseases (e.g. sorafenib in liver fibrosis [92–94], imatinib in liver and lung fibrosis [95–98]), but only nintedanib, which targets PDGFR, VEGFR and FGFR, has been approved for the treatment of pulmonary fibrosis.

Despite overwhelming scientific efforts, none of the numerous drugs currently in clinical development have been approved for the treatment of hepatic, renal or intestinal fibrosis. Moreover, the sole drugs that are clinically available – pirfenidone and nintedanib – only slowly down the progression of lung fibrosis, while the disease continues to progress and is ultimately fatal [1,99]. Hence, the resolution of organ fibrosis remains an urgent and unmet clinical need. Targeting both the inflammatory component and basic elements of the fibrotic program (i.e., growth factors, ECM, etc.) proved to be challenging. Indeed, a lot of attention has been given to therapeutics with an anti-inflammatory/antioxidant mode of action, highlighting the prominent role of inflammation, proinflammatory mediators and ROS in the progression of fibrosis. However, there is growing evidence that once fibrosis becomes self-sustainable, the mere control of inflammation is not enough to revert this process [14]. In addition, the clinical development of drugs that directly interfere with key players in the fibrotic process is hindered by the multifaceted nature of these factors and their role in maintaining total body homeostasis. A classic example of this is pharmacological modulation of TGFβ function. A plethora of therapies targeting TGFβ have been explored (reviewed in [18,58,100]), yet, only a few reached clinical trials and resulted in positive patient outcomes. Although systemic inhibition of the TGFβ pathway may ameliorate fibrosis, such a therapy might also block its anti-inflammatory function and lead to deleterious side effects in patients [100]. Instead of direct inhibition of TGFβ or its receptors, selective targeting of downstream TGFβ signalling mediators could abolish some safety concerns [18]. Furthermore, due to the lack of clinical success with compounds that directly targeted known drivers of fibrosis, drug discovery and development has shifted towards indirect antifibrotic agents, some of which are now entering phase I and phase II clinical trials, as reviewed elsewhere [89,101–105]. Moreover, emerging strategies of targeted drug delivery (by using nanoparticles, liposomes, cell-penetrating peptides, etc.), cell therapy (e.g. by using mesenchymal stem cells) and combination therapy comprise an exciting avenue of antifibrotic drug development [16].
RECIPE FOR SUCCESSFUL CLINICAL DRUG DEVELOPMENT

Before we dive into brewing a successful clinical trial, let’s take a look at our cooking pot. A simple PubMed database search tells us that for the past 40 years, more than 130,000 articles addressing fibrosis have been published, not counting reviews and articles dedicated to cystic fibrosis. Since 2016, each year is marked by more than 8000 of publications dealing with fibrosis. At the same time, more than 2000 clinical studies have been listed on clinicaltrials.gov with the keyword “fibrosis” (excluding those mentioning “cystic fibrosis”), of which over 660 are active/ongoing. A multitude of antifibrotic strategies that had promising effects in preclinical experimental models, proved inefficient and/or unsafe in human clinical trials. To date, clinical development yielded only two approved drugs that slow down the progression of fibrosis in a single organ, the lung, increasing the life expectancy for patients with lung fibrosis with a few months. Still, no therapy exists for any other type of organ fibrosis. Oh, tearful onions.

Much like in any cooking recipe, there are several key ingredients that are required to achieve the desired outcome, in our case, clinical trials that provide strong evidence for effective antifibrotic therapies. Critical factors must be considered in the design of clinical trials, including: 1) patient recruitment and selection; 2) identification of appropriate end-points, and 3) availability of biomarkers. Ideally, study subjects should be matched according to aetiology, age, gender, medication use and risk factors, and diagnosed with an intermediate stage of fibrosis, where dynamic changes are best detectable [101]. At present, most clinical trials continue to enrol a non-homogeneous population of patients with advance disease stages, multiple comorbidities and different pathogenic processes [102]. Diagnostically conclusive primary end-points are a major determinant of the outcome of clinical trials, but studies often rely on clinical parameters that do not fully reflect disease progression [106]. For example, renal fibrosis is poorly captured by glomerular filtration rate (GFR) or proteinuria, which are generally considered as primary end-points in almost all antifibrotic trials in kidney patients [85]. Similarly, liver function parameters, such as albumin or prothrombin time, are usually altered in the late stages of liver disease, and serum transaminases are not predictive [101]. The situation is even more complicated regarding intestinal fibrosis, since no clinically useful serum markers, nor patient-reported endpoints for assessment of therapeutic response are available [79,86]. The best read-out for a trial investigating a potential antifibrotic therapy would be fibrosis itself. Currently, tissue biopsy is considered the gold standard for assessing hepatic, renal and intestinal fibrosis. Unfortunately, such a procedure is invasive and prone to sampling error, limiting the usefulness of this approach [14,106]. Therefore, therapeutics that specifically target fibrosis in these organs, without affecting conventional clinical parameters, are especially challenging to investigate in clinical trials. This is in sharp contrast with trials in idiopathic pulmonary fibrosis, as there is a very reliable end-point, termed ‘forced vital capacity’. The vital capacity, measured by the amount of expelled air from the lungs after maximum inhalation, is a simple, non-invasive test and can be easily repeated with no risk for the patient [107]. Luckily, the field of liver fibrosis is catching up: the recently developed FibroScan® (vibration-controlled transient elastography) is a novel, non-invasive technique to assess hepatic fibrosis and steatosis that has been validated in chronic hepatitis B and C, and non-alcoholic fatty liver disease (NAFLD) [108]. Nonetheless, specific, sensitive and non-invasive biomarkers of fibrosis are essential for successful antifibrotic drug development, as they could replace the current gold standard - invasive biopsy - for assessing and staging fibrotic changes, predicting disease progression and revealing drug efficacy [14,89].

Even before we can mix these key ingredients, we have to address the core element of the recipe: adequate preclinical evaluation is undoubtedly the foundation of successful clinical drug development. The conventional steps in the identification process of new therapeutic targets and drug testing significantly rely on the use of in vitro systems, such as cell mono- and co-cultures, and in vivo animal models. However, the low clinical success of putative therapeutics argues for the poor translational efficiency of basic biomedical research using conventional preclinical tools.

While two-dimensional cell cultures comprise a simple, cost-efficient and potentially high-throughput method to study fibrosis, they lack cellular heterogeneity and relevant cell-cell and cell-ECM interactions, which are a prerequisite to mimic the multicellular character of fibrosis. To minimize the gap between cell culture and the in vivo situation, and to tackle the limited physiological relevance of most experimental models, new in vitro models are being developed, such as co-cultures, sandwich cultures and three-dimensional (3D) tissue engineered models (e.g. spheroids, decellularized organs, 3D bioprinting), reviewed in [109–111]. The latter are gaining more and more attention; however, these models do not fully recapitulate the structural organisation of human organs and still face many technical hurdles, limiting their use in fibrosis research [112].

Animal models, particularly rodents, are the gold standard for basic and preclinical research in medical and pharmacological science. From a regulatory perspective, their use is mandatory in the transition from preclinical to clinical studies. In the last decade, a wide variety of animal models, including surgical, genetic, toxic and nutritional models, have been established to study renal, hepatic and intestinal fibrosis [113–116]. However, animal testing raises many ethical and scientific concerns. A major criticism is that animal models do not fully replicate human pathology due to the distinct differences in anatomy, (patho)physiology, metabolic capacity and immunology [45]. In many widely used animal models of fibrosis, the disease is provoked by triggers that are rarely, if ever, seen in human disease (e.g. toxic injury by CCI, in liver or bleomycin in lung) [29]. The use of knockout and transgenic mouse models, in which genes/proteins that regulate fibrosis have been knocked out or overexpressed, also poses a challenge regarding clinical translation [5]. Furthermore, most in vivo studies initiate treatment before or at the time of disease induction, hindering extrapolation to the clinical situation [22]. In clinical practice, therapeutic interventions are frequently initiated during late stages of the disease, when fibrosis already exists. In addition, animal studies are more often than not performed in single-strain young male mice, in contrast to the heterogeneous population of patients enrolled in most clinical trials [106]. Collectively, current in vivo models have a limited predictive capacity for drug safety and efficacy in humans, as a staggering 9 out of 10 drugs that passed animal experiments ended up failing in human clinical trials [117]. Nevertheless, even though the translational value of
animal models is continuously questioned, most preclinical research is executed in rodents because human-based models are lacking.

In conclusion, the secret to brewing a deliciously successful antifibrotic therapy for humans entails that clinical drug development refines clinical trial design, establishes fibrosis-specific biomarkers and, most importantly, improves the translational aspect of preclinical tools, including the search for alternative, human-based models.

**PRECISION-CUT TISSUE SLICES – 5 MILLIMETER ORGAN MINIATURES**

In light of the great need for versatile, predictive and translational preclinical models, *ex vivo* precision-cut tissue slices (PCTS), a functional 3D organ model, has recently caught the attention of biomedical research. PCTS represent a more physiologically relevant model compared to most of the currently used *in vitro* systems. Each slice, only 5 mm in diameter, contains all cell types and acellular components of the whole organ in their original configuration, while preserving the intercellular and cell-matrix interactions. In other words, PCTS are miniaturized organs that retain native tissue architecture and intact cellular environment. Therefore, PCTS generated from rodent organs provide a link between currently used *in vitro* and *in vivo* models (Figure 4).

Such organotypic cultures have been established for various organs, including lung [118], liver [119], kidney [120,121], intestine [122], heart [123] and pancreas [124]. PCTS have emerged as a tool for pharmacological and toxicological research and have been proven useful in elucidating drug metabolism, drug transport, toxicity and drug-drug interactions [125–131]. As a more recent application, the PCTS model has found its use in elucidating the mechanisms of organ fibrosis and testing the efficacy of putative antifibrotic compounds [132–137]. Additionally, the use of PCTS has expanded into the fields of nanotechnology [138], virology [139] and gene silencing [140].

The most invaluable advantage of PCTS is the ability to use human tissue, which is considered to be more predictive for human responses than animal experiments. This not only increases the predictive capacity of the model, but also eliminates the need for animal-to-human extrapolation. Thus, PCTS provide a robust screening platform that reinforces preclinical evidence of potential therapeutic targets and compounds for future clinical trials. Therefore, human PCTS help to bridge the gap between animal models and the patient setting (Figure 4). Human material for the preparation of PCTS is usually obtained from surgical waste remaining from resection surgery or non-transplantable organs. Highlighting the versatility, PCTS can also be prepared from fibrotic tissue obtained from experimental animals or patients with severe fibrosis, allowing the study of advance disease stages and the effects of compounds in a pathological environment.

Other beneficial features of the PCTS model include reproducibility, simplicity in PCTS preparation and handling and the capacity to test several experimental conditions at once. Multiple slices can be obtained from a single organ, with an option to use various organs from the same animal. Therefore, murine and human PCTS greatly reduce animal use in accordance with the 3Rs (reduction, replacement and refinement). Still, as with any research model, PCTS culture is subjected to several limitations, which are addressed in detail in the general discussion of this thesis (Chapter 8).

![Figure 4. Precision-cut tissue slices (PCTS) in the drug development process.](image)

The PCTS model reflects the complex three-dimensional architecture of the whole organ and maintains cellular diversity – two unique features that can make *in vitro* drug testing more physiologically relevant. Hence, murine PCTS might be a translational link between currently used *in vitro* and *in vivo* models. In turn, the ability to use human tissues makes PCTS an indispensable tool in preclinical drug development that can bridge the translational gap between animal studies and human clinical trials.
AIM OF THE THESIS

Prompted by scarce therapeutic options for the treatment of organ fibrosis, the field of fibrosis research is in dire need of good translational human-based preclinical models. The ex vivo PCTS model promises to meet this urgent need. The aim of this thesis was threefold (Figure 5): to deepen our knowledge of the molecular processes that take place in PCTS cultures (Part I); to demonstrate the predictive value of PCTS as a screening platform for antifibrotic drugs (Part II); and to optimize current PCTS culture conditions (Part III).

The use of PCTS as preclinical screening platform for antifibrotic compounds is demonstrated in chapter 5, which details the antifibrotic efficacy of nintedanib in murine and human kidney PCTS, emphasizing the added value of using human tissues in the drug development process. Chapter 6 describes the use of an even more extensive panel of antifibrotics to showcase the predictive and translational power of PCTS. To this end, the ex vivo effects of pirfenidone, galunisertib (LY2157299) and imatinib were compared to the published in vivo effects and performance in clinical trials. One of the major limitations of the PCTS model is the relatively short viability of the slices during culture, which precludes the investigation of long-term drug toxicity and efficacy. The research presented in chapter 7 aimed to optimize the current culture conditions of intestinal PCTS by modifying the composition of the culture media leading to prolonged tissue viability. Lastly, chapter 8 summarizes and discusses the findings described in this thesis, and suggests future directions for ex vivo fibrosis research.

Although numerous molecular pathways are common in the development of organ fibrosis, justifying the term “core pathways of fibrosis”, each organ responds differently to injury and displays unique organ-specific features of fibrosis. Chapter 2 delineates the diversity in culture-induced fibrogenic responses in murine PCTS, as well as organ-specific responses to antifibrotic treatment. An incomplete understanding of culture-driven processes in PCTS hails the use of this model in preclinical research to its full potential. Therefore, chapter 3 provides a comprehensive characterization of the dynamic transcriptional changes in murine and human PCTS, originating from liver, kidney and gut, during culture. In chapter 4, the molecular processes that unfold in human PCTS from diseased organs are described and compared to human PCTS from healthy tissues, defining the role of culture and pre-existing pathology.
REFERENCES


CHAPTER 1

PART I

CHARACTERIZATION OF THE PCTS MODEL