Kidney Allograft Fibrosis: Diagnostic and Therapeutic Strategies

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Abstract. Interstitial fibrosis with tubule atrophy (IF/TA) is the response to virtually any sustained kidney injury and correlates inversely with kidney function and allograft survival. IF/TA is driven by various pathways that include hypoxia, renin-angiotensin-aldosterone system, transforming growth factor-β signaling, cellular rejection, inflammation, and others. In this review, we will focus on key pathways in the progress of renal fibrosis, diagnosis and therapy of allograft fibrosis. This review discusses the role and origin of myofibroblasts as matrix producing cells and therapeutic targets in renal fibrosis with a particular focus on renal allografts. We summarize current trends to use multiomic approaches to identify new biomarkers for IF/TA detection and to predict allograft survival. Furthermore, we review current imaging strategies that might help to identify and follow-up IF/TA complementary or as alternative to invasive biopsies. We further discuss current clinical trials and therapeutic strategies to treat kidney fibrosis.

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INTRODUCTION

Renal tubulointerstitial fibrosis is characterized by excessive accumulation of extracellular matrix proteins. It is the final common pathway of virtually any sustained kidney injury. Factors known to cause interstitial fibrosis (IF) in transplant are immune-mediated or nonimmune-mediated, including acute and chronic rejection, arterial hypertension, dyslipidemia, diabetes, infections, ischemia, and immunosuppressive agents, particularly calcineurin inhibitors (CNIs).[1]

The severity of tubulointerstitial fibrosis correlates well with kidney function[2-4] and is strongly associated with tubule atrophy (TA), and thus considered as a single parameter interstitial fibrosis (IF)/TA.[5] IF/TA, previously included in the term chronic allograft nephropathy, is common in kidney allografts. IF/TA is detectable in 25% of renal allografts at 1 y,[6,7] around 65% at 2–5 y[8,9] and 90% at 10 y after transplantation.[9] Of note, another recent study suggests that moderate or severe fibrosis might be less prevalent (17% by 5 y).[10] IF/TA, especially when accompanied by inflammation, correlates with decreased graft survival.[11-13] It has been reported that 10-y graft survival was 95% in patients without IF/TA, but only 41% in patients with IF/TA and transplant vasculopathy.[14] Within patients that return to dialysis or require retransplantation, the most common cause of allograft failure has been reported to be IF/TA irrespective of the underlying cause, followed by acute rejection and recurrent primary kidney disease.[15,16] IF/TA is commonly caused by a defined etiology such as calcineurin nephrotoxicity,[17] polyomavirus-associated nephropathy,[18,19] or as a consequence of allo-immune mechanisms, including both cellular (T-cell mediated [TCMR]) and antibody-mediated rejection (AMR).[20] Although risk of death declined over the past 20 y in transplant patients, still many die from cardiovascular diseases and infections early after transplantation, and from cancer and cardiovascular diseases at later time points.[21,22] Therefore, death remains an important cause of allograft failure.[23]

In this review, we will focus on key pathways, diagnosis, and therapeutic options in kidney allograft fibrosis. Fibrosis of native kidneys and IF/TA in kidney allografts likely share pathophysiology and mechanisms. Since most

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knowledge on kidney fibrosis comes from nontransplant rodent models or patients with chronic kidney disease (CKD), we will also highlight relevant work from the general kidney fibrosis field. Whenever possible, recent work conducted in the field of transplantation will be presented. For further information on kidney allograft fibrosis and CKD, the readers are referred to the following excellent reviews.[24-27]

KEY PATHWAYS INVOLVED IN RENAL FIBROSIS

The key mechanistic pathways that have been implicated in the pathogenesis of IF/TA are renin-angiotensin-aldosterone system (RAAS), hypoxia,[28] transforming growth factor (TGF)-β signaling, unrecognized cellular rejection and chronic inflammation, cellular senescence and cell cycle arrest,[29-31] and DNA methylation[32,33] among others. Some of these pathways are partially induced by immunosuppressive therapy (Figure 1).

Myofibroblasts Are Matrix Producing Cells in Renal Fibrosis

It is believed that the kidney consists of >43 cell types with even more cell-states of individual cell-types.[34] The complex crosstalk between epithelial (tubule and glomerular cells), inflammatory, and endothelial cells and many other cell types in response to sustained injury leads to activation of myofibroblasts, which are the cellular origin of kidney fibrosis.[27,35] We have shown by genetic lineage tracing analysis that tissue-resident, but not circulating, Gli1+ mesenchymal stem cell-like perivascular cells proliferate after kidney injury to generate myofibroblasts.[36] Gli1+ cells represent a relatively small subset of the platelet-derived growth factor (PDGF) receptor beta + kidney population, but expand considerably after injury and contribute to about half of all myofibroblasts. Peritubular capillary loss has been proposed to be the final common pathway for fibrotic renal disease. We could show that kidney injury leads to loss of capillary number and size[37] and that the severity of kidney injury determines the severity of capillary loss. Gli1+ cells are adjacent to the endothelium of renal capillaries and are also present in the adventitia of arterioles and arteries distant from endothelium. Upon injury, Gli1+ cells detach from endothelium to become interstitial myofibroblasts driving fibrosis.[38] Gli1+ cell ablation in kidney homeostasis resulted in capillary loss and hypoxic tubule injury,[38] suggesting that the detachment of Gli1+ cells from endothelium might indeed trigger endothelial cell-death, capillary destabilization, and loss with subsequent hypoxic tubule injury, inflammation, and fibrosis. A potential role of vascular endothelial growth factor, PDGF, and ephrinB2 signaling has been reported to be involved in perivascular cell activation and potentially detachment to drive the process of capillary loss.[35,39-43] Using single-cell RNA–sequencing of nephrectomies from patients with and without CKD we could demonstrate that myofibroblasts are primarily derived from distinct fibroblast and pericyte lineages, whereas other cell-populations only show very minor contribution to matrix deposition.[44] The combination of PDGF receptor-alpha and PDGF receptor beta allowed to mark the vast majority of kidney myofibroblasts in these human kidneys.[44] Our analysis further indicated that immune cells and injured tubule epithelium are the key crosstalk partners that might drive activation of fibroblasts and pericytes. Importantly, these data have not been confirmed in kidney allografts yet, but since we discovered very conserved mechanisms of fibroblast and pericyte to myofibroblast differentiation in rodent kidney fibrosis and human nephrosclerosis, we hypothesize that the mechanisms in allografts will be quite similar with very likely recipient immune cells driving activation of donor mesenchymal cells.

Is Epithelial-mesenchymal Transition Involved in fibrosis? Fibrosis?

There is still ongoing controversy about additional precursor cells of myofibroblasts, including circulating bone marrow-derived cells, or transition from either macrophage, epithelial (EMT), or endothelial cells.[45-49] In the kidney, EMT describes the transitioning and cell migration of polarized epithelial tubule cells through the basal membrane into apolar mesenchymal cells in the interstitium. The evidence for EMT is strong in studies performed in vitro, but in vivo studies argue mostly against at least "complete" EMT.[45,48] A study using a rat model of kidney transplantation suggested that EMT is involved in the development of IF/TA and it coexists with enhanced oxidative stress.[50] An association between EMT 3 mo after renal transplantation and the late IF/TA lesions observed 1 y after transplantation has been reported.[51] We have performed inducible genetic fate tracing using SLC34a1CreER;tdTomato mice subjected to kidney fibrosis[52] and could show that proximal tubule epithelium does not differentiate into interstitial myofibroblasts. Furthermore, our recent single-cell RNA–sequencing data indicate that epithelial cells do not contribute much to matrix gene expression in human CKD.[44] However, partial EMT, a mechanism in which epithelial cells dedifferentiate upon injury toward a mesenchymal phenotype and secrete various profibrotic factors, that activate resident interstitial mesenchymal cells to differentiate into myofibroblasts, might play an important role in fibrosis.[53,54] Given the strong evidence against complete EMT outside the transplantation field,[45-48] it is very likely that the reported phenomenon indicates dedifferentiation of tubule epithelium, which then activates interstitial cells in a paracrine fashion.

Inflammation and Immune-mediated Injury

Acute TCMR is often prevented with the use of immunosuppressive therapy, but if present it is linked to a higher risk of IF/TA development.[13,55,56] Samples with prevalent IF/TA and with acute rejection-affiliated genes as identified in 234 graft biopsy samples correlated with a higher risk of graft lost at 5 or more y.[57]

The Banff Classification of Allograft Pathology is the predominant classification system for the reporting of renal allograft biopsies.[58] Since the combination of IF/TA with inflammation of any degree was associated with a worse prognosis as compared to fibrosis alone,[11,13,59,60] the Banff lesion score inflammation (i)-IF/TA was introduced in 2015.[61] Profibrotic and proinflammatory cytokines, produced by epithelial and inflammatory cells, are members of the TGF-β superfamily, interleukin-6, interleukin-8, monocyte chemoattractant protein-1 (MCP-1/CCL2), endothelin-1, macrophage inflammatory protein-1, chemokine (C-C motif) ligand 5, complement C3,

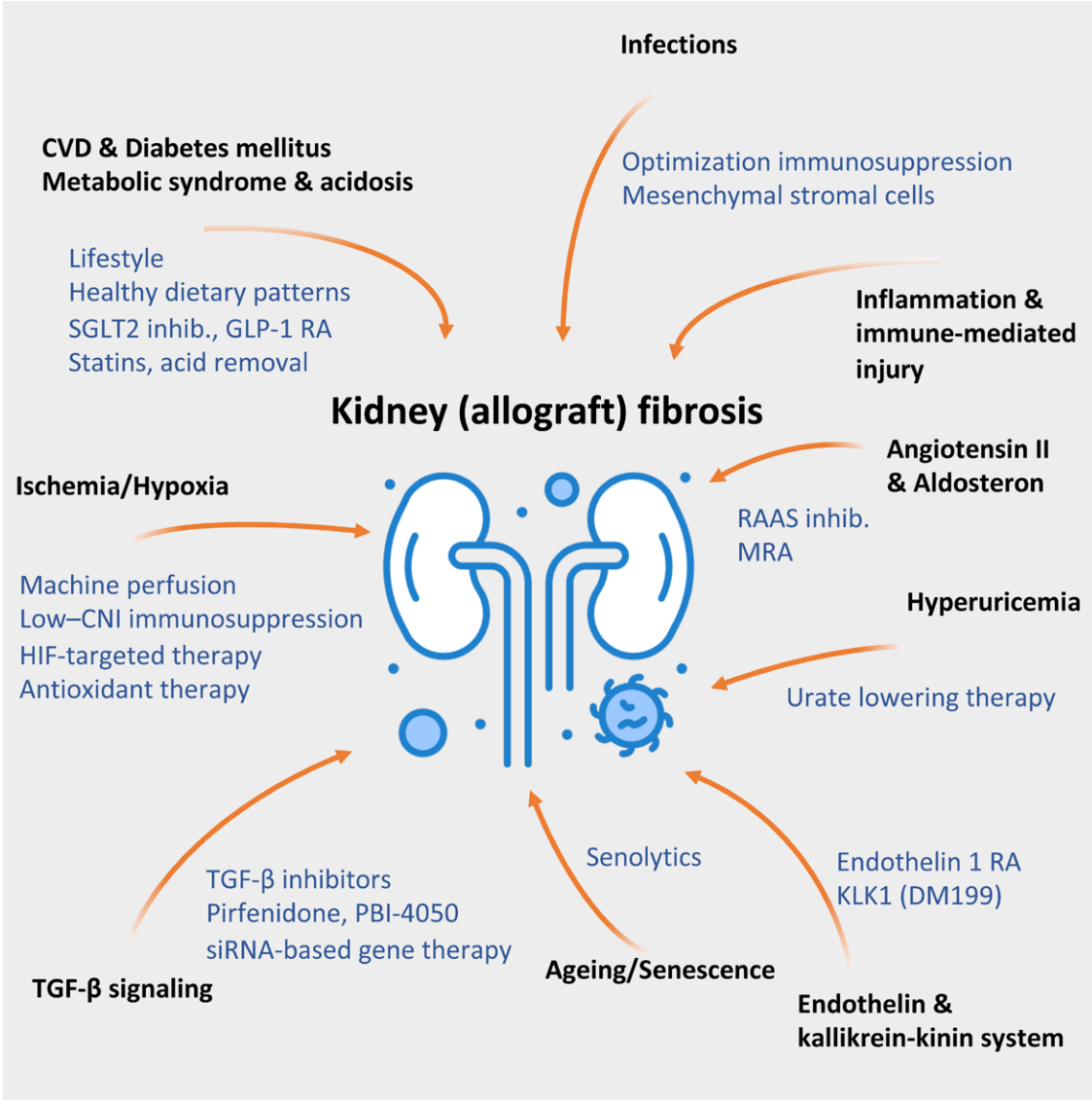

FIGURE 1. Risk factors for progression of kidney fibrosis and potential antifibrotic strategies. CNI, calcineurin inhibitor; CVD, cardiovascular disease; GLP-1, glucagon-like peptide 1 receptor agonist; HIF, hypoxia-inducible factor; KLK1, kallikrein 1; MRA, mineralocorticoid receptor agonist; RAAS inhib., renin-angiotensin-aldosterone system inhibitors; SGLT-2 inhib., sodium-glucose cotransporter 2 inhibitors; siRNA, small interfering RNA; TGF-β, transforming growth factor-beta.

macrophage migration inhibitory factor, and macrophage inflammatory protein-2.[62-66] i-IF/TA describes the extent of inflammation in the scarred cortex and is one of the criteria necessary for a diagnosis of chronic active TCMR.[5,67] Accelerated IF/TA progression by chronic TCMR inflammation is associated with allograft failure.[67,68] Molecular profiling indicated that IF/TA samples with or without inflammation have ongoing and often subclinical immune-mediated injury.[57] Borderline TCMR is a separate category of the Banff schema, which uses a lower diagnostic threshold and is usually considered as mild TCMR, although it has been also linked to IF/TA.[69-72]

AMR is associated with IF/TA and graft failure.[7,73-75] Diagnostic characteristics of AMR based on the Banff 2019 classification[76] are tissue injury in the absence of any other cause, evidence of recent or present endothelial reaction to antibodies, and presence of donor-specific antibodies (DSAs), in particular anti-HLA antibodies.[75] Of note, DSAs[77,78] are not always detected in AMR, but positive C4d staining and molecular classifiers are accepted as surrogate markers for DSAs.[76,79] In addition, recent evidence suggests that HLA-independent mechanisms can also trigger allo-recognition,[80,81] associated with early vascular intimal fibrosis and allograft survival.[82] Like TCMR, AMR can present with abrupt kidney dysfunction or can have a subclinical course.[83] Importantly, subclinical rejection (AMR without signs of acute kidney dysfunction, that is, rise in serum creatinine <20% or development of proteinuria <500 mg/d or per gram creatinine) has also long-term consequences for kidney allograft survival. An observational study showed a similar prevalence of subclinical TCMR and AMR, but patients with subclinical AMR had poorer graft survival at 8 y posttransplant (56%) compared to patients with subclinical TCMR (88%).[7] AMR with de novo DSA (occurrence after transplantation) is associated with lower allograft survival compared to AMR with preexisting DSA (present before transplantation).[84,85] Recently, a large study showed that preexisting

DSA resolved in half of the patients within the first 3 mo after transplantation, and patients with persistence of preexisting DSA had increased risk of 10-y graft loss.[86]

Hypoxia

Kidney transplants are exposed to several conditions that can cause hypoxia. The conditions include perioperative ischemia and reperfusion,[87-90] as well as postoperative factors including surgical complications, vasoconstriction by immunosuppression,[91] infections,[92,93] and vascular rarefaction as a consequence of fibrosis.[94,95] An epigenome-wide mapping study of kidney allograft biopsies showed that cold ischemia occurring during kidney transplantation induced DNA hypermethylation by loss of 5-hydroxymethylcytosine and predicted reduced allograft function 1 y after transplantation.[32] Methylation of DNA at cytosine bases of the CpG-rich sites reduced the expression of genes involved in suppressing kidney injury and fibrosis.[32,33] Hypoxia leads to oxidative stress and accumulation of reactive oxygen species,[50,96-98] which is a profibrogenic stimulus.[99-101] Hypoxia is a major risk factor for delayed graft function (DGF), which is usually defined by dialysis in the first week after transplantation. DGF is associated with the activation of the innate immune system triggered in particular by hypoxic proximal tubule injury, which in turn secretes proinflammatory cytokines, promoting migration of neutrophils and macrophages into kidney interstitium.[102,103] This cascade further stimulates cytokine synthesis, activation of dendritic cells and an adaptive immune response. Finally, both leukocytes and lymphocytes activate the complement system, contribute to endothelial dysfunction, and promote the formation of microthrombi, which further aggravates the hypoxia, causing a vicious cycle within the kidney.[104-106] Patients with DGF have higher risk for acute rejection and reduced allograft survival.[89,107] Although clinical and experimental studies have demonstrated that hypoxia and acute kidney injury (AKI) are independently associated with an increased risk of CKD, known as AKI-to-CKD transition,[108-113] the role of DGF on progression of fibrosis is less clear. A retrospective study showed similar progression of fibrosis between control and DGF group within the first year after transplantation.[114] Similarly, donated kidneys after circulatory death with severe AKI did not have increased fibrosis after 1 y of transplantation.[115] Another study showed that DGF only in combination with TCMR was associated with progression of fibrosis,[116] thus suggesting once again that prolonged inflammation is critical for development of allograft fibrosis.[12]

Cellular Senescence

The senescent cell is characterized by increased lysosomal content and a stable arrest in cell cycle resistant to apoptosis through activation of cyclin-dependent kinase inhibitors, including p16Ink4a, p14arf, p53, and p21Cip1/Waf1.[117-120] Cellular senescence can be induced by DNA damage, telomere shortening, and oxidative stress upon repetitive acute injuries and ongoing inflammation accumulated over time.[121] In mouse fibrosis models, it has been shown that tubule cells become arrested in G2/M of the cell cycle and adopt a profibrotic secretory phenotype,[122] which can be targeted pharmacologically using c-Jun N-terminal kinase inhibitor[122] or histone deacetylase inhibitor[123] to reduce fibrosis. The senescent cells obtain a senescence-associated secretory phenotype and secrete proinflammatory and profibrotic cytokines and chemokines as well as growth factors.[121,124] Cullin3 (Cul3) E3 ubiquitin ligase ubiquitinates, and thus regulates, a wide range of substrates involved in Wnt/β-catenin, Hedgehog/Gli, nuclear factor kappa-light-chain-enhancer of activated B cells, Notch, Kelch-like ECH-associated protein 1/nuclear factor erythroid 2-related factor 2 (Nrf2), collagen export, and cell cycle.[125-134] Cul3 mutations are believed to be involved in induction of cellular senescence.[135-137] We observed that tubule epithelial-specific deletion of Cul3 in mice led to DNA damage, increased expression of p21, and sustained tubule injury and fibrosis.[138] Similarly, Cul3-deficient liver cells undergo DNA damage and express senescence markers including p16 and p21.[139] Various studies targeted successfully p16Ink4a so far.[140-144] The amount of senescent cells, in particular of p16INK4a positive cells, in donor kidneys have been correlated with IF/TA.[29,145,146] In kidney biopsies, level of p16INK4a before transplantation correlated with donor age, telomere length, and serum creatinine levels 6 mo and 1-y posttransplant.[147,148] Targeting senescent cells using senolytics such as dasatinib and quercetin[144,149] might provide a promising approach in renal fibrosis and development of IF/TA in chronic allograft nephropathy.

DIAGNOSIS OF RENAL FIBROSIS

Routine clinical approaches to assess kidney health are measurements of serum creatinine, estimated glomerular filtration rate (eGFR), urine volume per day, or urinary excretion of protein or red blood cells. However, these parameters have its limitations to detect early stages of kidney fibrosis as significant loss of renal function is required before abnormalities can be observed during patient's visit.[150] In fact, substantial fibrosis can be found in some recipients with fairly normal eGFR values.[9,64,151] Early detection of renal allograft dysfunction is critical to implement renoprotective treatment to prevent progression into end-stage renal transplant failure reducing morbidity and mortality and improving quality of life. The lowest observed creatinine after transplantation is often presumed to reflect "baseline" renal function. However, whether this is the true baseline or a state in which a mild sustained injury is ongoing is often not known. Calculations to predict expected posttransplant kidney function by incorporating donor and recipient information might add relevant information to assess allograft function; yet whether deviation between expected and observed serum creatinine should trigger further diagnostic and therapy to avoid accelerated loss of kidney function is unknown.[152-154]

Renal Biopsy

Assessment of renal fibrosis by kidney biopsy is the gold standard in daily practice.[155,156] Even when clinical evaluation strongly suggests a particular cause of allograft dysfunction, biopsy is often still needed to clarify the severity of kidney injury to guide treatment. In addition to indication biopsies, some centers also perform protocol biopsies as part of their surveillance program. Protocol biopsies are helpful to detect subclinical chronic pathologies and to track progression of kidney fibrosis.[157,158] i-IF/TA can be

found in one-third of protocol biopsies,[68] and can be used as surrogate for allograft survival.[9,151] The Banff classification is the standard in the evaluation of kidney allograft biopsies and allows a good prediction of allograft survival.[5] However, new automated morphometric analysis may outperform the semiquantitative Banff classification in predicting allograft survival.[159-161]

Biomarkers

Renal biopsy suffers from its invasiveness with bleeding as the most common complication after biopsy,[162-164] thus noninvasive tools are highly desirable to monitor kidney function. Biomarkers may help to predict long-term allograft outcome or differentiate between chronic rejection and IF/TA of other origin. The list of potential biomarkers for kidney transplant injury is growing exponentially through access to high-throughput omics-technologies and extension to microRNA, cell-free DNA, metabolic products, etc.[165] Unfortunately, most biomarkers are often not organ-specific and reflect the inflammatory state or overall body homeostasis rather than the extent or progression of renal fibrosis. Further, increased levels of urine biomarkers might reflect either a less stringent glomerular filtration barrier or decreased uptake of "biomarkers" by tubule cells rather than reflecting the accumulation of extracellular matrix in the kidney.

The diagnostic value of the tubule injury marker neutrophil gelatinase-associated lipoprotein (NGAL) is well described in AKI and has been also studied in the setting of renal transplantation. Higher urine NGAL correlated with tacrolimus-induced chronic renal allograft fibrosis.[166] The CONTEXT trial showed that NGAL on day 1 after transplantation was associated with DGF, but it did not correlate with the eGFR at 12 mo after transplantation. Similarly, the study also found no correlation between eGFR and biomarkers, including liver-type fatty acid-binding protein, cystatin C, and YKL-4.[167] In contrast, the FAVORIT trial showed an association between high urine NGAL and risk for allograft failure.[168] However, this association was much weaker in comparison to that of a1-microglobulin and MCP-1.[169] Other studies have observed that a1-microglobulin and MCP-1 levels correlate with degree of IF/TA in protocol biopsies.[170-173] CXCL10 has been shown to be associated with TCMR in the first 12 mo after transplant, eGFR decline, and risk of allograft loss.[174] Urinary CXCL13 has also been found to be predictive of IF/TA and acute rejection.[174,175] Transglutaminase type 2 is an extracellular matrix crosslinking enzyme known to have a pivotal role in kidney fibrosis[176] and might be a potent biomarker for kidney allograft fibrosis.[166,177] A currently recruiting observational clinical study investigates urinary transglutaminase type 2 as a biomarker for kidney allograft fibrosis (NCT03487861). Mineral metabolism indices vitamin D, PTH, and serum calcification propensity (T50) associate with IF/TA, but do not show additive value to fibrosis prediction by eGFR and proteinuria in kidney transplant recipients.[2]

MicroRNAs control gene expression by affecting the translation of related mRNA. Several microRNAs have been reported to regulate pathways relevant for inflammation and development of fibrosis, including TGF-β and PDGF signaling.[178] MicroRNAs are stable in plasma and urine, making them interesting biomarker candidates. In urine of recipients of proven IF/TA, lower miR-200b, -211, and -204, and higher miR-21, -142–3p were found.[179-182] In serum, higher levels of miRNA-148a, -21, -150, and -423, and lower levels of miR-200b, -145-5p, and -148a were reported in recipients with IF/TA.[183-185] More miRNAs were discovered in kidney biopsies.[179,186-191] Since preclinical data highlighted miR-21 as a potential target in a mouse model of Alport syndrome, a current phase 1 clinical study has been completed that investigated miR-21 inhibitor RG-012 in Alport patients (NCT03373786); the results have been not reported yet.

Integration of 6 transcriptome datasets revealed 85 genes specific to IF/TA, which was used for computational drug repurposing analysis.[192] Here, kaempferol and esculetin were identified as potential therapeutics for IF/TA.[192] Kaempferol attenuated TGF-β1–mediated profibrotic signaling in vitro and in mice, whereas esculetin inhibited Wnt/β-catenin pathway in vitro and in vivo.[192] A transcriptome analysis of kidney biopsies within the Chronic Allograft Rejection study identified a set of fibroblast-specific genes that were independently predictive for the development of fibrosis at 1 y.[193] However, a recent study showed that transcripts in allograft biopsies added little additional predictive ability to clinical parameters regarding allograft survival.[194] The Banff Molecular Diagnostics Working Group created a commercially available Banff Human Organ Transplant panel covering 770 genes related to transplant-associated virus infection, immune response, rejection, tolerance, and drug-induced toxicity.[195]

Proteomic analysis of renal allograft biopsies identified coagulation factor XIII, actin-related protein 2/3, uridine phosphorylase 1, and cytochrome c oxidase assembly factor 6 homolog as highly associated with IF/TA.[196,197] Factors directly involved in extracellular matrix production and their turnover are attractive biomarkers for IF/TA.[198,199] CKD273 is a multidimensional urinary proteome classifier consisting of 273 protein fragments,[200] predicting the incidence and progression of CKD and/or albuminuria in type 2 diabetes on top of baseline albuminuria and eGFR.[201-206] These peptide markers were mostly different fragments of various collagens, blood proteins, and specific kidney-derived proteins (eg, uromodulin). A case-control study compared the urinary proteomic profile of pediatric renal transplant recipients with chronic active antibody-mediated rejection (cAMR) to that of pediatric renal transplant recipients with stable kidney function without cAMR.[207] Combination of cAMR-classifier consisting of 79 biomarkers with the CKD273-classifier increased the specificity of detecting cAMR.[207] Other currently recruiting studies are assessing urinary exosomal biomarkers of IF/TA (NCT03870542) and proteogenomic biomarker panels for acute rejection and IF/TA in blood, urine, and kidney tissue (NCT01531257).

Imaging

An alternative or complementary approach to detect fibrosis is imaging. Ultrasound technologies are at this time-point not convincing for detection of fibrosis and require further development.[208,209] Several different MRI techniques have been used to detect renal fibrosis.[210-215] A recent study combined 3 functional measures, which correlated with IF in kidney transplants.[216] First, blood oxygen level-dependent imaging was used

to detect less-oxygen requiring areas of fibrosis.[217,218] Second, diffusion-weighted imaging (DWI) quantified tissue water molecule free movement, which is restricted in areas with increased extracellular deposition.[219,220] Third, arterial spin labeling was used to measure tissue microvascular perfusion,[221] known to be decreased in native and kidney transplants with fibrosis.[222,223] Blood oxygen level-dependent has been frequently used for evaluation of kidney fibrosis, but mixed results have been reported because of many factors influencing blood flow and oxygenation.[215] Other techniques may better correlate with the deposition of collagen in fibrotic kidney tissue. For example, magnetic resonance elastography can be used to measure kidney transplant stiffness as a surrogate marker for fibrosis,[224-226] but requires external hardware and is only available in specialized centers. Magnetic resonance relaxometry (MRR) produces pixel-wise parametric maps of tissue-specific T1 and T2 relaxation times for assessment of edema or fibrosis.[227-229] A recent study suggested that higher cortical T1, obtained by MRR, and lower medullary fractional anisotropy, a parameter describing the direction of molecular motion within DWI, show the best correlation with eGFR and IF/TA.[213] Other studies reported similarly higher T1(rho)[230] and lower fractional anisotropy values in kidney allografts with impaired function.[231,232] Recommendation articles for standardization of MRR[233] and DWI[234] have been published to address the present inconsistency of imaging protocols. Other studies are ongoing to compare histologic findings with multiparametric renal MRI (NCT03780101) and magnetic resonance elastography (NCT03918161). However, the specificity of several imaging modalities to diagnose and to stage fibrosis have been questioned, as they might reflect impaired kidney function by, for example, altered blood flow rather than by renal fibrosis.[235-238] A recent report indicates that molecular imaging of the extracellular matrix protein elastin using the elastin-specific MRI contrast agent elastin-specific magnetic resonance imaging agent allowed for repetitive and reproducible assessment of renal fibrosis, and thus has the potential to become a fibrosis-specific imaging method to assess renal fibrosis.[239]

Optical Tissue Clearing

Although traditional 2D histopathology of kidney biopsies is crucial to guide nephrologists, it suffers from low sensitivity of sparse lesion and lack of spatial information. The combination of optical tissue clearing protocols with advanced light microscopy made 3D imaging and analysis of cellular structures in whole organs possible.[240-242] Optical clearing methods use so-called refractive index matching solutions to make organs translucent for a volumetric view on lesions. Several studies have shown its usefulness to detect multilayered structures in diseased kidneys[243-246] and renal allograft biopsies.[247] Optical clearing has the potential to expand into pathology practice, but further development and optimization of protocols are needed to facilitate the future in routine pathology with 3D examination.[248-250]

THERAPEUTIC OPTIONS

Arterial hypertension, diabetes, dyslipidemia, tobacco use, and obesity are risk factors for chronic allograft injury and cardiovascular disease. Thus, treatment and/or control of these risk factors are simple and powerful antifibrotic approaches. It is important that patients suspected of having chronic allograft injury leading to progression of IF/TA undergo biopsy, as treatment of some causes such as BK virus nephropathy could stop progression of fibrosis. Unfortunately, most biopsies will show IF/TA with or without other irreversible features consistent with chronic graft injury. Thus, the diagnosis of chronic allograft injury is a diagnosis of exclusion. In Table 1, we summarize all currently recruiting clinical trials in kidney fibrosis that were identified by a search of ClinicalTrials.gov.

Optimization of the Immunosuppressive Therapy

The immunosuppressive therapy with cyclosporine and tacrolimus has significantly improved allograft survival,[251,252] but long-term exposure to CNI can result in renal fibrosis. CNI causes vasoconstriction and oxidative stress, and also increases TGF-β, apoptosis, and macrophage infiltration.[17] Transition from high-dose cyclosporine therapy to lower-dose tacrolimus with mycophenolate supplementation helped to reduce CNI nephrotoxicity.[253] In creeping creatinine study, mycophenolate was substituted for cyclosporin A, and there were no differences in mortality, graft loss, acute rejection, infection, or blood pressure between the 2 groups after 12 mo.[254] The "Chronic Renal Allograft Failure" study replaced cyclosporin A with tacrolimus, and there was no difference in death or graft loss between both groups. However, less incident cardiac events were observed with tacrolimus.[255] Immunosuppressive agents that inhibit mechanistic target of rapamycin, such as sirolimus and everolimus, can be used to reduce the dose of CNIs; for review see.[256] Tacrolimus with mycophenolate compared to a 3-mo conversion to everolimus with low-exposure tacrolimus provided similar effects in terms of fibrosis, acute rejection rates, and graft function, but higher rates of BK and cytomegalovirus infections.[257] Mesenchymal stromal cells have emerged as potential candidates for immunomodulatory cell therapy with antifibrotic effects,[258-261] and a currently active phase 2 study is combining mesenchymal stromal cells with everolimus to facilitate tacrolimus withdrawal and to reduce fibrosis (NCT02057965).

Sodium-glucose Transporter 2 Inhibitors

The sodium-glucose transporter 2 (SGLT2) inhibitors canagliflozin, empagliflozin, and dapagliflozin were shown to have renoprotective effects in diabetic patients.[262-265] This led to the initiation of clinical studies investigating kidney disease events of empagliflozin and dapagliflozin in nondiabetic CKD patients (NCT03594110, NCT03190694). A recent study showed that patients with CKD who received dapagliflozin had significantly lower risk for kidney function decline or death than those who received placebo, independent of the presence or absence of type 2 diabetes.[266] These studies made SGLT2 inhibitors attractive as an antifibrotic drug to potentially treat millions of patients with kidney disease. SGLT2 inhibitors seem to be safe in diabetic renal transplant patients with a low rate of adverse events and urinary tract infection.[267] However, studies investigating the effect of SGLT2-inhibition on progression of IF/TA in kidney allograft recipients are lacking.

TABLE 1.

Currently recruiting clinical intervention trials in phases 2–4 investigating potential antifibrotic drugs in patients with CKD (only those listed on clinicaltrials.gov; search terms: chronic kidney disease, renal fibrosis, renal allograft loss, kidney transplant, and fibrosis)

Condition	Target	Drug	Primary endpoint	Secondary endpoint	ClinicalTrials.gov identifier: NCT
Phase 2					
eGFR >25 mL/min/1.73 m^2	Mineralocorticoid receptor antagonism	Spironolactone; Amiloride	Change in albuminuria, flow-mediated dilation, oxidative stress	Change in eGFR, potassium	02497300
CKD 1–4	Uric acid	Verinurad; Allopurinol	Change in UACR	Change in eGFR, cystatin C, serum creatinine	03990363
Diabetic nephropathy	GLP-1/glucagon agonist	Cotadutide	Change in UACR	Change in BP, adverse events, etc	04515849
CKD 3B/4	Pleiotropic	Adipose tissue-derived stem cells	Adverse events; change in eGFR	Change in several lab parameters	02933827
CKD 3–5	Pleiotropic	Adipose tissue-derived MSC	Adverse events; change in eGFR	Change in several lab parameters	03939741
Diabetic nephropathy	Pleiotropic	Umbilical cord MSC	Change in UACR	Change in eGFR, HbA1c, etc	04216849
eGFR 15–45 mL/min/1.73 m^2, DM	Senescent cells	Dasatinib; Quercetin	Senescence markers in skin, fat, and/or blood	MSC function, frailty index, change in eGFR	02848131
CKD 1–4	TGF-β	Pirfenidone	Fibrosis assessed by DW-MRI; urine α1M, PIIINP, and MCP-1	Changes in eGFR and UACR	04258397
Diabetic nephropathy	Vascular endothelial growth factor-b blockade	CSL346	Change in UACR	Change in eGFR, BP, adverse events, etc	04419467
eGFR 30–90 mL/min/1.73 m^2	Kallikrein	DM199	Adverse events, eGFR, albuminuria	Inflammation markers	04123613
CKD with eGFR 20–75 mL/min/1.73 m^2 and 200–5000 mg albumin/g creatinine	5-Lipoxygenase-activating protein inhibitor	AZD5718 vs dapagliflozin vs placebo	Change in UACR	Change in UACR, eGFR, systolic BP, adverse events	04492722
Phase 3					
eGFR ≥20 to <90 mL/min/1.73 m^2	SGLT-2	Empagliflozin	Composite: kidney disease progression or death	Hospitalization, death, etc	03594110
CKD 2–3B	Glucose	Metformin	Change in eGFR	Mortality, proteinuria, etc	03831464
eGFR 25–75 mL/min/1.73 m^2	GLP-1 receptor	Semaglutide	Composite: eGFR decline ESRD, death	Any component of primary outcome, etc	03819153
20–40 mL/min/1.73m^2	Acid removal	TRC101	≥40% reduction in eGFR; ESRD; renal death	Time to ESRD, eGFR slope, KDQOL, etc	03710291
Phase 4					
CKD 3–4	ACEi, calcium-antagonist	Lisinopril, Amlodipine	Increase in baseline creatinine	Adverse events, mortality	03195023
CKD 2/3	HMG-CoA reductase	Telmisartan with or without Rosuvastatin	Change in proteinuria	eGFR, MCP-1, type IV collagen, etc	03550859
CKD 1–4	Uric acid	Febuxostat or Allopurinol	Changes in eGFR and albuminuria, gout attacks	Cardiovascular mortality, nervous system	03336203
Diabetic nephropathy	Antihistamine	Fexofenadine vs placebo	Change in UACR	Urinary cyclophilin A and MCP-1	04224428
Diabetic nephropathy	Antihelminthic, pleiotropic	Niclosamide	Change in UACR and eGFR	Change in urinary MMP-8 and podocalyxin	04317430
CKD 3–4, T2DM	Phosphodiesterases; TNF-α; MCP-1	Pentoxifylline	Change in eGFR	Vitamin C, NT-proBNP, hs-CRP, SF36 questionnaire	03664414

α1M, alpha 1 microglobulin; ACEi, angiotensin-converting-enzyme inhibitor; BP, blood pressure; CKD, chronic kidney disease; DM, diabetes; DW-MRI, diffusion-weighted magnetic resonance imaging; eGFR, estimated glomerular filtration rate; ESRD, end-stage renal disease; GLP-1, glucagon-like peptide 1; HMG-CoA, β-Hydroxy β-methylglutaryl-CoA; hs-CRP, high sensitivity CRP; KDQOL, Kidney Disease and Quality of Life; MCP-1, monocyte chemoattractant protein-1; MMP-8, matrix metalloproteinase-8; MSC, mesenchymal stem cell; NT-proBNP, NT-probrain natriuretic peptide; PIIINP, N-terminal procollagen type 3 peptide; SF-36, 36-Item Short Form Survey; SGLT-2, sodium-glucose cotransporter 2; T2DM, type 2 diabetes; TGF-β, transforming growth factor β; TNF-α, tumor necrosis factor-alpha; UACR, urine albumin to creatinine ratio.

RAAS Inhibition and Endothelin 1 Receptor Antagonist Atrasentan

Angiotensin II promotes vasoconstriction and aldosterone release to increase blood pressure, but it also has several prooxidant, proinflammatory, and profibrotic effects.[268-271] The profibrotic effects are partially exerted through angiotensin II type 1 receptor and extracellular signal-regulated kinase to activate SMAD signaling.[272] RAAS inhibition leads to dilation of efferent arterioles, and thus decreases intraglomerular pressure and stress. RAAS inhibition has been shown to slow the progression of CKD.[273-275] Benefits of RAAS blockade in kidney transplant recipients remain controversial,[276-279] as RAAS blockade did not delay CKD progression after renal transplantation.[280,281] A recent meta-analysis concluded that a trial with >10 000 patients would be needed to answer whether RAAS blockade reduces kidney allograft loss.[277] The ineffectiveness of RAAS inhibition in kidney transplant recipients might be due to the alternative RAAS pathway, in which chymase and neprilysin cleave Angiotensin I to Angiotensin II even in the presence of angiotensin-converting enzyme inhibitors.[282]

Endothelins are potent vasoconstrictors and profibrotic growth factors.[283] The recent SONAR study demonstrated that the endothelin 1 receptor antagonist atrasentan delays CKD progression in diabetic CKD patients.[284] Sparsentan is a dual-acting angiotensin receptor blocker and highly selective endothelin type A receptor antagonist. Two clinical studies are currently investigating the renoprotective effects of sparsentan in IgA nephropathy (NCT03762850) and focal segmental glomerulosclerosis (NCT03493685).

Mineralocorticoid Receptor Antagonists

Several preclinical studies have shown that mineralocorticoid receptor antagonists (MRAs) such as spironolactone and eplerenone ameliorate or cure kidney injury and dysfunction in different models of kidney disease.[285] One study demonstrated reduced renal oxidative stress when spironolactone was initiated in kidney recipients.[286] The multicenter study EPURE is currently recruiting patients to assess the impact of eplerenone on 3-mo renal graft function.[287] In children with biopsy-proven IF/TA, eplerenone administration for 24 mo attenuated the progression of fibrosis.[288] The SPIREN trial is currently investigating the effect of spironolactone for 3 y on GFR, proteinuria, and IF in biopsies and cardiovascular events.[289] MRAs carry the risk to cause hyperkalemia, although good safety profiles have been reported for kidney transplant recipients.[290-292] The recent FIDELIO-diabetic kidney disease study showed that the MRA finerenone versus placebo met the primary endpoint in reducing the risk of CKD progression, kidney failure, or death from renal cause, in patients with CKD and type 2 diabetes (hazard ratio 0.82).[293] Adding Daiichi-Sankyo's MRA CS-3150, known as Esaxerenone, for 12 wk to an ongoing RAAS inhibition reduced albuminuria in patients with type 2 diabetes in a phase 2 study.[294] According to the website of Daiichi-Sankyo, a phase 3 clinical trial (ESAX-DN study) met recently its primary endpoint of remission to normoalbuminuria after treatment for 52 wk in patients with type 2 diabetes and microalbuminuria. In Japan, esaxerenone became clinically available for hypertensive patients as it lowered blood pressure in the phase 3 study ESAX-HTN.[295]

Lowering Urate

A study showed that higher uric acid level after kidney transplantation was associated with more severe IF/TA and lower graft survival over 10 y.[296] However, in patients with CKD, urate-lowering treatment with allopurinol did not slow the decline of eGFR as compared to placebo.[297] Similarly, the measured GFR after 3 y did not differ among patients with long-standing type 1 diabetes who took allopurinol or placebo.[298] However, data from another study suggest that early intervention with urate-lowering therapy in patients with better kidney function may have a positive effect.[299]

Reduction of Hypoxia

Considerable efforts have been made to limit hypoxia during transplantation. Here, machine perfusion of explanted organs holds the potential for improving transplant outcome.[300] The results from machine perfusion studies are difficult to compare owing to differences in several aspects of the protocol, such as perfusate temperature, oxygenation, and pressure.[301,302] However, machine perfusion seems to reduce DGF and overall graft survival compared to static cold storage.[303-307] The large multicenter EVNP trial is currently investigating the efficacy of normothermic machine perfusion compared to cold storage technique in kidney transplantation.[308]

Antioxidant Therapy

The strong association between oxidative stress and IF/TA led to several studies investigating different antioxidant drugs, including HIF targeted therapy.[309,310] In a randomized controlled trial, the effect of human recombinant superoxide dismutase, given intravenously during transplantation, showed a reduction of acute rejection episodes.[311] The study further reported an increased 4-y graft survival rate in treated patients to 74% compared to 52% in the control group.[311] Peroxisome proliferator-activated receptor-alpha (PPARα) is involved in the regulation of β-oxidation of fatty acid and has several antioxidant effects.[312] miR-21, a potential biomarker of IF/TA, mediates downregulation of PPARα in mouse fibrosis model.[313] A study suggested that fenofibrate (a PPARα agonist) might suppress fibrosis by ameliorating oxidative stress-induced EMT, as assessed by reduced amount or expression of reactive oxygen species, Snail, S100A4, and vimentin, in a rat kidney transplant model.[314]

N-acetylcysteine (NAC) is able to regenerate glutathione stores and scavenges oxygen-free radicals.[315,316] NAC treatment for 7 d after transplantation reduced serum levels of thiobarbituric acid reactive substances, a marker of oxidative stress, and was associated with higher mean of eGFR at 1 y after transplantation. Further, DGF and dialysis were lower among the group receiving NAC.[317] Similarly, patients receiving NAC from days 0 to 5 after kidney transplantation reduced urine NGAL levels injury, but NAC had no impact on early graft function.[318,319] In contrast, a randomized control trial could not observe any beneficial effect of NAC (2 single-dose i.v. administration in brain dead donors) on eGFR, mortality, or 1-y kidney graft survival of recipients.[315]

Sulforaphane is a natural Nrf2 activator derived from cruciferous vegetables, which alleviates fibrosis in rat renal allografts.[320] However, Nrf2 activator bardoxolone

methyl was terminated because of adverse cardiovascular side effects in patients with diabetic nephropathy, although it may preserve kidney function and delay onset of kidney failure in this cohort.[321] Other antioxidative strategies using NOX1/4 inhibitor GKT137831/setanaxib (NCT02010242) or ASK 1 inhibitor selonsertib (GS-4997)[322] did not attenuate progression of kidney fibrosis in patients with CKD.[323] A current phase 2 trial aims to evaluate the efficacy and safety of NOX1/4 inhibitor in adults with type 1 diabetes and persistently elevated urinary albumin excretion over a period of 48 wk.[324,325]

A recent study showed that APP-103, a molecule that specifically inhibits production of ROS, has beneficial effects on kidney function and allograft survival in rat renal transplant model and ischemia-reperfusion injury model.[326]

Anti-TGF-β Therapy

In general, the pathogenic role of TGF-β in CKD is well established. However, besides its profibrotic effects, it also has antifibrotic effects and various roles in tissue homeostasis and regeneration, preventing the therapeutic use of TGF-β inhibitors so far.[327-332] Bone morphogenic protein (BMP) 7, a member of the TGF-β superfamily, acts as an antagonist of TGF-β signaling. A study showed that the orally administered BMP7 agonist (THR-123), which acts via the BMP receptor activin-like kinase 3, attenuates renal fibrosis in mouse models.[333] Clinical studies with an anti-TGF-β antibody (fresolimumab) in patients with steroid-resistant focal and segmental glomerulosclerosis, as well as in diabetic nephropathy, have not been successful so far.[327,334] Another activator of TGF-β signaling is thrombospondin-1, and inhibition of thrombospondin-1 resulted in angiogenesis and reduction of fibrosis in mouse kidney fibrosis models.[335]

Urinary levels of Wnt/β-catenin modulator Dickkopf-3 predicted kidney function decline in patients with CKD.[336,337] Binding of β-catenin to T cell factor (TCF) promotes profibrotic pathways, including TGF-β/Smad, integrin/ILK, and Wnt/β-catenin pathways.[338] In contrast, binding of β-catenin to Foxo, might induce regulatory T cells, which are relevant to TGF-β's anti-inflammatory and antifibrotic function.[339,340] A study showed that inhibiting β-catenin/TCF interaction using ICG-001 reduced inflammation and fibrosis in mouse kidney fibrosis models.[341] Similarly, proximal tubular β-catenin activation requires the presence of FoxO3 to have protective effects upon kidney injury.[342] A recent study showed in 240 transplanted kidneys that the binding of β-catenin to TCF1 rather than FoxO1 in renal tubular cells was associated with a profibrotic response.[343] Overall, the preclinical data suggest that Wnt/β-catenin is protective in AKI and may be mediated by epithelial cells. In CKD, transcriptional signaling partners such as TCF and location of Wnt/β-catenin signaling in fibroblast might have detrimental effects.[344]

Pirfenidone (PFD) is an antifibrotic, antioxidant, and anti-inflammatory drug targeting TGF-β and tumor necrosis factor-alpha, and is approved for IPF therapy.[345] Potential beneficial effects for kidney fibrosis have been described.[346-349] In a rat renal transplant model, intragastric administration of PFD resulted in the alleviation of inflammation and renal fibrosis.[350] The phase 2 TOP-CKD study is currently recruiting to assess the effects of PFD in prevention of CKD progression (NCT04258397).

PBI-4050, an orally antifibrotic compound, binds to free fatty acid receptor 1 (GPR40) and GPR84[351] to inhibit TGF-β mediated fibrotic pathways. Indications being pursued for PBI-4050 include Alström syndrome and idiopathic pulmonary fibrosis (IPF). Of note, the company Liminal BioSciences might potentially initiate studies to test PBI-4050 in the field of CKD.

Preclinical data revealed Integrin αvβ6, which is involved in TGF-β activation, as a potential target in lung and kidney fibrosis.[352] However, a phase 2 study testing an antibody against integrin αvβ6 (BG00011/STX-100) in patients with IPF was stopped because of safety concerns (NCT03573505).

RNAi Gene Therapy

Given that a number of miRNAs have been associated with IF/TA, sequence-specific small interfering RNA (siRNA)-based gene therapy to eliminate specific mRNA transcripts has been applied in the field of kidney transplantation.[353] Several preclinical kidney transplant studies revealed a renoprotective effect of siRNA against caspase 3,[354,355] TGF-β,[356,357] soluble fibrinogen-like protein 2,[358] complement component C5A,[359] C3,[360] caspase 8,[361] CD40,[362] inhibitor of nuclear factor kappa-B kinase subunit beta,[363] RelB,[360,364] Fas,[360] SHARP-2,[365] p53,[366,367] and tumor necrosis factor-alpha.[368] However, a number of factors restricted the wide clinical use of siRNAs so far, such as serious off-target effects, short half-life, and tissue barriers.[369] Nevertheless, the U.S. Food and Drug Administration approved the first siRNA-based gene therapy Onpattro (patisiran) in 2018 to treat a form of amyloidosis. In the field of kidney transplantation, a phase III multicenter trial to evaluate the reduction of incidence and severity of DGF by p53 siRNA has been completed; results have been not reported yet (NCT02610296).

Diet

Adherence to a "healthy" diet is known to be associated with better kidney function and lower prevalence of comorbidities in patients with CKD.[370-372] Marine n-3 fatty acids may exert beneficial effects on inflammation, fibrosis, and endothelial function, which could preserve renal graft function.[373] Kidney transplant recipients receiving either marine n-3 fatty acids or olive oil for 44 wk had similar renal function, but n-3 fatty acids lowered plasma triglyceride and high-sensitivity C-reactive protein levels, and improved endothelial function.[374] A similar phase 3 study is currently recruiting to investigate the effect of marine omega-3 fatty acid supplementation on eGFR and fibrosis in renal transplantation (NCT03018041).

CONCLUSION

Kidney fibrosis is not only the final common pathway of virtually all progressive renal disease, but also highly prevalent and a potent predictor of kidney allograft outcome. Thus, it is not surprising that academic research and industry have a longstanding interest in identification of therapeutic targets and development of targeted therapeutics in fibrosis. The prediction of allograft survival remains difficult, but combination of clinical, biomarker, and pathology data together with artificial intelligence will likely improve diagnostic precision and therapeutic

guidance. So far, many potential antifibrotic pathways have been identified, but yet no specific antifibrotic drug has been approved in the kidney. The complexity of renal fibrosis makes drug designing challenging as many pathways overlap and compensate for each other. Further, the same molecule may have beneficial effects in the acute setting or in some renal cell types, but deleterious effects in sustained injury or when expressed in other cells such as myofibroblasts. Blocking several downstream pathways might quite effectively reduce accumulation of extracellular matrix, but could cause systemic toxicity. In addition, it remains unclear whether inhibition of matrix deposition without limiting tubule injury or inflammation is meaningful. Thus, kidney-specific or even kidney epithelial-specific drug targeting[375-377] might open the possibility to interrupt the maladaptive crosstalk between cells more effectively. Furthermore, a combination of an antifibrotic myofibroblast targeted intervention combined with an anti-inflammatory and/or tubule epithelial protective treatment might be promising. Further studies are needed to establish such a combined therapy or identify specific antifibrotic targets.

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