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Fibrometabolism – an emerging therapeutic frontier in pulmonary fibrosis

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Abstract

Fibrosis is the final pathological outcome and major cause of morbidity and mortality in a number of common chronic inflammatory, immune-mediated and metabolic diseases (1). Despite the growing incidence of fibrotic diseases and extensive research efforts, there remains a lack of effective therapies that improve survival. Over the last decade, the application of omics technologies has revolutionised our approach in identifying new therapeutic targets and potential disease biomarkers. The application of metabolomics to improve our understanding of disease pathomechanisms has garnered a wave of scientific interest with emerging evidence suggesting that alterations in metabolism is not only a feature but may play an influential role in the pathogenesis of fibrosis, most notably in idiopathic pulmonary fibrosis (IPF), the most rapidly progressive and fatal of all fibrotic conditions. This review will detail the role of key metabolic pathways, their alterations in the myofibroblast, the key effector cell of the fibrogenic response, and the potential this new knowledge offers for the development of novel anti-fibrotic therapeutic strategies.

Fibrosis – a major unmet medical need

Fibrosis, characterised by the excessive and disorganised deposition of a collagen-rich extracellular matrix (ECM) in response to acute or chronic tissue injury, is a major cause of morbidity and mortality in a number of chronic diseases, including pulmonary fibrosis, end-stage liver and kidney disease, heart failure, rheumatoid arthritis, scleroderma and Crohn's disease. Fibrosis has also been linked to promoting tumour progression as part of the stromal reaction in cancer (2). Despite its pervasive role in several disease states, there remains a pressing need to develop effective anti-fibrotic therapeutic agents that halt the fibrotic response and improve survival.

Of all the fibrotic conditions, idiopathic pulmonary fibrosis (IPF) represents the most rapidly progressive and fatal, with a dismal median survival of just 3.5 years from diagnosis for patients not receiving anti-fibrotic therapy. The aetiology of IPF remains incompletely understood but the current favoured hypothesis proposes that IPF arises as a result of repetitive injury to the alveolar epithelium in genetically susceptible and aged individuals. The ensuing wound healing programme is highly dysregulated and marked by an over-exuberant fibrotic response, characterised by the formation of hallmark pathological lesions, termed fibrotic or fibroblastic foci. These lesions comprise alpha smooth muscle actin positive (αSMA+) myofibroblasts embedded within a type I collagen-rich extracellular matrix, overlaid by a highly abnormal epithelium, exhibiting evidence of multiple phenotypic states, including apoptosis, senescence and hyperplasia. Myofibroblasts are considered the main effector cells responsible for the production of extracellular matrix (ECM) in several fibrotic conditions (3). The excessive accumulation and persistence of myofibroblasts at sites of injury, as a result of a failure of apoptosis, is considered fundamental to the relentless

progression of fibrosis in IPF and other conditions (4). Although multiple pro-fibrotic mediators have been implicated in promoting fibroblast to myofibroblast differentiation and extracellular matrix production, current evidence points to a key role for transforming growth factor-β (in particular the TGF-β_1 isoform) by signalling through canonical Smad and non-canonical pathways.

The approval of pirfenidone and the successful repurposing of the oncology drug, the triple tyrosine kinase inhibitor, nintedanib for the treatment of IPF, represented a watershed moment in the development of anti-fibrotic agents(5, 6). However, although these drugs slow lung function decline over time, they do not halt disease progression or significantly improve survival (5, 7), so there remains a pressing need to develop new anti-fibrotic strategies (8). In this article, we review the alterations that occur within the major metabolic pathways during fibrogenesis, with a focus on myofibroblasts in the context of IPF. These metabolic alterations are likely generalizable to stromal populations in multiple conditions associated with excessive deposition of extracellular matrix, including the stromal reaction in cancer. We also present emerging evidence that modulation of altered metabolic signatures may present multiple novel therapeutic opportunities for the treatment of IPF and potentially other fibrotic conditions. It is also worth commenting that persistent lung function abnormalities, including restrictive lung disease, decreased diffusing capacity, and fibrosis, are expected in COVID-19 patients who had a severe course, particularly those who required mechanical ventilation (9).

Metabolic reprogramming – lessons from cancer

Almost a century ago, Otto Warburg and colleagues made the landmark discovery that cancer cells dramatically increased the uptake of glucose and that despite normoxic conditions, glucose was largely converted into lactate rather than used for oxidative phosphorylation; a phenomenon known as aerobic glycolysis or the "Warburg effect"(10). Major advances in our understanding of this phenomenon over the last decade culminated in the recognition that altered metabolism, which is often now referred to as "metabolic reprogramming" is a hallmark feature of cancer. Metabolic reprogramming, in its simplest terms, describes the process by which cells rewire their metabolic networks to support the requirements of exponential growth and proliferation and protection against oxidative stress. Common adaptions include enhanced glycolysis and glutaminolysis, as well as changes in mitochondrial function and redox homeostasis. Beyond the setting of cancer, there is increasing recognition that metabolic reprogramming is critical for shaping inflammatory and immune responses by influencing innate and adaptive immune cell differentiation and function (11–15). There is also now growing evidence that metabolic reprogramming may contribute to the pathogenesis of important non-oncological conditions, including Alzheimer's disease, obesity, cardiovascular disease, diabetes, ageing and notably, fibrosis (16–18). It is also appreciated that there is considerable mechanistic overlap, including some

shared metabolic signatures observed between cancer and IPF (19, 20). This article will now focus on the emerging evidence for metabolic reprogramming in the context of pathological fibrosis, focussing in particular on pulmonary fibrosis, where altered metabolism has most extensively been investigated.

Glycolysis

Glycolysis describes the ten-step process through which glucose is broken down into pyruvate, with the free energy released used to generate the high-energy molecule, adenosine triphosphate (ATP), and the coenzyme, reduced nicotinamide adenine dinucleotide (NADH) (Figure 1)(21). In the presence of oxygen, pyruvate is usually oxidised in the mitochondria to form ATP and CO_2 through the process of oxidative phosphorylation (OXPHOS). In contrast, under hypoxic conditions, pyruvate is reduced to lactate in the cytosol, a process referred to as anaerobic glycolysis. Despite OXPHOS generating approximately 18 times more ATP per glucose molecule compared to glycolysis, rapidly proliferating cancer cells even in the presence of sufficient oxygen demonstrate high rates of glucose uptake and lactate secretion (aerobic glycolysis)(10, 22). Otto Warburg proposed that this feature of aerobic glycolysis in cancer cells was a consequence of primary mitochondrial defects, which could be overcome by increasing glycolytic flux. However, this hypothesis has now been largely refuted based on the evidence that cancer cells have been shown to have intact mitochondrial function and generate ATP from both glycolysis and oxidative phosphorylation, with the majority coming from the latter (23). Indeed, it was widely held that the high rates of glycolysis observed in cancer cells allows for a plentiful supply of glycolytic intermediates to feed macromolecular biosynthesis pathways in rapidly proliferating cells (10, 13). However a recent carbon labelling study in non-small cell lung cancer called this view into question by revealing that amino acids rather than glucose account for the majority of carbon mass in proliferating cells and that glucose represents an important source of ribose for nucleic acid replication and contributes to biomass by providing non-carbon material, such as energy and reducing equivalents (24). Additionally, there is now evidence supporting the “reverse Warburg phenomenon” in cancer cells, where surrounding stromal cells, notably cancer associated fibroblasts (CAFs), exhibit high rates of aerobic glycolysis resulting in the secretion of carbon rich intermediates, including lactate, to neighbouring cancer cells and thereby fuel OXPHOS and anaplerotic reactions (25).

The glucose-addicted nature of cancer cells has been exploited clinically with the use of ^{18}F-FDG PET/CT scans to detect certain cancers and monitor treatment response by coupling the positron emitting ^{18}F to a glucose analogue (FDG) which is taken up by cells but not subject to further metabolism. The first clue indicating that enhanced glycolysis may also be a feature of IPF came from studies performed at University College London (UCL) demonstrating that ^{18}F-FDG uptake is increased in the lungs of patients with IPF. The signal was further localised to areas associated with

honeycombing, a radiological hallmark feature of IPF on high-resolution computed tomography (HR-CT)(26), suggesting that these areas are metabolically active and may share the glycolytic phenotype of cancer. Interestingly, the FDG signal was subsequently also found to be higher in non-fibrotic areas of IPF lungs compared to control lungs, suggesting a global metabolic change may occur in IPF before radiological features of fibrosis are apparent (27). Further work revealed that ^{18}F-FDG PET uptake can potentially be used as a clinical biomarker to assess therapeutic response in fibrosis. For instance, pirfenidone was found to significantly reduce ^{18}F-FDG uptake in the murine bleomycin model of lung fibrosis (28). Moreover, ^{18}F-FDG PET uptake was found to predict progression-free survival in IPF and was independently associated with increased risk of mortality (29, 30). In contrast, ^{18}F-FDG uptake in the lungs of IPF patients did not appear to change over 3 months of currently approved anti-fibrotic treatment (nintedanib or pirfenidone) (28). The interpretation of these findings however remains at the centre of an interesting debate around the potential confounding effect of increased lung density on the measured PET signal (tissue-fraction effect or TFE). Different methods of TFE correction have produced opposing observations with a recent study using dynamic imaging reporting that the ^{18}F-FDG PET signal in the fibrotic areas of IPF patients is reduced when corrected for TFE (31, 32). In contrast, a recent experimental medicine study focused on assessing the tolerability of the pan-PI3K/mTOR inhibitor, omipalisib, revealed an exposure-dependent reduction in ^{18}F-FDG uptake in fibrotic areas of the lung (33). Further work is now required to improve current understanding of the signal and biological process underlying the uptake of ^{18}F-FDG PET in IPF and to identify which cell type(s) are responsible for the enhanced ^{18}F-FDG PET signal. It is also worth commenting that enhanced ^{18}F-FDG PET uptake is not limited to lung fibrosis, but has also been observed in liver, skin and retroperitoneal fibrosis (34, 35), indicating that altered glycolysis might represent a hallmark feature of multiple fibrotic conditions. These clinical observations therefore prompted considerable research efforts aimed at furthering current understanding of the underlying mechanisms responsible for altered glycolysis, with a particular focus on (myo)fibroblast function.

Glucose uptake

Glucose uptake marks the first rate-limiting step of glycolysis, which is enhanced by the increased expression of glucose transporters. In vitro studies in human lung fibroblasts revealed that upon stimulation with the major pro-fibrotic mediator TGF-β_1, glucose uptake and the expression of glucose transporter 1 (GLUT1) are increased at both the mRNA and protein levels. GLUT1 expression has also been reported to be raised in lung tissue from IPF patients and in the bleomycin model of lung fibrosis (36–38). The necessity for glucose in mounting a fibroblast fibrogenic response was subsequently demonstrated in studies showing that TGF-β_1-induced myofibroblast differentiation (based on the de novo induction of the marker protein, αSMA) and collagen synthesis were inhibited in the absence of extracellular glucose (37, 38).

IPF is classically described as a characteristic disease of the aging population, where overlap of perturbed cellular processes in aging and IPF contribute to accelerated fibrogenesis. Pulmonary fibrosis is enhanced in response to bleomycin injury in aged versus young mice and this was shown to be associated with increased GLUT1 mRNA and protein levels (39–41). Loss-of-function studies based on silencing GLUT1 (*SLC2A1*) with shRNA or by pharmacological inhibition using the GLUT1 inhibitor, GLUT inhibitor II and sodium-coupled glucose transporter inhibitor, phloretin, showed that TGF-β_1-induced αSMA expression was inhibited. Phloretin was additionally found to inhibit the production of both collagen and fibronectin by TGF-β_1-stimulated fibroblasts as well as attenuate lung fibrosis in the bleomycin model (36, 41).

Glycolytic enzyme expression

Enhanced glycolysis is often facilitated by an increase in the expression of glycolytic enzymes. Increased mRNA levels of the key rate-regulating glycolytic enzymes, hexokinase 2 (HK2), phosphofructokinase-1 (PFK1) and pyruvate kinase muscle isoenzyme M2 (PKM2) have been reported in TGF-β_1-stimulated control and IPF fibroblasts (37, 38, 42). Protein levels of the enzyme 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3), which catalyses the conversion of fructose-6-phosphate to fructose-2,6-bisphosphate, an allosteric activator of PFK1 and a potent stimulator of glycolysis, was shown to be increased in response to TGF-β_1 stimulation in control and IPF lung fibroblasts. Moreover, PFKFB3 levels have been reported to be elevated in pulmonary epithelial cells, macrophages and in fibroblastic foci in IPF lungs, as well as in experimental models of pulmonary fibrosis (42). HK2 protein levels are also increased in response to TGF-β stimulation in control and IPF-derived lung fibroblasts. Pharmacological inhibition of PFKFB3 (using 3PO) and HK2 (using 2-deoxyglucose and lonidamine) abrogated TGF-β_1-induced myofibroblast differentiation, collagen production and contractility (42–44). Furthermore, HK2 inhibition by either siRNA silencing or pharmacological inhibition using lonidamine, abrogated the TGF-β induced activation of known transcriptional regulators of key profibrotic mediators, YAP and TAZ (YAP/TAZ) (44). Pre-clinical in vivo studies support the potential therapeutic utility of targeting glycolysis in the setting of fibrosis, in that 3PO and lonidamine treatment also attenuated the development of fibrosis in the murine bleomycin and TGF-β_1-induced lung fibrosis models, with lonidamine also improving lung function (42, 44).

Lactate production

Glycolysis culminates in the production of pyruvate, which can either be shuttled into the TCA cycle or converted into lactate by lactate dehydrogenase (LDH). Lactate excretion is therefore used as a surrogate marker of enhanced glycolytic flux and can be measured by commercially available kits, mass spectrometry or nuclear magnetic resonance (NMR). Extracellular flux analysis (e.g. Seahorse Bioscience platform) allows the simultaneous analysis of extracellular acidification and oxygen consumption

as proxy measures of glycolysis and OXPHOS and has been used in multiple fibroblast studies. Extracellular acidification and enhanced lactic acid production have been extensively reported to be a key feature of TGF-β_1-activated fibroblasts and critical for myofibroblast differentiation and collagen synthesis (37, 38, 42, 45). Lactate levels are increased in IPF lung tissue (45–47) and in the murine model of bleomycin-induced fibrosis (42, 48); however, whether this reflects increased lactate production and glycolysis by myofibroblasts, at least in part, is not known.

LDH5, one of the five LDH isoenzymes with the highest efficiency to promote the conversion of pyruvate to lactate (49), was reported to be increased at both the mRNA and protein levels during TGF-β_1-induced myofibroblast differentiation in control and IPF derived lung fibroblasts, as well as in IPF lung tissue (45). Moreover, non-selective pharmacological inhibition of LDH using gossypol and siRNA silencing of LDH5, attenuated the TGF-β_1-induced increase in αSMA protein synthesis in control and fibrotic lung fibroblasts (50). Gossypol not only prevented the development of fibrosis but also halted progression of fibrosis in the murine model of bleomycin-induced fibrosis (48). The study investigators proposed that increased lactate production may provide a feed-forward loop for the activation of latent TGF-β_1 via a pH-dependent mechanism to drive myofibroblast differentiation (45). However, it is worth commenting that in anti-cancer trials, gossypol was found to exert unspecific cytotoxic and genotoxic effects in mammalian cells (51, 52). A subsequent study examining the effect of a specific small molecule inhibitor of LDH5, Tool Compound 408 (Genentech) on TGF-β_1-stimulated differentiation of primary human lung fibroblasts(53), revealed that selective inhibition of LDH5 did not decrease fibronectin, collagen and αSMA expression, despite inhibiting the TGF-β_1-induced increase in lactate production. Additionally, neither siRNA knockdown of *LDHA* nor of *LDHB* (which encode gene products in varying combinations to generate all LDH1-5 isoforms) inhibited TGF-β_1-induced fibroblast differentiation. In contrast, gossypol appeared to exert its anti-fibrotic effect via a detrimental effect on cell viability rather than through a specific effect on LDH5.

De novo serine-glycine production

Current evidence suggests that lactate accumulation may not be critical for myofibroblast differentiation and collagen production, but rather might be representative of enhanced glycolytic flux. This in turn enables increased glycolytic intermediates to be made available for biosynthetic pathways, including de novo amino acid synthesis. Recent studies have highlighted the importance of adapting de novo synthesis of glycine to meet the demands of fibrogenesis. Glycine is a non-essential amino acid that occupies every third position of the collagen triple helical region. The glycolytic intermediate, 3-phosphoglycerate, produced by phosphoglycerate kinase is the feeding substrate into de novo serine and glycine biosynthesis. Evidence from function blocking studies of TGF-β_1-stimulated fibroblasts and the bleomycin model presents strong support that the increased requirement for glycine during fibrogenesis

is provided by the increased expression of the key glycine biosynthetic enzymes, phosphoglycerate dehydrogenase (PHGDH), phosphoserine aminotransferase 1 (PSAT1), phosphoserine phosphatase (PSPH) and serine hydroxymethyltransferase 2 (SHMT2) (37, 38, 54). Furthermore, enhanced expression of PHGDH and SHMT2 was observed within IPF fibrotic foci (37) and evidence from gas chromatography-mass spectrometry (GC-MS) profiling further reported an increase in glycine abundance in IPF lung tissue (47).

The critical role for glycine biosynthesis during fibrogenesis was further established by demonstrating that pharmacological blockade (using CBR-5884 and NCT-503) as well as genetic inhibition of PHGDH abrogated TGF-β_1-induced increases in αSMA and collagen expression in human lung fibroblasts in vitro and further that pharmacological inhibition of PHGDH with NCT-503 attenuated bleomycin-induced lung fibrosis (37, 38, 54). Moreover, studies using ^{13}C-labelled glucose have shown increased incorporation of glucose-derived carbons into serine, glycine and collagen in TGF-β_1-stimulated lung fibroblasts in vitro(37, 55). Further work from our laboratory demonstrated that extracellular glycine supplementation was able to partially rescue the inhibitory effect of glucose deprivation on TGF-β_1-stimulated collagen synthesis (38). Taken together current evidence therefore indicates that glucose is integral to the synthesis of collagen by contributing carbon to the de novo glycine synthesis pathway (38). Enhanced glycolysis therefore plays a key role in fibrogenesis by providing key intermediates for collagen synthesis.

It is worth commenting that increased glycolysis is not just limited to actively synthesising myofibroblasts in the lung. Indeed, a switch to a glycolytic phenotype has also been observed in fibroblasts derived from fibrotic skin and myofibroblast-like hepatic stellate cells in the setting of liver fibrosis. Genome wide transcriptomic profiling of fibrotic human skin and TGF-β stimulated skin fibroblasts provided evidence for increased expression of glycolytic genes, with genetic and pharmacological manipulation of glycolysis inhibiting ECM production in skin fibroblasts. Furthermore, the metabolic changes that occur during the transdifferentiation of quiescent hepatic stellate cell (Q-HSC) into myofibroblastic hepatic stellate cells (MF-HSC) have similarly been shown to include enhanced glycolysis to meet the requirements associated with myofibroblast differentiation and collagen production (reviewed in (56)).

It should be highlighted that although the majority of studies support the notion that TGF-β_1 stimulation of fibroblasts leads to an overall increase in glycolysis, this was not a universal finding. Indeed, one study demonstrated that IPF-derived senescent fibroblasts have a tendency towards reduced glycolysis, assessed by measuring the extracellular acidification rate (ECAR) of the surrounding tissue culture media, and demonstrating a reduced rise in ECAR upon TGF-β_1 stimulation when compared to normal lung fibroblasts (57, 58). However, whether this observation is restricted to a specific senescent IPF fibroblast population within the IPF lower lung lobes from which

these particular fibroblasts were isolated remains unknown. It is also not clear if the conflicting data obtained in different laboratories might be reflective of cells representing different stages of the disease. By combining metabolomic and microarray analysis, a recent study recently reported that levels of glycolytic metabolites, including fructose 1,6-bisphosphate and phosphoenolpyruvate, were lower in IPF whole lung tissue relative to control lung and was accompanied by a decrease in the expression of PFK and PFKFB3. The same study reported increased lactate levels, suggesting that glycolytic products might be funnelled into lactate production. Conversely, using a mass spectrometry-based metabolomics approach, others reported increased levels of glycolytic intermediates, which would suggest that there is either increased glycolysis or decreased glucose derived carbon utilisation in the IPF lung (46, 47). Further investigation involving approaches such as metabolic flux analysis, will be needed to reconcile these somewhat disparate findings. It is also worth bearing in mind that whole lung tissue studies in IPF represent a combined profile of resident and recruited immune cells, so that spatial-temporal heterogeneity of the disease process and the contribution of individual cell types, may be important factors influencing the data obtained using this approach.

The currently available evidence supports the overall conclusion that enhanced glycolysis is a feature of myofibroblasts regardless of the organ of origin. This raises the prospect that targeting their common synthetic vulnerabilities could potentially lead to shared anti-fibrotic strategies across different fibrotic pathologies. In this regard, there are currently several glycolytic inhibitors in phase I/II clinical trials in the cancer setting and several of these agents have already been reported to have good safety and tolerability profiles in humans (59–61).

Glutamine metabolism

Glutamine is the most abundant amino acid in plasma and tissue and its breakdown by glutaminolysis provides an essential carbon source for a number of fundamental cellular reactions. Glutamine plays an important role in replenishing tricarboxylic acid (TCA) cycle intermediates, such as alpha-ketoglutarate (α-KG), by anaplerosis, thereby contributing to macromolecular synthesis and energy generation. It also acts as a nitrogen donor for nucleotide and amino acid synthesis and is required for glutathione production and redox homeostasis, as well as being involved in the activation of signalling pathways and epigenetic transformation (62). As these processes have all been implicated in the development of fibrosis, the role of glutaminolysis in fibrogenesis has been the focus of several recent studies. The first key enzymatic step during glutaminolysis is the conversion of glutamine to glutamate via glutaminase (GLS) (Figure 2). Current evidence suggests a potential role for GLS1 in the context of fibrogenesis, in that this isoform has been reported to be increased in TGF-β_1-stimulated IPF and non-IPF derived fibroblasts (63–65), as well as in IPF lung tissue and in the bleomycin model of lung fibrosis (65). In accord with enhanced GLS1 expression, there is a concomitant increase in intracellular glutamate levels and

extracellular glutamine consumption by myofibroblasts (63–65). Furthermore, a metabolic profiling study using mass spectrometry has also revealed increased glutamate abundance in IPF lung tissue (47).

Data obtained from in vitro studies of glutamine deprivation, pharmacological inhibition (using either CB839 or BPTES), as well as gene silencing of GLS1, support a critical role for glutaminolysis during the fibrogenic response by inhibiting TGF-β_1-induced collagen and α-SMA synthesis (63–65). Pharmacological inhibition of GLS1 did not lead to de-differentiation of the myofibroblast but did limit α-KG production, which is critical for proline hydroxylation and collagen structural integrity. GLS1 inhibition therefore led to accelerated collagen degradation in response to TGF-β_1 (65). The importance of GLS1 was further confirmed in vivo, in that conditional and selective ablation of GLS1 in fibroblasts resulted in attenuated bleomycin-induced lung fibrosis. This was confirmed by pharmacological inhibition with the GLS1 inhibitor, CB839 (66), which is currently being evaluated in clinical trials either alone or as a combination therapy in the cancer setting (e.g. NCT02071927; NCT02861300).

A role for the enzymes involved in the conversion of glutamate to α-KG during the second step of glutaminolysis has also been implicated in fibrogenesis. TGF-β_1 has been shown to increase the expression of alanine aminotransferase 2 (GPT2), aspartate aminotransferase (GOT1/2) and PSAT1 with a decrease in glutamate dehydrogenase 1 (GLUD1) expression in human lung fibroblasts (37, 38, 64). The global amino transferase inhibitor aminooxyacetate (AOA), inhibited TGF-β_1-induced collagen production in myofibroblasts, however genetic silencing of the aminotransferases, GPT1/2 and GOT1/2, had no effect on collagen production. Conversely, silencing of the glutamate-utilising enzyme, PSAT1, which additionally converts 3-phosphohydroxypyruvate (3-PHP) to 3-phosphoserine (3-PS) during de novo glycine synthesis, significantly inhibited collagen production in TGF-β_1-stimulated normal and IPF-derived human lung fibroblasts. Furthermore, PSAT1 expression is enhanced in IPF lung tissue and in the bleomycin model of lung fibrosis (64). Taken together, these data suggest that PSAT1 requires glutamate to promote 3-PS production for glycine synthesis during fibrogenesis but is also potentially required to generate α-KG for α-KG-dependent reactions, such as collagen hydroxylation as described above and for potentially replenishing the TCA cycle.

Glutamine-dependent anaplerosis is known to drive the TCA cycle and OXPHOS in the setting of cancer but there is conflicting evidence whether this occurs in the context of fibrosis. One study has shown that neither glutamine deprivation, GLS inhibition nor the use of the pan-aminotransferase inhibitor, AOA, impact the TGF-β_1-induced increase in OXPHOS as determined by an increase in the mitochondrial oxygen consumption rate (OCR) (64). However, a second study demonstrated that glutamine deprivation causes a decrease in OCR and postulates that the observed increase in the TCA intermediates, succinate and fumarate are formed through glutamine-derived anaplerosis to stabilise HIF1α (63). As previously mentioned, conflicting data obtained

in different laboratories might be reflective of different fibroblasts being used (adult lung versus fetal lung fibroblasts). A recent labelling study, however has shed more light; TGF-β_1 enhanced glutamine consumption by lung fibroblasts was associated with increased carbon labelling of citrate and downstream TCA metabolites (glutamate, malate and aspartate) from glutamine suggesting increased glutamine consumption and glutaminolysis are required to provide anapleurotic substrates for the TCA cycle (55). The same study showed that TGF-β_1 preferentially directs the flux of glutamine carbons into proline biosynthesis by seven-fold compared to only two-fold into TCA cycle metabolites (55). Glutamate is an important precursor for proline, which makes up 23% of the collagen molecule (67). Labelling studies in TGF-β_1 stimulated pulmonary fibroblasts, demonstrated enhanced collagen production, proline abundance and incorporation of glutamine derived carbons into proline. Proline is synthesised in the mitochondria from glutamine derived glutamate via two steps. First Δ-1- pyrroline 5 carboxylate synthetase (P5CS encoded by *ALD18A1*) catalyses the ATP- and NADPH-dependent phosphorylation and reduction conversion of glutamate to glutamate-γ-semialdehyde (GSA), which is in equilibrium with Δ-1-pyrroline-5-carboxylate (P5C). P5C reductases (*PYRC1,2* and *L*) then reduce P5C to proline, utilising NADH in the process (55). TGF-β_1 has been shown to increase the expression of all the enzymes involved in the proline synthetic pathway. P5CS and P5C reductases (*PYRC1,2* and *L*), with increased expression of P5CS are also reported in IPF lung tissue and in experimentally-induced pulmonary fibrosis in mice (46, 47, 55, 64). Furthermore, genetic inhibition of *ALDH18A1* depleted proline levels and reduced collagen production in TGF-β_1-stimulated human lung fibroblasts (55, 64). In terms of clinical evidence it is worth highlighting that markers of lung function; forced vital capacity (FVC) and diffusion capacity coefficient (DLCO) correlated inversely with the expression of P5CS in IPF lung tissue (55).

Accumulating evidence also suggests that epigenetic reprogramming plays an important role in the pathogenesis of IPF through the modulation of fibroblast differentiation, collagen synthesis and apoptosis. Glutaminolysis has been reported to alter histone methylation and thereby influence anti-apoptotic gene expression in IPF fibroblasts. The apoptosis-resistant phenotype of myofibroblasts is widely acknowledged to be a key feature contributing to the progression of lung fibrosis in IPF. X-linked inhibitor of apoptosis (XIAP) has been reported to be increased in IPF as well as enhance apoptosis susceptibility in lung fibroblasts(68). JMJD3 histone demethylase has been shown to bind to the promoter of the anti-apoptotic gene, *XIAP*, in a glutamine- and α-KG-dependent manner, indicating that glutaminolysis is required for the epigenetic regulation of anti-apoptotic genes in IPF-derived fibroblasts (69).

A picture is emerging that glutaminolysis promotes a number of processes that are critical for the pro-fibrotic phenotype of myofibroblasts in IPF. These observations have been mirrored in the context of liver fibrosis, where glutaminolysis also promotes the transdifferentiation of HSCs into MF-HSCs (56). GLS1 inhibitors are currently being trialled in phase I and II cancer trials, either alone or in combination with other

therapeutic agents (NCT02861300) (Table 1). GLS1 inhibitors, either alone or in combination with other anti-fibrotic approaches might therefore also represent a potential opportunity to interfere with fibrogenesis in multiple fibrotic pathologies.

Lipid metabolism

There is a wealth of evidence demonstrating that an imbalance of lipid mediators, such as prostaglandins and lysophospholipids, can drive fibrogenic responses in the setting of IPF. This evidence was detailed in a recent review (70). However, the reprogramming of the lipid synthesis pathways in the context of fibrosis has been less well studied. Fatty acid synthase (FASN) is a multifunctional homodimeric enzyme that generates long-chain fatty acids from malonyl-CoA through the de novo fatty acid synthesis pathway (Figure 3). In response to TGF-β_1, FASN is increased at the mRNA and protein levels in human lung and murine fibroblasts, as well as in bleomycin-challenged murine lung. Silencing FASN decreases the expression of collagen I, connective tissue growth factor (CTGF) and fibronectin with pharmacological inhibition (C75) decreasing lung fibrosis and stabilising lung function decline in mice (71). The mechanism through which FASN mediates its pro-fibrotic effects remains unclear, and further work is required to determine whether increased long chain fatty acids are generated to fuel the TCA cycle or potentially provide precursors for lipid mediated signalling pathways. It is worth commenting that aberrant FASN expression is also observed in many cancers with phase I trials of agents targeting FASN, showing favourable tolerability profiles in patients with solid tumours (72, 73).

In the context of skin fibrosis, a recent study revealed that while enhanced glycolysis drives ECM production in fibrotic skin and skin that was abundant in ECM, fatty acid oxidation, conversely was suppressed. A reduction in fatty acid oxidation in fibrotic skin fibroblasts was accompanied by a downregulation of the expression of genes associated with fatty acid oxidation. Furthermore, pharmacological and genetic inhibition of fatty acid oxidation enhanced fibroblast ECM production in dermal fibroblasts (35). However, it is unclear why the downregulation of fatty acid oxidation would promote ECM production, although it has been speculated that limiting other ATP generating sources permits glycolysis to function more efficiently. Our understanding of the alterations that occur within the lipid metabolic networks during fibrogenesis remains incomplete. Future studies aimed at characterizing these alterations are needed and may identify potential novel therapeutic approaches, including targeting FASN.

Mitochondrial metabolism

Mitochondria are the powerhouses of the cell, capable of producing ATP, biosynthetic intermediates and dictating differing biological outcomes, such as programmed cell death. The mitochondria contain the ATP-producing machinery, which comprises the

514 tricarboxylic acid (TCA) cycle and oxidative phosphorylation. Substrates including
515 pyruvate, fatty acids and glutamine enter the TCA cycle, and lead to the production of
516 NADH and $FADH_2$ to drive ATP production by the electron transport chaing (ETC).
517 Mitochondria are also important sites of production of key intermediates, such as
518 oxaloacetate and α-KG for macromolecule biosynthesis, while acetyl–CoA and α-KG
519 are also important for epigenetic regulation of gene expression (62)(Figure 3).
520
521 There is growing interest in the role of dysfunctional mitochondrial metabolism in
522 driving age-related diseases, such as diabetes and cancer (74, 75) and more recently
523 the reconfiguration of mitochondrial function has also been implicated in the
524 pathogenesis of IPF. Age is a significant risk factor for the development of IPF and this
525 predisposition has been attributed to increased sensitivity of key pulmonary structural
526 cells, in particular alveolar epithelial cells, to cellular stress. There is now evidence
527 suggesting that abnormal mitochondrial phenotype, including enlarged mitochondria,
528 increases in mitophagy, reactive oxygen species (ROS) production and cell death
529 pathway activation, enhances cellular susceptibility to stress and vulnerability to
530 develop fibrosis (reviewed in (58)).
531
532 Deficient autophagy, including mitophagy, has also been implicated in IPF and the
533 development of pulmonary fibrosis in response to injury (76). Mitophagy is a highly
534 conserved adaptive response that targets healthy and damaged mitochondria for
535 turnover, thereby regulating the number of mitochondria to match cellular energy need,
536 as well as avoiding potential cellular stress by mitochondrially-produced ROS
537 (mtROS). Mitophagy is mediated through the PTEN-induced kinase 1 (PINK1)-Parkin
538 signaling pathway, where PINK1, a serine threonine kinase, detects partial
539 mitochondrial membrane depolarization and recruits Parkin, a cytosolic E3 ubiquitin
540 ligase, to the outer mitochondrial membrane. Parkin in turn ubiquitinates dysfunctional
541 mitochondria, marking them for degradation in the autophagasome. TGF-β has been
542 shown to decrease PINK1 mRNA levels and associated mitochondrial recycling in
543 myofibroblasts. Reduced PINK1 protein expression has also been reported in IPF lung
544 biopsies, mouse models of fibrosis and in aged murine models (77). A recent study has
545 further shown that lowered PINK1 and Parkin (*PARK2*) expression in lung fibroblasts
546 causes a decrease in mitophagy and thereby leads to mtROS production and promotes
547 signalling through the pro-fibrotic PDGF receptor to drive increased fibroblast
548 proliferation and myofibroblast differentiation. In support of these in vitro findings,
549 lung fibrosis is augmented in *Parkin*-deficient mice following bleomycin challenge
550 (78). Moreover, the licenced anti-fibrotic agent, pirfenidone, has been reported to
551 increase PARK2-mediated mitophagy, which may, in part, explain how it mediates its
552 anti-fibrotic effects (79).
553
554 ROS can activate multiple signalling pathways, including hypoxia-inducible factor 1-
555 alpha (HIF1α), p53 and NF-κB, which, in turn, leads to increased expression of
556 cytokines involved in tissue injury and fibrosis (80–82). Several pathways contribute
557 to ROS production, including the ETC and NADPH oxidase 4 (NOX4). In the context

of fibrogenesis, TGF-β has been demonstrated to induce the generation of mtROS by complex III of the electron transport chain in human lung fibroblasts and that this is required for TGF-β-mediated gene transcription of αSMA and CTGF downstream of the phosphorylation and nuclear translocation of Smad3 (83). Furthermore, the earlier reported TGF-β induced increase in glutaminolysis supports TCA anapleurosis, resulting in increased mitochondrial respiration in human lung fibroblasts. Enhanced OXPHOS can therefore lead to increased mtROS production that can stabilise HIF1α and lead to HIF1α mediated metabolic reprogramming in fibrosis (55).

NOX4 also contributes to ROS production and has been shown to localise to the mitochondria in murine embryonic fibroblasts (84). Enhanced NOX4 expression is further observed in the IPF lung. TGF-β_1-induced ROS production by NOX4 has been reported to promote fibroblast to myofibroblast differentiation and extracellular matrix production in IPF-derived mesenchymal cells, whereas pharmacological (using Cpd 88) and genetic inhibition of NOX4 (using siRNA) prevented bleomycin-induced lung fibrosis (85, 86). Additionally, inhibition of TGF-β-induced mitochondrial ROS generation and gene expression by mitochondria-targeted antioxidants has been reported in fibroblasts derived from IPF patients (83).

Despite a sustained increase in mitochondrial respiration in response to TGF-β in myofibroblasts, the early increase in ROS production has been reported to decline over time. Enhanced proline biosynthesis during fibrogenesis, has been demonstrated to act as an effective vent for redox stress, by utilising redox equivalents, NADP and NADPH and thereby reduce excessive mtROS production from increased OXPHOS in response to TGF-β (55). Furthermore, genetic silencing of mitochondrial NADPH and NADH oxidases reduced proline accumulation in lung fibroblasts while inhibition of the ETC and ATP synthesis inhibited proline production, suggesting that an intact mitochondrial ETC is critical for maintaining proline levels (55).

Oxidative damage caused by ROS produced by the mitochondria can in turn cause mitochondrial DNA (mtDNA) damage and the release of mtDNA into the extracellular environment. Extracellular mtDNA release has been shown to be increased in IPF fibroblasts and in TGF-β_1-induced primary HLFs, with mtDNA alone triggering αSMA expression in unstimulated HLFs (87). mtDNA has CpG-rich regions that can act as damage-associated molecular patterns (DAMPS) that trigger a toll-like receptor 9 (TLR9) regulated immune response which has also been implicated in driving fibrosis (88, 89). Increased circulating mtDNA is also observed in IPF bronchoalveolar lavage fluid (BALF) and correlates with enhanced disease progression and reduced event-free survival (87).

The sirtuins SIRT-3 and -7, which are major NAD^+-dependent deacetylases that control mitochondrial metabolism (including limiting mitochondrial ROS levels), have also been reported to be decreased in IPF lung tissue (90). Silencing of SIRT-3 or -7 (90, 91) in human lung fibroblasts promotes Smad3 transcription, collagen I protein

synthesis, with overexpression of SIRT-3 and -7 decreasing αSMA and collagen I protein abundance in TGF-β_1-stimulated human lung fibroblasts. SIRT-3 and -7 protein abundance is decreased in lung fibroblasts derived from murine models of aging and pulmonary fibrosis; with SIRT3-deficient mice being more susceptible to bleomycin-induced pulmonary fibrosis (91).

Recent evidence suggests that IPF myofibroblasts and TGF-β_1-stimulated human lung fibroblasts not only assume a glycolytic phenotype but also increase mitochondrial respiration, albeit to a lesser degree (42, 43, 55). Increased mitochondrial respiration provides ATP, TCA carbon intermediates and ROS to support a number of fibrogenic responses (55). A recent study demonstrated that increased mitochondrial respiration was accompanied by enhanced mitochondrial biogenesis facilitated by a p38 mitogen activated protein kinase (p38 MAPK)-dependent increase in the phosphorylation of the mitochondrial biogenesis transcription factor, peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC1α), and the downstream mitochondrial transcription factor A (TFAM). Silencing TFAM decreased αSMA protein production but had no effect on collagen IA1 or fibronectin mRNA levels (43). Furthermore, the ETC complex I inhibitor, rotenone and TFAM knockdown both inhibited TGF-β_1-induced myofibroblast contractility and αSMA protein synthesis (43). However, work in our laboratory showed that inhibiting ETC complexes I and III with rotenone and antimycin A did not affect TGF-β_1-induced collagen production (38). These data therefore favour the notion that enhanced OXPHOS may therefore be predominantly required for maintaining the contractile phenotype of the differentiated myofibroblast, rather than for collagen synthesis.

Finally, the TCA cycle in the mitochondria is important for cataplerosis, supplying a number of intermediates that can be utilised by enzymatic reactions for cell growth and proliferation. Two recent mass spectrometry-based studies demonstrated that TGF-β_1 enhances the production of the TCA metabolites: succinate, α-KG, fumarate, malate and citrate (55, 63). Succinate is also increased in TGF-β_1-stimulated lung myofibroblasts and in the murine model of bleomycin induced lung fibrosis (42). Metabolic profiling of IPF lung tissue additionally revealed increased mRNA levels of succinyl-CoA synthetase, which is responsible for converting succinyl-CoA to succinate in the TCA cycle (46). In normoxia, succinate stabilises the master regulator of hypoxia, HIF1α, by inhibiting the prolyl hydroxylases responsible for its degradation (92). A potential role for HIF1α in IPF was suggested by the identification of a HIF binding site within the promoter of the αSMA (*ACTA2*) gene (42). Furthermore, succinate has been shown to enhance the TGF-β_1-induced increase in HIF1α stabilisation and αSMA protein expression in myofibroblasts (42).

Increased TCA cycle activity may therefore enhance succinate-induced stabilisation of HIF1α and thereby facilitate the myofibroblast differentiation programme. HIF1α is also a key transcriptional regulator of metabolic reprogramming in other cell types and has been shown to promote lactate secretion in TGF-β_1-stimulated IPF fibroblasts (93).

One mechanism by which HIF1α increases lactate production is by increasing pyruvate dehydrogenase kinase 1 (PDK1) expression. PDK1 is located in the mitochondrial matrix and inhibits the pyruvate dehydrogenase complex, which attenuates entry of glucose carbons into the TCA cycle as acetyl-CoA. PDK1 expression is increased in TGF-β_1-stimulated fibroblasts and knockdown of PDK1 and the pharmacological inhibitor, DCA, decreased the TGF-β_1-induced increase in lactate and αSMA production in IPF fibroblasts, and also attenuated the development of fibrosis in the bleomycin model(93).

There is therefore now an emerging body of evidence that in addition to producing ATP, mitochondria promote a pro-fibrotic environment via several mechanisms, including ROS production and by reducing mitophagy. In terms of therapeutic implications, the use of the non-targeted antioxidant, N-acetyl cysteine (NAC), to increase glutathione levels in IPF, was recently shown to have no significant clinical benefit (94). Although the use of targeted mitochondrial antioxidants have not yet been tested in the context of IPF, the NOX1/4 inhibitor, GKT13781, is currently being evaluated in a phase II clinical trial in IPF patients (NCT03865927).

Kinase regulators of metabolism: mTOR and AMPK

Mechanistic target of rapamycin (mTOR) and AMP-activated protein kinase (AMPK) acts as key nutrient and cellular energy sensors and master regulators of cell metabolism. The interplay between these two signaling pathways is increasingly recognized to play a pivotal role in directing the reconfiguration of metabolic networks in the context of multiple conditions, including cancer, autoimmune disease, metabolic disease, neurological disorders with increasing evidence now also emerging in the setting of fibrosis, including IPF.

We and others have shown that in addition to signalling via the canonical Smad pathway, TGF-β_1 also activates the mTOR signalling pathway in control and IPF derived lung fibroblasts (95–97) and further that mTOR signalling is central to mediating the fibrogenic effects of TGF-β_1. This observation, together with evidence of PI3K pathway activation in human IPF lung tissue (98) underpinned a recently completed proof of mechanism trial of the potent pan-PI3K/mTOR inhibitor, omipalisib, in IPF (33). Deconvolution of the mechanisms by which omipalisib exerts its inhibitory effects on TGF-β_1-induced collagen deposition further revealed that these effects are exclusively mediated through the mTORC1-4E-BP1 axis (97). More recent data from our laboratory further identified a critical role for this axis in fine-tuning the metabolic programme in TGF-β_1–induced myofibroblasts in that TGF-β_1-was found to increase the production of activating transcription factor 4 (ATF4), the transcriptional master regulator of amino acid metabolism, to supply glucose-derived glycine to meet the amino acid requirements associated with enhanced collagen production. TGF-β_1 induces ATF4 in a transcriptional and translation manner, with the latter depending on the activation of the mTORC1-4E-BP1 axis. ATF4, in turn, promotes the transcription

of genes encoding enzymes of the de novo serine-glycine biosynthetic pathway and glucose transporter 1 (GLUT1), which are integral to TGF-β_1-induced collagen synthesis as described earlier (38, 99) (Figure 4). Furthermore, cell-labelling studies with ^{14}C-glucose, revealed that the ATP-competitive mTOR inhibitor, AZD8055, prevents glucose-derived carbons from being incorporated into collagen (38). In terms of the potential translational significance of these findings, omipalisib was found to induce an exposure-dependent reduction in ^{18}FDG uptake in fibrotic areas of IPF lungs (33).

The upstream activation of mTORC1 during fibrogenesis is still poorly understood. mTORC1 integrates a diverse range of upstream signals, including growth factor signalling, energy status, nutrient availability and oxygen levels. The most well-characterized phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) pathway promotes mTORC1 activation by inactivating the tuberous sclerosis complex (TSC) (100). However, recent in vitro studies from our laboratory demonstrated that although the TSC2/Rheb axis plays a critical role in regulating TGF-β_1-induced mTORC1 activation and collagen I deposition, the PI3K/AKT pathway was dispensable (97). It is also worth commenting that, although these data contrast with other studies that report a role for PI3K signalling during TGF-β- induced collagen synthesis (101, 102), these earlier studies were performed using, first generation, PI3K inhibitors with broad actions on PI3K associated kinases, including mTOR (103). More recent data have identified a role for α-KG, a known activator of mTORC1, in promoting fibroblast collagen production (63, 65). As described earlier, glutaminolysis is an important source of α-KG and inhibition of the glutaminolysis enzyme, GLS with either CB839 or glutamine depletion prevents TGF-β_1-induced mTORC1 activation and α-KG production in myofibroblasts(65).

In contrast to mTOR, which is activated in energy-replete conditions and promotes ATP-consuming anabolic reactions, AMPK is phosphorylated and activated in response to a state of energy depletion to promote ATP production and decrease ATP utilisation. Low AMPK activity is characterised by decreased phosphorylation of the AMPK α-subunit at Thr172 which has been observed in TGF-β_1 stimulated HLFs, IPF-derived fibroblasts as well as associated with fibrotic regions in IPF lung tissue and the bleomycin model (41, 104). AMPK is a critical upstream inhibitor of mTORC1 signalling through the phosphorylation of the TSC. In IPF fibroblasts, a decrease in AMPK phosphorylation is associated with increased mTORC1 signalling and metabolic reprogramming, including HIF1α stabilisation and downstream lactate production (104). Metformin, is a commonly prescribed biguanide for the treatment of type II diabetes and mediates AMPK activation through its inhibition of ETC complex I and a subsequent increase in AMP:ATP ratio, while AICAR is an AMP mimetic and directly activates AMPK. AICAR and metformin, have been reported to prevent αSMA and ECM protein production in TGF-β_1-stimulated fibroblasts and reverse αSMA expression after 24 hours of TGF-β_1 stimulation (104, 105). Potential mechanisms

733 through which AMPK activation promotes anti-fibrogenic effects are via increased
734 autophagy, enhanced mitochondrial biogenesis, greater sensitisation of fibroblasts to
735 apoptosis and increased ECM turnover (104). Furthermore, as well as preventing the
736 development of experimental fibrosis, metformin promotes the resolution of fibrosis.
737 The latter observation has been linked to the ability of metformin to decrease NOX4-
738 derived ROS generation and subsequently inhibit Smad phosphorylation and
739 myofibroblast differentiation (105). However, a post-hoc analysis of metformin versus
740 non-metformin in the placebo arms of three IPF trials revealed that this agent had no
741 significant effect on IPF disease outcomes, including disease progression, forced vital
742 capacity (FVC) decline and mortality (106), so that the therapeutic potential of
743 metformin in IPF remains to be established.
744

745 **Future directions for fibrometabolism**

746 Fibrometabolism is an emerging and exciting avenue of research. Exploiting key
747 metabolic vulnerabilities of stromal cells could potentially open up promising new
748 therapeutic strategies to interfere with fibrogenesis in the context of multiple fibrotic
749 conditions. Current evidence suggests that fibroblasts, regardless of organ of origin,
750 fine-tune their cellular metabolic networks to support the synthetic requirement of
751 myofibroblasts in response to multiple stimuli, including genomic alterations, the
752 microenvironment and metabolic stress. Shifts in metabolic networks allow the cell to
753 both respond to a specific stimulus and also directly influence cellular phenotype. In
754 addition, optimism is now growing for the implementation of metabolism-targeting
755 therapeutic strategies with a number of recent trials reporting good tolerability and
756 efficacy in the oncology setting (Table 1 (107–109)). Several of these agents may
757 therefore represent repositioning opportunities for fibrosis, either as potential
758 standalone therapeutics or, as in cancer, as adjuvant therapies, which could potentially
759 sensitise fibroblasts to currently approved and emerging therapies. It is worth
760 highlighting that, fibrosis is often a chronic process so that restoring metabolic
761 homeostasis as a novel anti-fibrotic approach will require long term treatment. Fine-
762 tuning suppression of fibrosis to maximise therapeutic benefit while avoiding
763 interfering with tissue homeostasis will be critical for this approach to be successful.
764 This will be a particularly important consideration during periods of tissue injury and
765 repair; where for example both fibroblasts and endothelial cells are known to fine-tune
766 their metabolic networks to promote tissue reparative responses (110).
767
768 Metabolomics, a field that has recently seen a rejuvenation due to major analytic
769 advances, particularly in mass spectrometry, further holds considerable potential to
770 uncover specific metabolic ‘fingerprints’ of different pathologies thereby offering
771 possible applications for disease therapeutics and precision-based medical care.
772 Reports of the application of metabolomic techniques, including mass spectrometry and
773 NMR, are only just beginning to emerge in the setting of fibrosis and have thus far only
774 assessed the static metabolic changes associated with fibrogenesis. A more

775 comprehensive understanding of the complex multi-directional metabolic networks
776 underlying fibrogenesis, will require the adoption of an equally dynamic and multi-
777 layered metabolomic approach. In the cancer setting, in vitro and in vivo tracing of
778 metabolic pathways using stable isotope-labelled metabolites (e.g. ^{13}C glucose) in
779 combination with computational modelling, centred on network and pathway-based
780 integrative methods (e.g. weighted gene co-expression network analysis (WGCNA),
781 gene set analysis) to characterise the utilisation of different metabolites during
782 tumorigenesis, have been transformational in terms of identifying novel metabolic
783 vulnerabilities for therapeutic targeting. Matrix-assisted laser desorption ionisation
784 (MALDI) imaging allows in situ simultaneous mapping and quantification of
785 metabolites to anatomical structures without the loss of tissue integrity and has equally
786 been informative in the cancer setting. This, or other MS-based imaging techniques,
787 have not yet been widely applied in the context of fibrosis but a proof-of-mechanism
788 study assessing the feasibility of using MALDI for the detection of metabolites in a
789 mouse model of pulmonary fibrosis and treatment response to the approved anti-fibrotic
790 agent, pirfenidone, has recently been reported. The investigators demonstrate a clear
791 separation of metabolic profiles between treatment groups, warranting further
792 investigation of this approach in more highly powered studies (111).
793
794 It is important to emphasize that we are only just beginning to uncover and understand
795 the aberrant metabolic pathways that are critical to fibrogenesis. The time is now right
796 for the fibrosis community to begin to incorporate metabolomics into a systems biology
797 approach which will combine high-dimensional data from multiple omic platforms,
798 including transcriptomics and metabolomics, to identify the pathways underlying the
799 development of fibrosis in order to develop novel diagnostic and prognostic biomarkers
800 and importantly to provide potential novel solutions to target these pathways
801 therapeutically for therapeutic benefit
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807 **References**
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Figure Legends

Figure 1

Schematic overview of pharmacologically targeting glycolysis and its metabolic intersection with the de novo glycine biosynthetic pathway during TGF-β_1-induced fibrogenesis. Inhibiting glycolytic flux, potentially reduces the siphoning of glycolytic intermediates into biosynthetic pathways, including the de novo glycine biosynthetic pathway to supply glycine to meet the biosynthetic requirements of increased collagen production. GLUT 1; glucose transporter 1. HK2; Hexokinase 2. G6P; glucose-6-phosphate. F6P; fructose-6-phosphate. PFKFB3; 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase. F-2,6-BP; fructose-2,6-biphosphate. PFK1; phosphofructokinase. FBP; fructose-1,6-bisphosphate. 3PG; 3-phosphoglycerate. PEP; phosphoenolpyruvate. PKM2; pyruvate kinase isoenzyme M2. LDH; lactate dehydrogenase OXPHOS; oxidative phosphorylation. PHGDH; phosphoglycerate dehydrogenase. 3-PHP; 3-phosphohydroxypyruvate. PSAT1; phosphoserine aminotransferase 1. 3-PS; 3-phosphopserine. PSPH; phosphoserine phosphatase (PSPH). SHMT2; serine hydroxymethyltransferase 2. Metabolic inhibitors are marked in red. Glut II; GLUT inhibitor II. 2DG; 2 deoxyglucose.

Figure 2

A schematic overview of glutaminolysis and its metabolic connections with glucose derived glycine biosynthesis and collagen production**.** Glutaminolysis is critical for collagen synthesis by providing proline as well as α-KG for TCA cycle replenishment, mTOR activation, proline hydroxylation and epigenetic regulation. Metabolic inhibitors are marked in red. GLS; glutaminase. PSAT1; phosphoserine aminotransferase 1. 3-PHP; phosphohydroxypyruvate. 3-PS; 3-phosphoserine. P5CS; δ1- pyrroline 5 carboxylate synthetase. PYRC; P5C reductase. GPT; alanine aminotransferase. GOT; aspartate aminotransferase. GLUD; glutamate dehydrogenase. NH4+; ammonium. α-KG; alpha-ketoglutarate. BPTES; bis-2-(5-phenylacetamido-1,3,4-thiadiazol-2-yl)ethyl sulphide. AOA; aminooxyacetate. NAD+; nicotinamide adenine dinucleotide. NADH; reduced nicotinamide adenine dinucleotide. NADP+; nicotinamide adenine dinucleotide phosphate. NADPH; reduced nicotinamide adenine dinucleotide phosphate.

Figure 3

The ATP producing machinery of the electron transport chain and the tricarboxylic acid cycle (TCA) in the mitochondria promote fibrogenesis through ATP production, supply of TCA intermediates for biosynthetic pathways, redox and epigenetic regulation. Mitochondrial dysfunction, including reduced mitophagy and increased ROS production promote profibrotic signalling pathways. Inhibiting fatty acid synthesis may also potentially mediate antifibrotic effects through an inability to replenish the

TCA cycle or provide precursors for lipid mediating signalling. Potential anti-fibrotic strategies aimed at targeting mitochondrial and lipid metabolism are highlighted in red. PDH; pyruvate dehydrogenase complex. PDK1; pyruvate dehydrogenase kinase 1. α-KG; alpha-ketoglutarate. *SUCLA2*; succinate-CoA ligase. NADH; reduced nicotinamide adenine dinucleotide. $FADH_2$; reduced flavin adenine dinucleotide. OAA; oxaloacetate. mtDNA; mitochondrial DNA. NOX4; NADPH oxidase 4. ROS; reactive oxygen species. TFAM; mitochondrial transcription factor A. PINK1; PTEN-induced kinase 1. DCA; dichloroacetate. ACC; acetyl CoA carboxylase. FASN; fatty acid synthase.

Figure 4 ATF4-mediated metabolic and biosynthetic network reprogramming to support enhanced collagen biosynthesis in TGF-β_1–stimulated myofibroblasts. TGF-β_1–induced activation of the TGF-β_1 receptor complex leads to a Smad3-dependent increase in ATF4 mRNA abundance and mTOR activation. Activated mTORC1–4E-BP1 signalling, in turn, promotes ATF4 protein production through a translational mechanism. ATF4 subsequently promotes the transcription of key serine-glycine pathway genes and *SLC2A1* and, therefore, an increase in the abundance of the *SLC2A1* gene product, GLUT1. The serine-glycine biosynthesis enzymes and GLUT1 act together to promote glucose-derived glycine biosynthesis to support enhanced collagen synthesis rates in activated myofibroblasts. AMPK is a critical upstream inhibitor of mTORC1 signalling and metformin and AICAR act as AMPK activators which decreases mTORC1 signalling by phosphorylating the tuberous sclerosis complex (TSC). G6P, glucose 6-phosphate; 3-PG, 3-phosphoglycerate; 3-PHP, 3-phosphohydroxypyruvate; 3-PS, 3-phosphoserine; OXPHOS, oxidative phosphorylation.

Table 1: Metabolic inhibitors demonstrating anti-fibrotic pre-clinical effects in IPF and tested in cancer clinical trials.

Target	**Metabolic pathway**	**Drug**	**Pre-clinical data in IPF**	**Clinical trials**
HK2	Glycolysis	2DG Lonidamine Ketoconazole Posaconazole	• ↑mRNA in TGF stimulated HLFs • 2DG: ↓ αSMA and collagen in vitro • Lonidamine: ↓ αSMA and collagen in vitro ↓fibrosis and lung function decline in vivo	• Lonidamine: Phase III clinical trials in breast cancer and lung cancer (94) • 2DG: Phase I/II trials in advanced solid tumours and in combination with radiotherapy in cerebral gliomas (95,96) • Ketoconazole, posaconazole in advanced gliomas (NCT03763396)
PFKFB3	Glycolysis	3PO PFK158	• ↑mRNA and protein in TGF stimulated HLFs, IPF fibroblasts, IPF lung tissue • 3PO: ↓ αSMA and contractility in vitro ↓fibrosis in vivo	• PFK158: Phase I trial in advanced solid malignancies (NCT02044861)
PKM2	Glycolysis	TLN-232	• ↑mRNA in TGF stimulated HLFs	• TLN-232: Phase II studies in metastatic renal cancer and metastatic melanoma (NCT00422786, NCT00735332)
NOX4	Mitochondria	Cpd-88 GKT137831	• Cpd-88: ↓fibrosis in vivo • GKT137831 to be tested in phase II IPF trial	• GKT137831: Not tested in cancer but phase II trials in diabetic nephropathy (NCT02010242) and primary biliary cholangitis (NCT03226067).
PDK1	Mitochondria	DCA	• ↑PDK1 in TGF HLFs • DCA: ↓αSMA in vitro ↓fibrosis in vivo	• DCA: Phase II trials in metastatic NSCLC and breast cancer (NCT01029925), head and neck cancer (NCT01163487), brain cancer (NCT00540176), in combination with cisplatin and radiotherapy in head and neck (NCT01386632).
GLS1	Glutaminolysis	CB-839, BPTES,	• ↑mRNA, protein in TGF stimulated HLFs, IPF fibroblasts, IPF lung tissue • CB839, BPTES:↓αSMA and collagen in vitro ↓fibrosis in vivo	• CB-839: Phase I/II Clinical trials in solid and hematologic malignancies (NCT03047993, NCT03798678, NCT03163667, NCT03428217, NCT02071888, NCT02071927, NCT02771626,

				NCT02071862, NCT03875313, NCT03944902, NCT03872427, NCT03528642, NCT03263429 NCT03831932, NCT03057600).
FASN	De novo lipid synthesis	C75 TVB-2640	• ↑mRNA and protein in TGF stimulated HLFs • C75: ↓fibrosis in vivo	• TVB-2640: Phase I in solid tumours (NCT02223247), Phase II trials recruiting in NSCLC (NCT03808558), colon cancer (NCT02980029), breast cancer (NCT03179904), astrocytoma (NCT03032484).
AMPK		AICAR Metformin (ETC1 inhibitor)	• ↓pAMPK in TGF stimulated HLFs • Metformin: ↓ αSMA, collagen in vitro ↓fibrosis in vivo	• Metformin is licensed for the use in Type II DM Phase III trial in breast cancer
mTOR		AZD8055 AZD2014 MLN0128	• ↑mTOR phosphorylation of 4E-BP1 and S6K in TGF stimulated HLFs • AZD8055: ↓collagen in vitro	• AZD8055: Phase I trials in recurrent gliomas (NCT01316809), liver cancer (NCT00999882), advanced tumours (NCT00731263). • AZD2014: Phase I trials in glioblastoma multiforme (NCT02619864,NCT03071874), phase II in meningiomas (NCT02831257), high risk prostate cancer (NCT02064608) • MLN0128: Phase I in advanced solid tumours (NCT02719691) and Phase II in sarcoma (NCT02987959)