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Plasma proteome signatures of ASK1 inhibition by selonsertib associate with efficacy in the MOSAIC randomized trial for diabetic kidney disease

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Abstract

Oxidative stress is a driver of acute and chronic kidney injury. Selonsertib is a clinical stage antagonist of ASK1 (MAP3K5), a serine/threonine kinase that is a mediator of oxidative stress signaling pathways. Selonsertib has demonstrated promising effects on preserving kidney function in the Phase2b Diabetic Kidney Disease (DKD) MOSAIC trial. However, little is known about the biological effects of ASK1 inhibition by selonsertib and its potential mechanism of action in DKD. We identified a plasma proteome signature of selonsertib activity that implicates numerous signaling pathways that regulate fibrosis, inflammation and oxidative stress response demonstrating translation of non-clinical models to the clinic. We further demonstrate that the effects of selonsertib on the plasma proteome are most pronounced in a subset of patients with poor baseline kidney function but who respond well to selonsertib treatment. This observation has implications for the future development of ASK1 inhibitors in a distinct patient population with DKD.

Trial registration

MOSAIC (NCT04026165 registered July 17, 2019).

Keywords Diabetic kidney disease, Selonsertib, ASK1, Proteome, Apoptosis, Inflammation, SomaScan, eGFR

Introduction

Diabetic kidney disease (DKD) is among the most common chronic disorders globally, affecting up to 40% of patients with type 2 diabetes mellitus (T2DM), and a leading cause of chronic kidney disease (CKD) [1]. In the United States, it was estimated that more than 38 million people suffered from diabetes, a number that has steadily increased over the past few decades {National Diabetes Statistics Report 2021, CDC}. Projections indicate that this figure will rise to over 60 million by the year 2060, largely due to the rise in obesity rates [2]. End-Stage Renal Disease (ESRD), a major complication

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of DKD, is diagnosed in over 120,000 new patients in the US annually and requires kidney transplant and/or dialysis to manage. In 2020, Medicare spending for all recipients diagnosed with CKD surpassed $85 billion. This amount accounted for nearly 25% of the total Medicare fee-for-service spending. On an individual basis, annual Medicare expenditures per patient were as high as $19,739 for those with both CKD and diabetes [3]. The enormous burden of DKD on individual, social, and economical level demands urgent development of novel treatment strategies focused on preserving renal function in diabetics.

The pathogenesis of DKD is a complex process involving numerous signaling pathways and molecular events. The onset and progression of the disease is driven by multiple factors including insulin resistance, glycolipid metabolism disorder, hemodynamics alteration, inflammatory response, cytokines, oxidative stress, and genetic factors [4]. Oxidative stress, one of the fundamental drivers of the DKD, is triggered by high glucose concentrations ultimately leading to reactive oxygen species (ROS) generation. In this context, ROS generation is mediated through increased activity of polyol, advanced glycation end products (AGE) and receptor for advanced glycation end products (RAGE), hexosamine, nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, and the protein kinase C (PKC) pathways [5]. Intracellular renal accumulation of ROS activates a wide array of molecular pathways, ultimately leading to endothelial cell apoptosis, glomerulosclerosis, inflammation, and tubulointerstitial fibrosis [6, 7].

ROS-related signaling is one of the stimuli that induces the mitogen-activated protein kinases (MAPKs) activity. The MAPK pathway functions as a cascading series of serine/threonine phosphorylations catalyzed by three classes of kinases: MAPK, MAP2K, and MAP3K. These kinases phosphorylate and activate each other in a sequential order; MAP3Ks phosphorylate and thereby activate MAP2Ks, and activated MAP2Ks phosphorylate and activate MAPKs [8]. Apoptosis signal-regulating kinase 1 (ASK1, MAP3K5) is a ubiquitously expressed redox-sensitive MAP3K that is activated by a wide range of stimuli, including oxidative stress. ASK1 transmits the downstream signaling through the MAPK cascade ultimately leading to apoptosis, necrosis, inflammation, and fibrosis [9]. Under normoxia, ASK1 is bound and kept inactive by antioxidant proteins, thioredoxin 1 in the cytoplasm and mitochondrial thioredoxin 2 [10]. Under oxidative stress conditions, thioredoxin oxidizes and dissociates from ASK1, leading to trans-autophosphorylation of ASK1 homodimers increasing ASK1's catalytic activity and promoting phosphorylation of downstream MAP2Ks, which then phosphorylate and activate the MAPKs p38, and c-Jun N-terminal kinase (JNK) [11].

The role of p38 kinase in kidney disease was tested in Mkk3 (MAP2K3)−/− mice that show suppressed p38 activity as compared to wild type (WT) animals when subjected to insults that produce renal toxicity. In unilateral ureteric obstruction (UUO) and db/db models of renal disease, Mkk3−/− mice were protected from renal injury, apoptosis, tubular fibrosis, and tissue inflammation [12, 13]. ASK1 directly induces p38/JNK activation that contributes to renal fibrosis in the UUO mouse model [6]. ASK1−/− mice show improved renal function as measured by blood urea nitrogen and serum creatinine levels compared to WT mice following ischemia/reperfusion-induced acute kidney injury [14]. Exposure to GS-444217, a small molecule inhibitor of ASK1 activity, protected auranofin-treated rats from renal injury, tubular apoptosis and necrosis, and similarly reduced tubular epithelial cell death, interstitial inflammation, and kidney fibrosis in the rat UUO model [15]. In a db/db eNOS$^{-/-}$ mouse model of DKD, GS-444217 treatment improved glomerular filtration rate (GFR) decline and albuminuria, along with key pathological manifestations associated with progressive kidney disease [15–18]. Together, this body of evidence suggested that ASK1 is a potential therapeutical target in patients suffering from DKD.

Selonsertib, a highly selective small-molecule inhibitor of ASK1, has been tested in two Phase 3 clinical trials for severe nonalcoholic steatohepatitis (NASH; NCT03053050 and NCT03053063) and was found to be ineffective [19]. A Phase 2 trial of selonsertib in pulmonary arterial hypertension (PAH; NCT02234141) also did not achieve its primary endpoint, nor did a Phase 2 trial in severe alcoholic hepatitis (AH; NCT02854631). In contrast, selonsertib was tested in a Ph2a DKD clinical trial (NCT02177786) that was suggestive of clinical benefit. The predefined study end point, a significant difference from placebo of the mean estimated glomerular filtration rate (eGFR) change from baseline to 48 weeks after initiation of therapy, was not achieved. The interpretation of eGFR readout, however, was confounded by an unexpected off-target effect of selonsertib on creatinine transporters. A post-hoc analysis, adjusted for the acute effect of selonsertib on serum creatinine levels, suggested a significant, dose-dependent amelioration of renal function decline, and highlighted the need for further testing in a larger patient cohort [20]. Recently, the Phase 2b study of selonsertib in DKD (MOSAIC, NCT04026165) achieved the primary endpoint of a reduction in the slope of decline of creatinine-based eGFR, suggesting that ASK1 inhibition is a valid target in DKD [21]. In addition, it was noted that the efficacy of selonsertib appeared to be greater in participants with lower eGFR values at baseline.

Though clinical activity of selonsertib has been demonstrated in the MOSAIC trial, little is known about

the pathways modulated by selonsertib in the context of human disease. In order to address this question, we have characterized the plasma proteome of participants in the MOSAIC trial. We identify numerous biomarkers of disease status and selonsertib activity and demonstrate that the impact of selonsertib on key biomarkers of inflammation, fibrosis, and cell death is consistent with the enhanced efficacy observed in trial participants with more severe disease. Moreover, our data supports the observation that a more pronounced effect of selonsertib on eGFR slope was detected in DKD patients with severe eGFR loss.

Results

Inhibition of ASK1 activity by selonsertib

Previous data from the Phase 2a clinical study NCT02177786 demonstrated a dose-dependent inhibition of phosphorylation of p38 MAPK, a kinase downstream of ASK1 in the signaling cascade. Observed suppression of phosphorylated p38 was 50–60% of baseline levels, consistent with the fact that p38 phosphorylation is not exclusively mediated by ASK1 signaling [20]. To directly assess the impact of selonsertib on ASK1 activity, an assay that detects the autophosphorylated form of ASK1 was developed and used to characterize the activity of selonsertib in whole blood lysate samples from NCT02177786. In this assay, 18 mg selonsertib treatment reduced pASK1 levels by >92%, indicating that selonsertib is a highly effective inhibitor of ASK1 kinase activity (Fig. 1). The observed pharmacodynamic effect demonstrates robust reduction of ASK1 activity upstream of p38 MAPK.

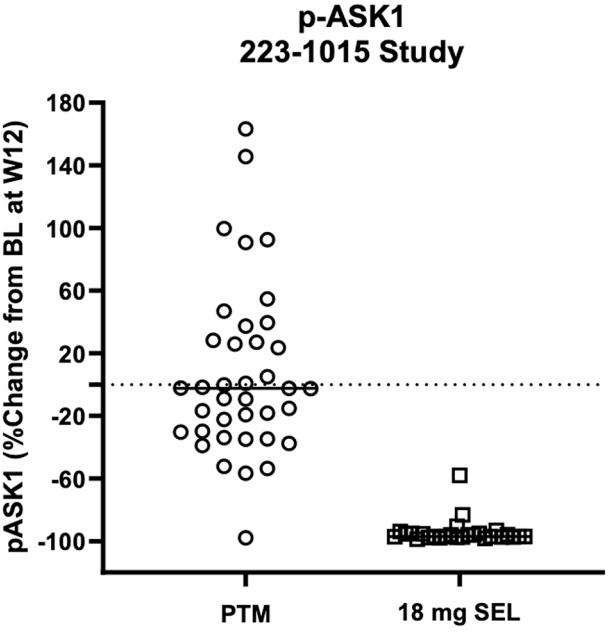


Fig. 1 pASK1 levels are reduced after selonsertib treatment. pASK1 levels were measured in whole blood lysates by pASK1 ELISA at baseline and week 12, and plotted as a relative change from baseline [median values: -2.46 (PTM, $n=38$), -96.97 (18 mg selonsertib, $n=27$); $p<0.001$]

Study design and samples analyzed

The MOSAIC trial evaluated efficacy and safety of selonsertib in over 300 randomized subjects with type 2 diabetes and moderate to advanced DKD (Supplemental Fig. 1). Due to a previously identified effect of selonsertib on creatinine transporters [20], the study included a run-in period, during which all participants received selonsertib for at least four weeks. After completing the run-in, participants were randomized and received a single daily dose of selonsertib (18 mg) or placebo to match (PTM) orally until study end. For subjects in the selonsertib arm, the baseline eGFR was calculated based on the post-run-in time point (Day 1), after the effect on creatinine transport had set in, while for subjects in the PTM arm the baseline eGFR was calculated based on pre-run-in values (Visit B). The study achieved its predefined primary endpoint using the eGFR slope as the efficacy measurement [21].

Here we report on a cohort of 225 patients (112 assigned PTM) from the MOSAIC trial that had complete SomaScan data with week 48 eGFR and SomaScan sample collection dates within one week of one another. Data were discarded from three patients with missing baseline eGFR and, for disease progression analyses, from an additional five patients with missing week 48 eGFR (2.2% attrition at week 48). Final sample sizes of 222 complete cases (110 assigned PTM) and 217 complete cases (108 assigned PTM) were retained for main treatment effect and disease progression analyses, respectively.

The plasma proteome from trial samples was interrogated using the SomaLogic 7 K platform. We analyzed plasma samples collected at baseline (visit B) and at the end of the study (week 48) from all patients. Additionally, we analyzed post-run-in samples (Day 1) from the patients in the selonsertib cohort (Supplemental Fig. 1). We analyzed the circulating proteome data to identify biomarkers that associate with the eGFR slope and to identify biomarkers of selonsertib activity that associate with efficacy.

Identification of biomarkers that associate with eGFR and eGFR slope

A proteomic analysis of plasma from DKD patients should identify known and potentially new biomarkers of DKD, in particular biomarkers associated with eGFR [22]. We thus performed an analysis to evaluate associations between eGFR and the plasma proteome as a validation of the quality of the experiment. We evaluated associations between circulating analyte concentrations and baseline eGFR, as well as associations between

the change in analyte levels by week 48 and eGFR slope in patients who received PTM, using robust regression methods (see Methods). We identified 461 and 614 proteins that were significantly associated with baseline eGFR and eGFR slope, respectively (318 proteins in the intersection). These included previously established biomarkers of renal function in DKD such as TNFR1, TNFR2 [23], Cystatin-C [24], Endostatin [25], VEGFA [26], and ApoC-III [27] (Supplemental Fig. 2). We next focused on a Kidney Risk Inflammation Signature (KRIS), a subset of proteins previously identified using the SomaScan platform that showed high prognostic value for the risk of ESRD, and a negative correlation with the eGFR slope in type 2 diabetes patient cohorts [28]. The majority of the KRIS proteins were also represented in our analysis of association between the plasma proteome and baseline eGFR, as well as change in proteome and eGFR slope (Fig. 2). Taken together, our results are consistent with previous reports of eGFR-associated plasma proteins and support the validity of our methodology and experimental approach.

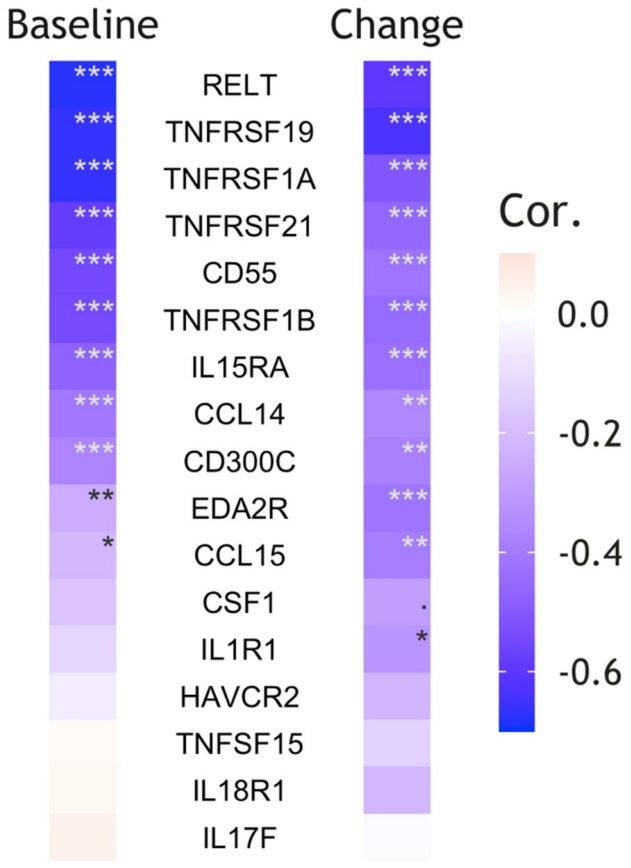


Fig. 2 KRIS proteins are inversely correlated with eGFR and eGFR slope. Baseline levels of KRIS [28] proteins in patients that received PTM were tested for association with baseline eGFR (left column), or the relative change in individual proteins of KRIS signature between the end of the trial and baseline was tested for association with eGFR slope (right column) ($P < 0.1$., $P < 0.05$*, $P < 0.01$**, and $P < 0.001$***)

Identification of a selonsertib activity signature

We next sought to identify the effects of selonsertib on the circulating proteome to better characterize selonsertib's downstream effects and mechanism of action in DKD. We identified proteins showing significant change in their levels between visit B and the end of the study (week 48) in the selonsertib-treated cohort vs. placebo-treated patients using robust regression (see Methods). We found 57 proteins that were strongly down regulated by selonsertib after 48 weeks of treatment and only one protein (APLP1) that was significantly upregulated (Fig. 3A). We also analyzed the selonsertib-modulated proteins during the 4-week run-in period to ascertain whether detected changes in the protein levels manifested early in the treatment period. 56 out of the 58 selonsertib modulated proteins identified at week 48 were also modulated to a similar magnitude and direction at week 4, suggesting that modulation by selonsertib occurs early in the treatment period, and that our estimated selonsertib effects are relatively reproducible within the dataset we examined (Fig. 3B). Numerous other proteins also appeared modulated at week 4; however, in the absence of a placebo group to compare to at week 4, we did not further evaluate these findings. Most of the proteins in the identified signature were significantly associated with eGFR at baseline (93%), as well as with eGFR slope (84%) when tested for change during the study.

Genes encoding selonsertib activity signature proteins are expressed in the kidney and elevated in disease

To determine whether the proteins in the Selonsertib Activity Signature (SAS) could originate from the kidney, we examined the overlap between components of the selonsertib signature and previously published databases of RNA expression profiles in healthy and diabetic kidneys. We utilized a published single nucleus RNA sequencing (RNAseq) dataset [29] to identify SAS gene expression patterns in the kidney cell types and assigned components of the signature to different compartments of the kidney. The majority of the genes encoding the SAS proteins (47/58) were highly expressed in kidney nuclei, suggesting that proteins downregulated by selonsertib may plausibly be derived from kidneys. Exploring the same database, we find that most of the SAS genes (35/47) were upregulated in nuclei derived from tissues of DKD patients. We also interrogated two additional datasets, a database of differentially expressed genes in DKD kidneys as compared to healthy kidneys generated by bulk RNA-seq [30], as well as a dataset of differentially expressed genes in injured kidney tissue scored by quantitative morphometric analysis [31]. We detected a substantial

	(%)		(%)		(%)
ALDH1A3	-17.80	CXCL6	-18.35	PDGFB	-33.71
ANGPT1	-23.99	DKK1	-19.53	PDGFD	-29.18
ANXA6	-24.34	DKK2	-18.92	PDYN	-16.03
APLP1	15.34	DKK4	-17.87	PF4	-31.83
APLP2	-11.72	DLL3	-13.82	PI3	-9.88
APP	-24.08	DSG3	-30.37	PPBP	-27.94
ASB9	-9.17	FUT5	-9.17	PRL	-12.80
BDNF	-32.24	FUT8	-11.79	PROK2	-26.49
CCL5	-24.69	GP1BA	-7.23	PRSS27	-9.79
CCN4	-13.16	HBEGF	-29.07	S100A12	-14.81
CDSN	-12.79	IL1RAP	-5.92	SERPINE1	-22.75
CEL	-19.79	INSL5	-12.27	SERPINE2	-31.17
CGB2	-22.21	KLK13	-13.37	SPARC	-24.91
COCH	-25.07	LOXL3	-23.67	SPINK7	-10.23
COL2A1	-17.71	MMP1	-24.34	SYT6	-18.77
CPXM1	-18.12	MMP9	-21.66	THBS1	-32.00
CRISPLD2	-23.55	NQO2	-11.57	TIMP3	-20.69
CST7	-17.62	OLR1	-14.42	TP53I13	-24.11
CXCL11	-18.38	PDGFA	-26.44	TRABD2A	-16.54
CXCL2	-23.58				

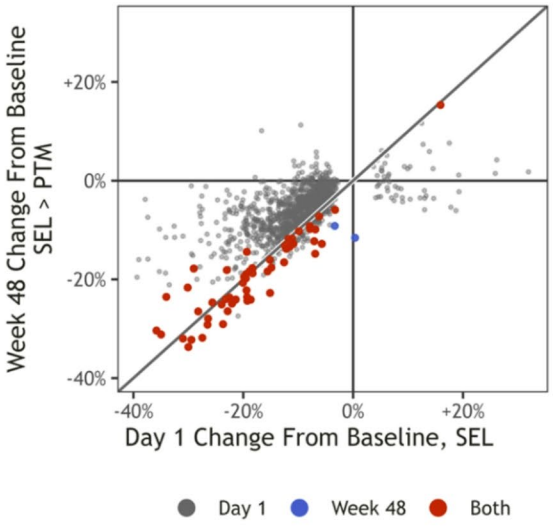


Fig. 3 Plasma protein signature of selonsertib activity. Change (%) in relative concentrations of 58 circulating proteins significantly modulated by selonsertib treatment (i.e. Selonsertib Activity Signature) between the end of study and baseline (**A**), and the intersection between the relative change in analytes over the whole study (y axis) and the short run-in period (x axis) (**B**)

overlap between SAS proteins and corresponding genes highly expressed in DKD in both databases (Fig. 4), further suggesting that proteins downregulated by selonsertib may originate from kidneys and contribute to pathophysiology of DKD.

SAS proteins reflect the molecular pathology of kidney disease

Pathway analysis revealed that the SAS is enriched in proteins belonging to inflammatory (OLR1), chemokine (C-X-C motif) ligand family (CXCL2, CXCL6, CXCL11); fibrotic (MMP1, MMP9, PDGFA, PDGFB, PDGFD, THBS1); apoptotic (SPARC, RANTES, TIMP3); and oxidative stress (HB-EGF, PAI-1, NQO2) pathways (Fig. 5). These pathways have been implicated in the onset and progression of DKD and are also modulated by ASK1 [8]. A number of the SAS proteins are involved in the activation of innate and adaptive immunity (e.g., OLR1, CXCLs, PF4, S100A12), another important component of DKD etiology and a process demonstrated as a target of ASK1 activity [32, 33].

Changes in levels of SAS proteins associate with efficacy of selonsertib

The MOSAIC trial utilized the difference in eGFR slope between selonsertib treated and placebo treated participants for its primary end point and resulted in a lower eGFR decline (slope = -2.29 ml/min/1.73m^2/year) in the selonsertib-treated group compared to placebo (slope = -3.49 ml/min/1.73m^2/year) [21]. In addition, a sensitivity analysis was performed to evaluate whether the baseline level of kidney dysfunction influenced the efficacy of selonsertib. Baseline eGFR ranges were categorized as Severe (≥ 15 to < 30 mL/min/1.73 m^2), Moderate (≥ 30 to < 45 mL/min/1.73 m^2), or Mild (≥ 45 mL/min/1.73 m^2). Participants treated with selonsertib whose baseline eGFR was Severe had higher slope (better response to selonsertib treatment) than subjects in the Moderate or Mild baseline eGFRs when compared to placebo-treated participants [21]. We explored the SAS proteins as a potential drug response biomarker that may support the eGFR slope findings in the sensitivity analysis. We determined whether the greater benefit of selonsertib treatment in trial subjects with low baseline eGFR could be reflected in the impact of selonsertib on the SAS biomarkers. We examined the SAS biomarkers and observed that the greatest difference in biomarker values between selonsertib-treated and placebo-treated participants occurred in the Severe eGFR group. In this group, SAS biomarker levels tended to rise over the 48-week period in placebo-treated participants in contrast to selonsertib-treated participants whose biomarker values remained at the baseline level or declined (Fig. 6). Thus, the observed stabilization in eGFR slope in the Severe eGFR group is associated with slower increase of the SAS biomarkers over time. The pattern of change of the biomarkers is consistent with the activity of selonsertib preserving kidney function and highlights a biological response to selonsertib that is consistent with the protective effect on eGFR slope in selonsertib-treated subjects in the Severe group. It is notable that in the groups with Moderate or Mild eGFR at baseline, little elevation in the SAS was observed over 48 weeks, consistent with a slow progression of disease.

Next, we sought to determine the association between the change in the SAS biomarkers and the progression

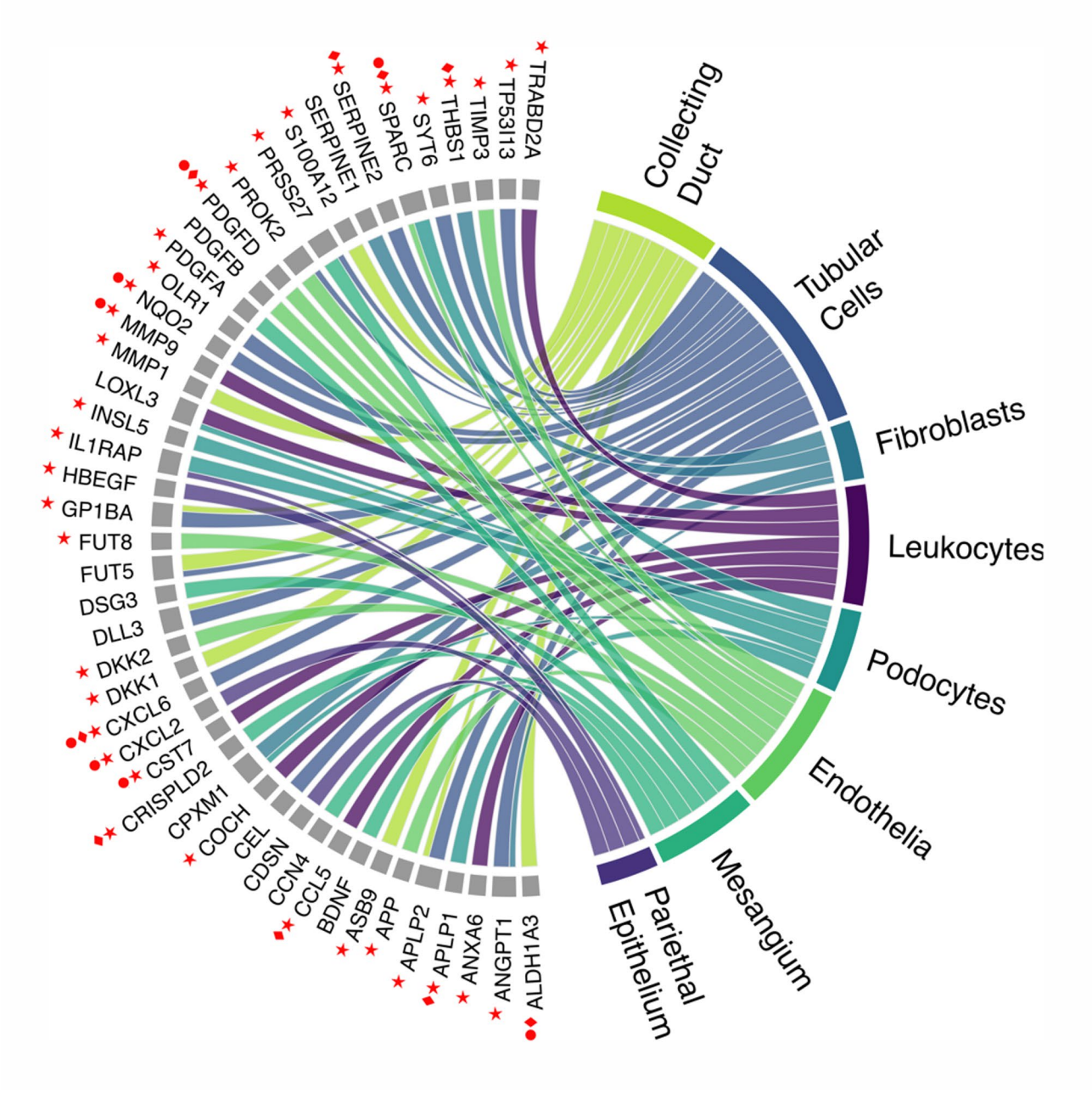


Fig. 4 Genes encoding SAS proteins are expressed in different kidney cell types and dysregulated in kidney disease. Signature proteins were assigned to separate kidney subregions according to single nuclei RNAseq data from an established database [29] and plotted in a chord diagram, the widths of connecting lines correspond to relative expression levels. Additional annotation denotes detected dysregulation of individual genes in different published datasets derived from DKD kidney tissue (star – [29]; diamond – [30]; circle – [31])

of the disease in the subgroup of patients in the Severe or Moderate eGFR categories at baseline. We analyzed patients in the placebo arm alone, and we plotted the change in biomarker levels on Y axis, against the change in eGFR on X axis. A large majority 44/57 of SAS biomarkers showed strong inverse correlation between the change in biomarker levels and the change in eGFR in the low eGFR patient subgroup. The observed correlation was pronounced when contrasted to the same analysis in moderate eGFR subgroup. Representative plots for the 10 biomarkers that showed the greatest increase over the course of the study in the placebo arm are shown in Fig. 7A.

To determine whether a quantitative relationship exists between the change in SAS biomarkers driven by disease progression and the effect of selonsertib treatment on these biomarkers we plotted the mean change in analyte level per 10-point change in eGFR on the X-axis

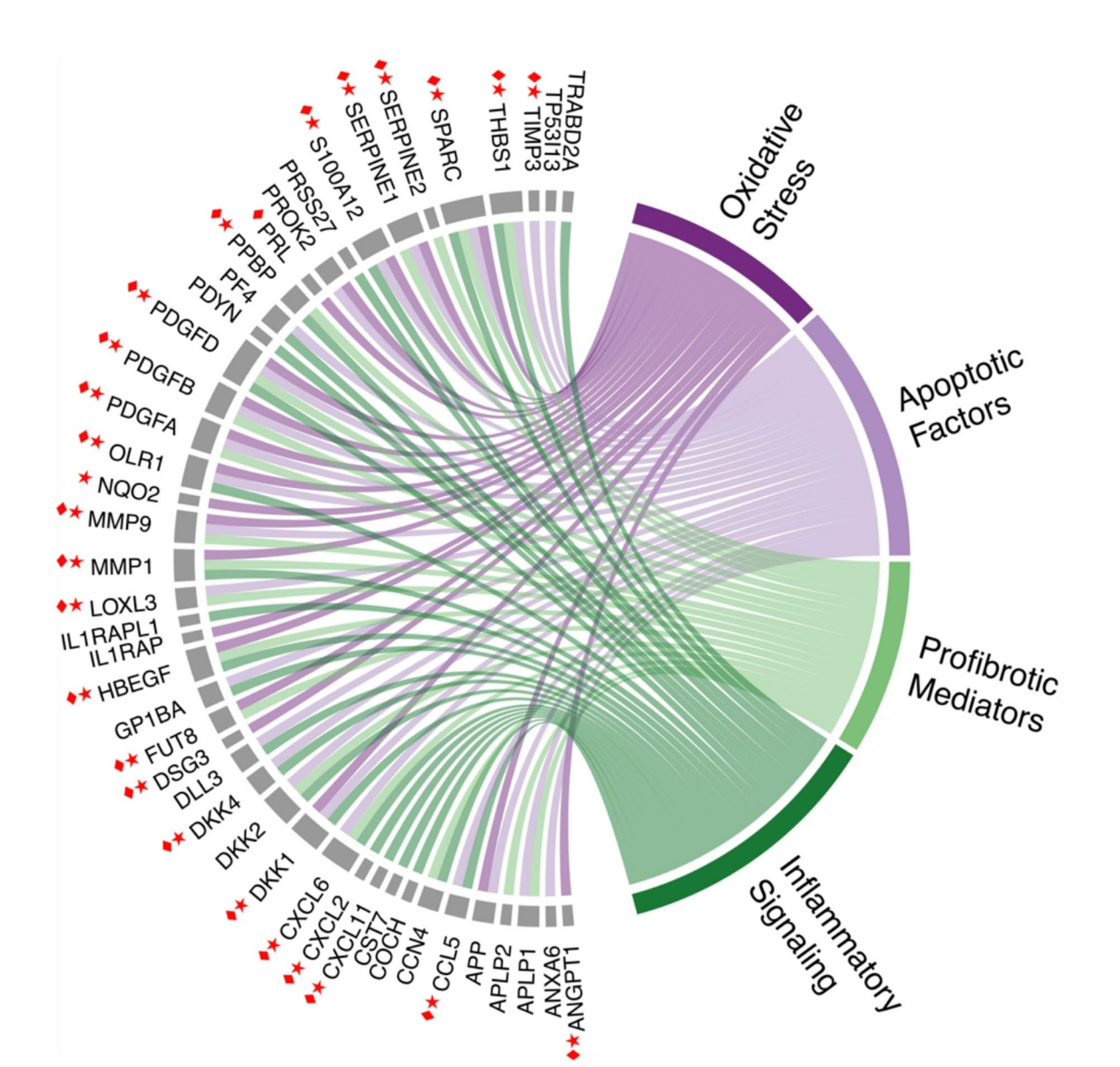


Fig. 5 Genes encoding SAS proteins are involved in regulation of key processes driving DKD. The signature proteins are categorized by major pathways that contribute to the onset and progression of DKD according to current literature [5, 33, 37, 46, 51, 80] and plotted in a chord diagram. Additionally, denoted are cases of known protein expression within kidney (star); and known involvement in the DKD pathology (diamond)

and mean change in SAS protein level with selonsertib treatment on the Y-axis (Supplemental Fig. 3). Consistent with the findings in Figs. 6 and 7a, we observed that the proteins downregulated by selonsertib in the severe eGFR baseline subgroup positively associated with the reduction in eGFR levels, and in many cases selonsertib treatment reduced the levels of the protein by roughly the same magnitude driven by the 10-point decrease in eGFR. The representative values for 10 biomarkers that showed the greatest increase over the course of the study in the placebo arm are shown in Fig. 7B. For example, PDGFD increases by 27% per 10-point reduction in eGFR and selonsertib treatment ameliorates the elevation of PDGFD by approximately the same magnitude. Similar results were observed for PF4, CCL5, ANXA6, PDGFA, PPBP, and CGB2. The quantitative relationship between the change in specific analyte levels and change in eGFR suggests that these analytes could be useful circulating biomarkers to monitor drug response and progression

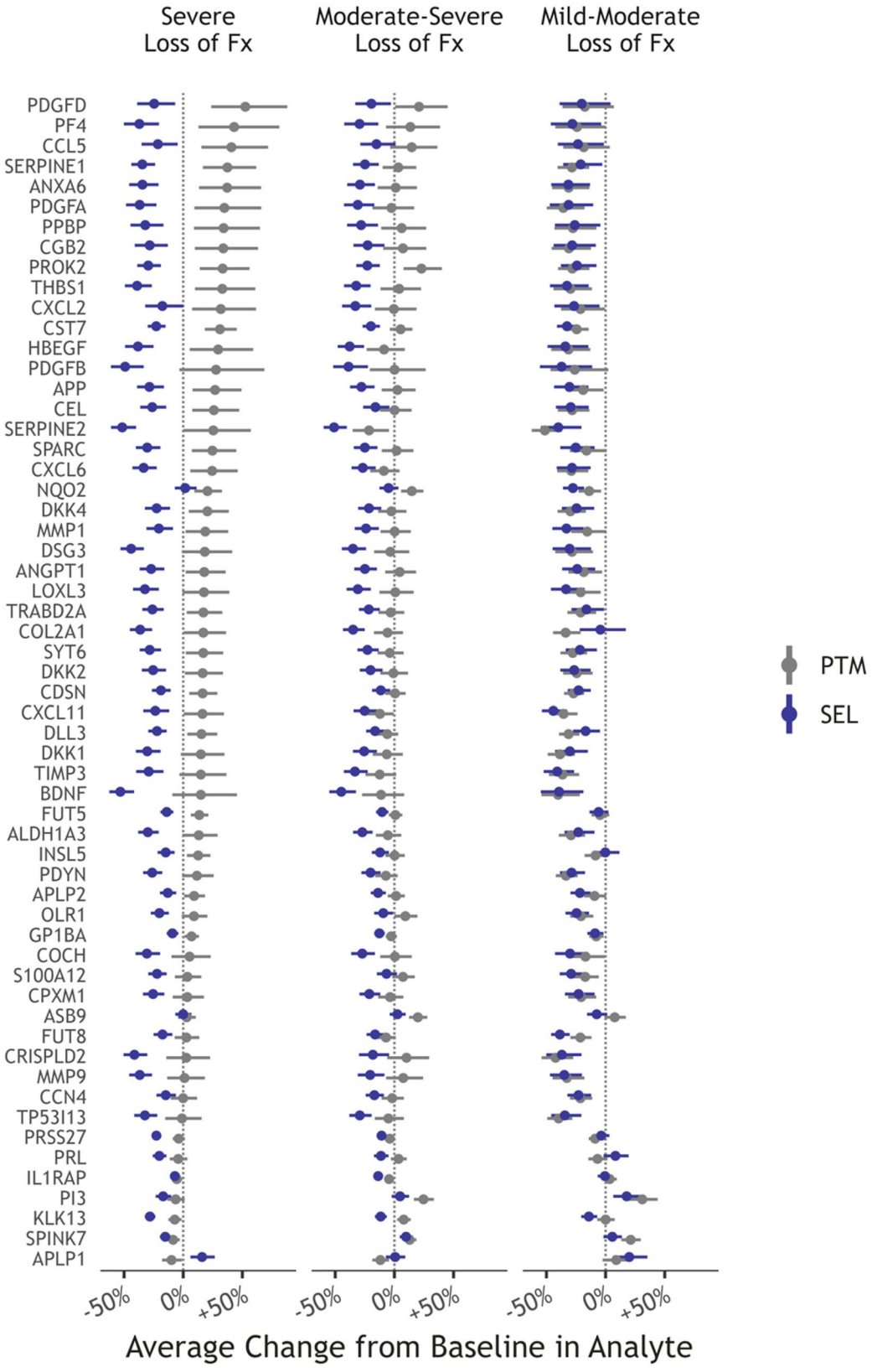
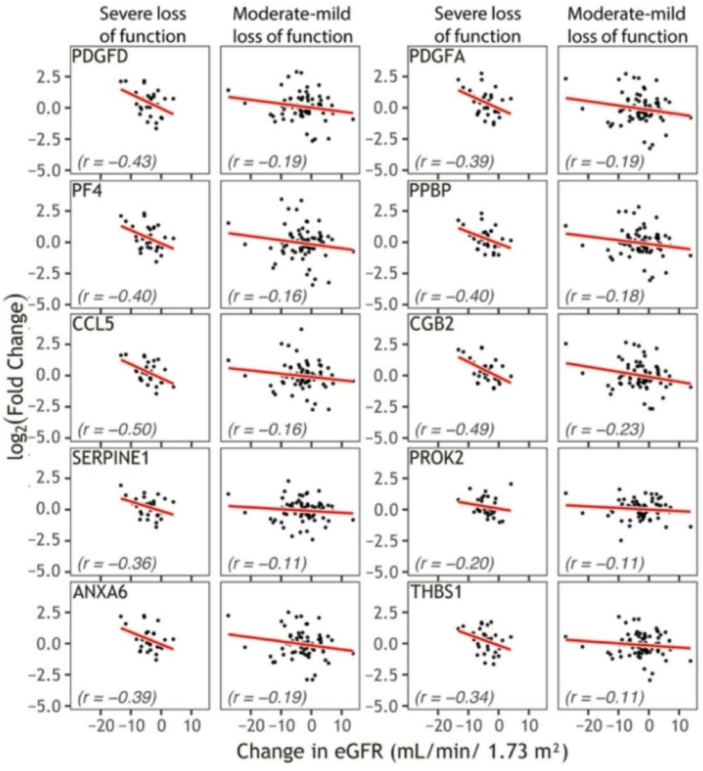


Fig. 6 SAS proteins are highly modulated by selonsertib in patients with low baseline eGFR. Trial participants were grouped based on baseline eGFR levels and the mean change in biomarker levels between the end of the study (week 48) and baseline was plotted for selonsertib and placebo to match (PTM) cohorts separately



	Change in analyte per 10-point decrease in eGFR (%)	Change in analyte with SEL treatment (%)
PDGFD	27.5967083	-27.935621
PF4	25.201874	-31.708348
CCL5	20.2253572	-23.482554
SERPINE1	10.2509865	-22.394279
ANXA6	22.7135498	-25.370488
PDGFA	22.7109606	-26.049115
PPBP	19.0970498	-27.489806
CGB2	28.9031512	-23.384078
PROK2	10.1376467	-26.179768
THBS1	6.33754453	-31.576004

Fig. 7 Majority of selonsertib activity signature analytes are inversely correlated with eGFR. Fold change in SAS proteins (y axis) was plotted against the change in eGFR (x axis) in the Severe or Moderate to Mild baseline eGFR subgroups of placebo-treated participants (**A**). Comparison of calculated percent change in individual SAS biomarkers per 10-point eGFR decrease (left column), and mean change induced by selonsertib during the course of the study (right column) is suggestive of a close inverse correlation of change in eGFR and SAS biomarkers (**B**)

of DKD patients treated with selonsertib or other ASK1 inhibitors.

Discussion

We examined over 7,000 circulating analytes in selonsertib MOSAIC study and identified a 58-protein SAS enriched in inflammatory, pro-fibrotic, pro-apoptotic, and oxidative stress-related factors. Our findings suggest that the SAS proteins contribute to processes driving the onset and progression of the kidney disease.

There has been considerable interest in the ASK1 signaling node as a potential therapeutic target, particularly in a number of fibrotic diseases [34, 35]. Selonsertib was the first small molecule ASK1 inhibitor tested in the clinic. Selonsertib was not effective in NASH, PAH, or AH [19, 36] and consequently no biomarker insights were obtained to better understand pathways and biological processes that ASK1 inhibition may impact in the context of human disease. Thus, the molecular effects of ASK1 inhibition on downstream signaling remain poorly understood in the clinical setting. The positive results from the MOSAIC trial afforded us a unique opportunity to interrogate the biological effects of ASK1 inhibition.

This study identifies a novel signature of circulating proteins associated with ASK1 inhibition in human subjects in the context of DKD. Individual markers of the signature contribute to different pathways that are known to promote cellular injury and inflammation of the kidney. The dominant pathways associated with the SAS proteins are known drivers of kidney disease, including inflammatory, pro-fibrotic, oxidative stress-related, and pro-apoptotic factors. Below we discuss the biology of the SAS proteins and how the processes they regulate relate to kidney disease.

Inflammation

Among the inflammatory factors identified in the signature, the members of the chemokine (C-X-C motif) ligand family CXCL2, CXCL6, and CXCL11 are known to contribute to inflammation in kidney disease. CXCL2 plays a critical role in neutrophil recruitment and infiltration, and immunoneutralization of CXCL2 in vivo reduced neutrophil migration into kidney glomeruli by 85% in an experimental model of toxin-induced renal injury [37]. A separate study demonstrated that mice with reduced expression of CXCL2 experienced an attenuated decrease in eGFR following lipopolysaccharide

(LPS)-induced kidney injury [38]. The same study also found that CXCL2 levels were regulated by the MAPK pathway activity, and that inhibition of p38 and JNK suppressed CXCL2 expression. CXCL6 and CXCL11 are known to attract neutrophils and T-lymphocytes, respectively, and were both found to be highly elevated in the serum of patients that received a kidney allograft transplantation and experienced subsequent transplant rejection [39]. The study suggests that these chemokines are important drivers of the recruitment of immune cells to kidney and are likely produced by renal epithelial cells.

Oxidized low-density lipoprotein receptor 1 (OLR1) is the main receptor for oxidized low-density lipoprotein (oxLDL) on endothelial cells, macrophages, and smooth muscle cells, and is upregulated by a number of pathological stimuli including pro-inflammatory cytokines and high glucose levels [40]. Upon binding to oxLDL, OLR1 initiates a pro-inflammatory cascade, and can also mediate the increased production of intracellular ROS [41]. OLR1 was found to be upregulated in the kidneys of mice following renal injury caused by myocardial infarction. OLR1−/− mice showed reduced production of renal pro-inflammatory cytokines, as well as improvement in renal function and histopathological features of the kidney as compared to WT animals [42].

APLP1 (amyloid precursor like protein 1), the only marker in the SAS that was upregulated by selonsertib, has been shown to localize to neuronal synapse region and play a key role in the development of Alzheimer's disease [43]. Available data outlines the role of APLP1 in the maintenance of neuronal function and synaptic transmission, but further studies are needed to describe its potential involvement in the signaling pathways regulating the onset and progression of DKD.

Fibrosis

The Thrombospondin 1 (THBS1) is an adhesive glycoprotein that mediates cell-to-cell and cell-to-matrix interactions. Among the many processes it regulates, cell migration and activation of the latent transforming growth factor beta 1 (TGFβ1) directly contribute to immune modulation and fibrotic changes. THBS1 is highly expressed at sites of kidney injury and inflammation by different resident renal and inflammatory cell types, in both human disease and a variety of experimental models [44]. THBS1 has been identified as a key driver of fibrosis in the rat model of diabetic nephropathy through its ability to activate latent transforming growth factor beta (TGF-β), while blocking this interaction led to a decrease in glomerular extracellular matrix accumulation and proteinuria [45].

Matrix metalloproteinases (MMPs) are zinc-dependent proteolytic enzymes that degrade various proteins in the extracellular matrix, regulating cell repair and tissue remodeling, but also affect cell function through modulation of activity of specific membrane receptors. MMPs are known to play an important role in kidney fibrosis through the induction of tubular cell epithelial-mesenchymal transition (EMT) [46]. MMP-9, which specifically cleaves type IV collagen and laminin, major constituents of tubular basement membrane, contributes to tubular cell EMT via the disruption of tubular cell membrane integrity enabling the newly transformed mesenchymal cells to migrate and invade the interstitial space and contribute to the development of fibrosis through extracellular matrix deposition [47]. TIMP3 is the most highly expressed tissue inhibitor of metalloproteinase (TIMP) in the kidney, but its contribution to renal disease is poorly understood. TIMP3 expression was increased in the renal arteries and proximal tubules of patients with diabetic nephropathy [48]. By regulating matrix composition, TIMP3 affects a wide range of physiological processes such as cell growth and migration, angiogenesis, and fibrosis. TIMP3 was also shown to induce apoptosis in cultured human endothelial cells by triggering a caspase-independent cell death pathway and targeting a focal adhesion kinase (FAK)-dependent survival pathway [49].

Platelet-derived growth factors (PDGFs) are stored and released by platelets upon activation, and regulate cell growth, division and migration of many cell types including fibroblasts. The members of the PDGF family are among the best characterized factors driving renal fibrosis, independent of the underlying kidney disease [50]. They are upregulated in mesangial cells, parietal epithelial cells, endothelial cells, tubular cells, and interstitial cells in rodent kidney injury models and their corresponding human diseases [51]. Upon binding to their receptors, PDGFs induce downstream signaling mainly via the JAK/STAT, phosphoinositide 3-kinase, PLC-γ, or RAS-MAPK pathways, promoting gene expression and mediating the biological functions of the PDGF isoforms, such as fibrosis, migration, and proliferation.

Oxidative stress

Heparin-binding epidermal growth factor-like growth factor (HB-EGF) is an autocrine growth factor that is primarily produced by monocytes and macrophages, and regulates cell division and migration. HB-EGF is upregulated in response to oxidative stress in different tissues and cell types by a process dependent on p38 MAPK activity [52]. Expression of HB-EGF, both in pro- and mature forms, increases after renal ischemia/reperfusion injury, especially in the outer medulla and distal tubular epithelial cells, and is believed to be involved in proliferation of tubular epithelial cells and repair of the kidney [53].

Plasminogen activator inhibitor-1 (PAI-1) is a multifunctional serine protease inhibitor with primary

function in regulation of extracellular matrix (ECM) degradation. It was shown that PAI-1 has a pivotal role in development of glomerulosclerosis and tubulointerstitial fibrosis of the kidney. Although not normally produced in kidneys, PAI-1 is expressed by both resident and intrarenal inflammatory cells in several acute and chronic kidney disease conditions, including progressive renal disease [54]. PAI-1, also implicated in many other complications of diabetes, was shown to be induced by both insulin stimulation and oxidative stress through activation of JNK, and the combined treatment increased the PAI-1 expression additively [55].

S100 calcium-binding protein A12 (S100A12) is a small calcium binding protein secreted predominantly by neutrophils and macrophages/monocytes exerting various extracellular activities. It has been shown that hexamers of S100A12 bind to RAGE activating downstream NFκB and MAPK signaling pathways that ultimately contribute to innate immune responses such as chemotactic activity and activation of pro-inflammatory cytokine production, in parallel with induction of oxidative stress [56, 57]. Plasma levels of S100A12 were found to be elevated in patients with moderate to advanced stage of CKD. In stage 5 CKD patients, increased concentration of circulating S100A12 was associated with higher inflammation and presence of cardiovascular disease and diabetes, as well as 32% higher all-cause mortality risk [58].

Cytosolic protein quinone reductase 2 (NQO2) is a flavoprotein that catalyzes the two-electron reduction of quinone substrates. NQO2 has been shown to mediate ROS production and is predominantly expressed in liver and kidneys [59, 60]. Deletion of NQO2 in cells from NQO2−/− mice prevented TNF-mediated activation of JNK, p38, and p44/p42 MAPK, as well upregulation of pro-fibrotic MMP-9 [61]. MMP-9, in turn, was also identified in our selonsertib signature.

Apoptosis

Secreted protein acidic and rich in cysteine (SPARC) is an antiproliferative calcium-binding matricellular glycoprotein that can interact with specific cytokines and growth factors such as PDGFs. SPARC is involved in glomerular remodeling and repair as well as in various renal diseases including diabetic nephropathy [62]. Under pathological conditions, SPARC expression is elevated in the kidney glomerulus to control the excessive proliferation of mesangial cells, which is induced, among other factors, by PDGFs [63]. A study on autosomal dominant polycystic kidney disease demonstrated that SPARC is involved in cell cycle regulation and promotes apoptosis in renal epithelial cells by regulating mRNA expression of apoptosis-related gene Bcl-2 [64].

RANTES (regulated upon activation, normal T cell expressed and secreted), also known as CCL5, is a chemokine active in several renal diseases, including CKD and diabetic nephropathy. RANTES attracts T lymphocytes, monocytes, natural killer cells, basophils, and eosinophils, and can also activate immune cells [65]. A study on human T cells demonstrated that RANTES induces cell death through the cytosolic release of cytochrome c, the activation of caspase-9 and caspase-3, and poly (ADP-ribose) polymerase (PARP) cleavage [66].

Annexin A6 (ANXA6) is a multifunctional scaffold protein, known to interact with various signaling proteins including the regulators of EGFR/Ras pathway [67]. ANXA6 also interacts with poly-(ADP-ribose) polymerase 1 (PARP1) which is one of the main regulators of mitochondrial pathway of apoptosis. Expression of ANXA6 in models of cardiac hypertrophy increased cellular susceptibility to apoptosis [68]. ANXA6 mRNA was also found to be upregulated in kidneys of mice that underwent unilateral renal artery clamping to induce renal ischemia-reperfusion injury. This type of injury induced apoptosis in different regions of kidneys from treated animals as confirmed by histo-pathological analysis and the TUNEL assay. ANXA6 was consistently upregulated in each of the kidney samples taken at 3, 12, and 24 h post injury [69]. The same study also identified *PDGFA*, a known pro-fibrotic factor, as another gene expressed in this model, suggesting its involvement in induction of renal apoptosis as well.

Successful treatment of DKD poses a challenge due to heterogeneous clinical presentation in patients, as well a large diversity of molecular processes involved and the complexity of their interplay. The majority of SAS proteins identified in this study are implicated in key processes known to drive the onset and progression of DKD. This suggests that selonsertib may improve multiple fundamental aspects of DKD on a molecular level, consistent with the efficacy observed in a clinical setting.

Taken together with observations from the MOSAIC clinical study, our analyses suggest that ASK1 inhibition may be best used in patients with severe disease, marked by poor kidney function and low eGFR. However, longer duration studies are needed to properly evaluate the treatment benefit that ASK1 inhibition provides in patients with severe, mild or moderate disease. The pronounced effect of selonsertib on eGFR in patients with advanced disease may be due to severe patients having an active disease process during the trial that can be modified by selonsertib. Participants in the placebo group with severe baseline disease demonstrated greater change in biomarker values over time compared to those with less severe baseline disease, indicative of a more active disease process that selonsertib may act upon.

The signature of circulating proteins identified in this study has a potential utility in monitoring the therapeutic effect of ASK1 inhibitors in clinical trials. A blood-based

test measuring an analyte, or a subset of analytes identified here, could be established as a quick, affordable, and a minimally invasive test of ASK1 inhibitor effect in DKD patients during drug development and perhaps even in clinical use should an ASK1 inhibitor be fully developed in DKD.

Clinical and biomarker data in DKD studies indicate that ASK1 inhibition attenuates eGFR decline. This suggests that ASK1 inhibition could also be active in other diseases driven by oxidative stress, such as cardiovascular diseases (atherosclerosis, ischemia, hypertension, cardiomyopathy, cardiac hypertrophy, and congestive heart failure [70–73]), neurological diseases (Parkinson's disease, Alzheimer's disease, amyotrophic lateral sclerosis, multiple sclerosis, depression, and memory loss [74–76]), respiratory disease (asthma and chronic obstructive pulmonary disease [77–79]), and rheumatoid arthritis [80–82].

Conclusions

In this report we provide the first in depth investigation of the effects of selonsertib on disease associated biomarkers. Our biomarker findings support the clinical findings by associating changes in numerous circulating protein markers with loss of kidney function and demonstrate the ability of selonsertib to blunt those changes in concert with the effect of selonsertib on slowing kidney function loss. These data extend the understanding of how selonsertib exerts its effects in DKD and translate preclinical observations to the clinical setting. We also provide biomarker data that supports the observation that patients with severe loss of kidney function benefit the most from selonsertib treatment and underscores the potential for patient selection based on kidney function for selonsertib treatment. In a more general sense, our results provide motivation to continue investigating ASK1 inhibition for other diseases where oxidative stress is a prominent driver.

Star methods

Trial and data

Data used in this study originate from the Phase2b MOSAIC trial (GS-US-223-1017; clinicaltrials.gov identifier NCT04026165). This trial evaluated the efficacy and safety of selonsertib versus placebo in a randomized cohort of patients with moderate to severe diabetic nephropathy [21]. Briefly, patients were enrolled beginning July 2019; the trial officially completed in September of 2021. One hundred-eleven sites across Australia (3), Canada (8), Japan (24), New Zealand (5), and the United States (71) participated in data collection. Three hundred eighty-four adult patients (aged 18–80 years) with diagnosed type-2 diabetes mellitus and moderate to advanced diabetic nephropathy were recruited into the trial. To be enrolled, participants had to meet any of the following criteria at screening: (i) estimated glomerular filtration rate (eGFR) between 45 and 60 mL/min per $1.73\ \mathrm{m}^2$ *and* urinary albumin to creatinine ratio (UACR) between 600 and 5000 mg/g; or (ii) eGFR between 30 and 45 mL/min per $1.73\ \mathrm{m}^2$ *and* UACR between 300 and 5000 $\mathrm{mg/g}$; or (iii) eGFR between 20 and 30 mL/min per $1.73\ \mathrm{m}^2$ *and* UACR between 150 and 5000 $\mathrm{mg/g}$. eGFR was calculated using the creatinine-based CKD-EPI formula [83]. All screening samples were collected prior to baseline and processed by a central laboratory.

Participants who met the eligibility criteria were randomized 1:1 into either an experimental (selonsertib) or a placebo comparator (PTM) arm. Randomization was stratified by (i) screening eGFR (stage 4 kidney disease, eGFR between 15 and 30 mL/min per $1.73\ \mathrm{m}^2$; stage 3b kidney disease, eGFR between 30 and 45 mL/min per $1.73\ \mathrm{m}^2$; stage 3a kidney disease, eGFR between 45 and 60 mL/min per $1.73\ \mathrm{m}^2$, (ii) screening albuminuria (UACR between 150 and 5000 $\mathrm{mg/g}$), and (iii) use of sodium-glucose co-transporter 2 inhibitor medications (SGLT2i).

The trial was designed with an initial run-in period of at least five weeks: patients received placebo-to-match (PTM) administered orally, once daily (po/qd) for the first week. This was followed by four weeks where every patient received 18 mg selonsertib po/qd. Following the run-in period, participants were randomized to receive either 18 mg selonsertib versus PTM po/qd for at least 48 weeks. Throughout this manuscript, we use "baseline" to reference patient records measured at the start of or just prior to the four-week selonsertib run-in block (also "visit B;" see Supplemental Fig. 1), and "week 48" to reference records measured after 48 weeks of randomized selonsertib versus PTM treatment (52 total weeks from baseline).

This trial was approved by the institutional review boards or independent ethics committees at all participating sites and conducted in compliance with the Declaration of Helsinki, GCP guidelines, and local regulatory requirements. The trial was designed and conducted according to protocol by the sponsor (Gilead Sciences, Inc., Foster City, CA) in collaboration with independent principal investigators. The sponsor collected the data, monitored study conduct, and performed the statistical analyses. An independent data monitoring committee reviewed the progress and provided oversight of the trial. The investigators, participating institutions, and sponsor agreed to maintain confidentiality of the data.

SomaScan assay and normalization

To study effects of selonsertib treatment on the circulating proteome, we used the SomaScan® (Gold et al. 2010; Schneider et al. 2022) (version 4.1) high throughput assay

system from SomaLogic, Inc. (Boulder, CO). SomaScan is a highly multiplexed, sensitive, aptamer-based assay platform that can simultaneously measure thousands of human proteins in small volumes of biologic samples (SomaLogic, Inc. 2021). The assay platform relies on protein capture technologies based on slow off-rate modified aptamer (SOMAmer®) reagents [84]. SomaScan assays have been validated for select markers of end-stage renal disease [85, 86], and have enjoyed increasingly frequent adoption in disease-linked biomarker discovery studies (e.g [87]).

Here, plasma samples were obtained from a subset of 256 trial participants for their baseline and week 48 study visits.

The SomaScan version 4.1 assay platform used in this study measures analytes corresponding to over 7,000 different SOMAmers. Analytes in turn correspond to different but not necessarily unique proteins and peptides measured in relative fluorescence units (RFUs). For this study, we selected SomaLogic's *plate-scale* normalization pipeline to help remove technical variation in the assays [88]. This normalization stream consists of three sequential steps: *hybridization control normalization* to adjust for nuisance variation in individual plate wells; *median signal normalization on calibrators*, a within-plate normalization step designed to adjust for nuisance variation due to overall protein concentration, etc.; and *plate-scale normalization* proper, designed to adjust for nuisance variation in total signal from plate to plate. In our data plate-scale normalization was consistently most similar (after simple linear rescaling) to available plasma analytes measured with enzyme-linked immunosorbent assays. This observation is consistent with published suggestions that relatively minimalist normalization streams (like the plate-scale sequence) may be preferred in cases where study samples can be expected to deviate broadly from a healthy proteome [86, 88].

Following normalization, we filtered the SomaScan panel down to 6,124 analytes associated with unique UniProt (UniProt Consortium 2015) human protein or peptide identification codes. In cases where multiple SOMAmers mapped to single UniProt codes, the analyte with the largest median absolute deviation at baseline was retained as representative for that protein or peptide, and data from the others was discarded.

Statistical methods

Analyses presented in this manuscript are all based on analysis of covariance using robust "Student-*t*" linear models (Lange, Little, and Taylor, 1989) estimated with maximum likelihood. Robust regression is an attractive competitor to standard linear regression when the outcome data contain occasional extreme values and there is no a priori reason to believe these measurements were made incorrectly. Regression parameters in the robust model proposed by Lange, Little, and Taylor (1989) have the same interpretation as those in ordinary least squares regression and yet their estimation is relatively less sensitive to apparent outliers. In all cases in the main text, we used one-at-a-time modeling of individual SomaScan analytes, taking the week 48 measurement as the response variable and conditioning on the baseline measurement as a covariate. Analyte measurements were log (base two)-transformed prior to analyses so that model coefficients could be interpreted as changes in analyte log geometric means given unit changes in corresponding predictors.

The analyses presented here can be grouped into those intended to estimate (geometric) mean selonsertib treatment effects or to characterize effects of kidney disease progression (controlling for selonsertib treatment) on the circulating proteome. All analyses were adjusted for treatment arm and baseline covariates patient age, UACR (natural log-transformed), eGFR, sex at birth, and SGLT2i use. We additionally included estimated linear changes in eGFR from baseline to week 48 as a covariate in disease progression analyses. Linear eGFR changes (eGFR "slopes") were computed using best linear unbiased predictor estimates from a linear mixed model used in the primary clinical analysis previously described [20]. In the present analysis, non-linear relationships between the week 48 analyte and the baseline analyte, UACR, and eGFR were modeled using restricted cubic regression splines (Stone and Koo, 1985). Estimated relationships between week 48 analytes and baseline patient age were generally found to be nearly linear for this cohort and observation period and so spline terms for age were ultimately discarded for simplicity. Restricted cubic splines are constrained to estimate functions that are linear outside of specified boundary knot locations and are thus naturally somewhat resistant to over-fitting. Interactions between treatment arm and baseline eGFR, and treatment arm and eGFR slope were also included in our analyses. Interactions were restricted to be only between treatment and linear eGFR terms (higher order interactions with spline terms were not considered here). See the Supplementary Materials for additional details.

Statistical significance was computed for each analysis using analyte-wise likelihood ratio tests, adjusted for multiple testing using the Benjamini-Hochberg procedure (Benjamini and Hochberg 1995), and assessed at a two-sided 10% level. Under certain assumptions about the tests performed, the Benjamini-Hochberg procedure limits the expected false discovery rate to be no more than q(where we set $q = 0.1$). For a study of the operating characteristics of the Benjamini-Hochberg procedure under varying assumptions about testing dependence, see e.g. Green and Diggle (2007). In our

analyses, we performed multiplicity adjustment in the following manner: (i) selonsertib treatment effect analyses were adjusted at a SomaScan-wide level (adjusted against thousands of tests). Given (i), we then asked if any selonsertib treatment-associated analytes were also significantly linked to kidney disease progression in the selonsertib arm. To address this question, we tested for (ii) PTM arm-specific effects of eGFR slope limited to analytes in (i) that were found significant (adjusted against hundreds of tests).

Abbreviations

ASK1	Apoptosis signal-regulating kinase 1
DKD	Diabetic Kidney Disease
T2DM	Type 2 diabetes mellitus
CKD	Chronic kidney disease
ESRD	End-Stage Renal Disease
ROS	Reactive oxygen species
AGE	Advanced glycation end products
RAGE	Receptor for advanced glycation end products
NADPH	Nicotinamide adenine dinucleotide phosphate
PKC	Protein kinase C
MAPK	Mitogen-activated protein kinase
JNK	c-Jun N-terminal kinase
WT	Wild type
UUO	Unilateral ureteric obstruction
GFR	Glomerular filtration rate
NASH	Nonalcoholic steatohepatitis
PAH	Pulmonary arterial hypertension
eGFR	Estimated glomerular filtration rate
AH	Alcoholic steatohepatitis
PTM	Placebo to match
KRIS	Kidney Risk Inflammation Signature
SAS	Selonsertib Activity Signature
RNAseq	RNA sequencing
LPS	Lipopolysaccharide
TGF-β	Transforming growth factor beta
MMP	Matrix metalloproteinase
EMT	Epithelial-mesenchymal transition
TIMP	Tissue inhibitor of metalloproteinase
FAK	Focal adhesion kinase
PDGF	Platelet-derived growth factor
ECM	Extracellular matrix

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12882-025-04166-4.

Supplementary Material 1

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n/a.

Author contributions
V.P. and A.N.B. designed experiments and wrote the manuscript. A.W. and V.A.M performed statistical analyses. S.K. and M.P. conducted additional analyses. G.B. provided input on manuscript draft. All authors reviewed the manuscript.

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Data availability
The datasets generated and/or analyzed during the current study are available in the github repository, https://github.com/gilead-BioInfo/SEL-SomaScan/tree/main/summary.

Declarations

Ethics approval and consent to participate
The trial was conducted in accordance with the Declaration of Helsinki and the International Council on Harmonisation tripartite guideline on the ethical principles of Good Clinical Practice. The study was conducted across 111 centers in Australia, Japan, New Zealand, and North America (United States and Canada), and each investigator was responsible for obtaining separate site IRB approval. The protocol was approved by institutional review boards or ethics committees at all participating sites, and all patients signed informed consent.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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