Urinary Dickkopf-3 (DKK3) uncovers inapparent progressive kidney injury: an observational study and experimental validation

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Abstract

CKD represents a global public health problem with high disease-related morbidity and mortality. Since the etiology of CKD is heterogenous, early recognition of patients at risk for progressive kidney injury is important. We evaluated the tubular epithelial-derived glycoprotein dickkopf-3 (DKK3) as a urinary marker for the identification of progressive CKD in a non-CKD cohort of patients with chronic obstructive pulmonary disease (COPD) and in an experimental model.

In COSYCONET, a prospective multicentre trial comprising patients with stable COPD (N=2,314, follow-up 37.1 months) urinary DKK3, proteinuria and estimated glomerular filtration rate (eGFR) were measured at baseline and tested for its association with trajectories of eGFR, forced expiratory volume in one second (FEV1), and 6-minute walking distance (6-MWD). We explored the impact of DKK3 in wild-type and Dkk3-/- mice subjected to cigarette smoke-induced lung injury combined with a CKD model (CS-CKD model).

Urinary DKK3, but not proteinuria or baseline eGFR identified patients with declining kidney function during follow-up (OR: 1.55, 95% CI: 1.16-2.08). In particular, DKK3 was associated with a significantly higher risk for declining eGFR in patients with eGFR >90ml/min/1.73m^2 and proteinuria <30mg/g. DKK3 was also associated with declining FEV1 (OR 3.36, 95% CI: 2.22-5.08) and 6-MWD (OR 1.56, 95% CI: 1.09-2.22). In the CS-CKD mouse model, genetic abrogation of DKK3 resulted in reduced pulmonary inflammation and kidney fibrosis, and preserved kidney function.

These data highlight DKK3 as a tubular marker for the early identification of patients with inapparent progressive CKD and above that with adverse outcomes in patients with COPD.

Introduction

Chronic kidney disease (CKD) represents a global public health problem affecting hundreds of millions of patients worldwide (1). Between 2005 and 2015, the global prevalence of CKD has increased by 26.2 % (2). In the general population, even a slightly reduced kidney function is already associated with higher mortality (3), and the mortality rate is almost five-fold higher in patients with severely impaired kidney function compared to the non-CKD population (4). The etiology of CKD is heterogenous, however, and early recognition of patients at risk for progressive kidney injury is therefore important (1). Furthermore, the individual course of CKD may be highly variable with patients showing long-term stably reduced glomerular filtration rate (GFR) without any signs of progression and others being characterized by a rapidly declining GFR (5). Accordingly, risk estimation equations have been developed and validated in patients with CKD in stage G3 to G5 to predict their risk of CKD progression. Thereby, the prediction of CKD progression is mainly based on GFR and albuminuria – a putative marker of glomerular injury (6, 7), a concept lingering in nephrology for several decades.

However, recent evidence suggests that a substantial proportion of CKD patients shows disease progression in the absence of proteinuria or albuminuria (8), so-called non-proteinuric pathways of CKD progression. In patients with type 2 diabetes included in the third National Health and Nutrition Examination Survey (NAHNES III), in only 19 % of patients with an estimated GFR (eGFR) lower than 60 ml/min/1.73 m^2 macroalbuminuria was present (9). Moreover, the annual decline of eGFR in type 2 diabetic patients was similar in subjects without albuminuria as compared to those with microalbuminuria (10). A longitudinal study, which followed patients with type 2 diabetes for 15 years, found that albuminuria started to increase when eGFR declined below 60 ml/min/1.73m^2, which indicates that albumin loss might be rather a consequence than the cause of CKD in these patients (11). In the community-based ARIC cohort, 19 % of the participants with type 2 diabetes developed incident CKD, of whom 58 % had normoalbuminuria (12). Importantly, large scale observations of US adults with diabetes from 1988 through 2014 document that the prevalence of CKD in diabetic patients significantly increased from 1988 to 2014, whereas the prevalence of albuminuria (i.e. albumin-

creatinine-ratio, ACR ≥30 mg/g) significantly decreased in the same time (13). Notably, non-proteinuric pathways of CKD progression are not restricted to patients with diabetes, but also of relevance for the progression of hypertensive, cystic and interstitial kidney diseases (14). These findings indicate that besides glomerular injury other compartments such as the tubulo-interstitial compartment of the kidney might play a pivotal role in the progression of CKD.

Recently, we identified Dickkopf-3 (DKK3), a member of the evolutionary conserved Dickkopf protein family, which is involved in Wnt/β-catenin signaling (15), to be a key driver in tubulointerstitial injury and fibrosis (16). DKK3 is released from “stressed” tubular epithelial cells in the kidney and can be quantified in the urine (17). Measurement of DKK3 in the urine allows identification of CKD patients with short-term risk of GFR loss as well as patients at high risk for postoperative acute kidney injury (AKI) and its transition into CKD, independent of kidney function and proteinuria (18).

The aim of the present study was to explore the impact of the tubular stress marker DKK3 in comparison to proteinuria as a marker for glomerular injury for the identification of patients at risk for kidney dysfunction in a primarily non-CKD cohort. We quantified DKK3 and proteinuria at baseline in the large-scale, multicentre COSYCONET study of patients with stable chronic obstructive pulmonary disease (COPD) and explored their association with kidney and lung function during long-term follow-up. In addition, we explored the impact of DKK3 in wild-type and Dkk3-/- mice subjected to cigarette smoke (CS)-induced lung injury combined with a CKD model (CS-CKD model).

Methods

Animal experiments

Details on the animal experiments can be found in the **Supplement**.

Study design, setting, and participants of the COSYCONET study population

The COPD and SYstemic consequences-COmorbidities NETwork (COSYCONET) is a German multicentre, prospective observational trial, which recruited 2,741 patients aged ≥40 years with diagnosis of COPD between 2010 and 2013 in 31 study centres. The study protocol has been previously described in detail (19). COPD was defined according to the spirometric Global Initiative for Chronic Obstructive Lung Disease (GOLD) criteria (20) as having a ratio FEV_1/FVC <70 % determined in standardized post-bronchodilator spirometry. 2,314 patients with baseline urine samples available were included in the present analyses. Six, 18, 36, and 54 months after enrolment, patients were invited to participate in follow-up visits, in which lung function testing was performed. All-cause mortality was assessed over a median follow-up period of 37.1 months. Three participants were lost to follow-up. COSYCONET was approved by the ethics committees at all participating sites and all participants provided written consent. The study is registered at clinicaltrials.gov (NCT01245933).

Lung function and exercise capacity in COSYCONET

Airway obstruction was quantified by determining the post-bronchodilator FEV_1 at baseline as well as during follow-up visits. Moreover, exercise capacity was determined by the measurement of the 6-MWD.

Laboratory measurements in COSYCONET

Creatinine measurements were calibrated to the gold standard, an isotope dilution mass spectrometry. eGFR at baseline, after 6 months, and after 18 months was calculated using the CKD-EPI equation (21). DKK3 concentrations in urine were measured using a commercially available ELISA (DiaRen, Homburg, Germany) as described previously (17, 18). The inter-

assay test variability was 4.7 % in the lower detection range and 5.1 % in the higher detection range. Any cross-reactivity with other Dickkopf proteins was excluded. Urinary DKK3 concentrations were normalized to urinary creatinine concentrations to account for urine dilution. Urinary protein concentrations were determined using a Beckman Coulter AU480 clinical chemistry analyzer.

Statistics

Continuous variables are presented as mean ± standard deviation (SD) when normally distributed or as median (interquartile range). Statistical differences between continuous or categorical variables were established using One-way ANOVA, Kruskall-Wallis test, or $\chi 2$ test where appropriate. In animal experiments, One-way ANOVA was followed by Dunnett's test for pairwise comparisons.

To assess the association between baseline urinary DKK3 and changes of FEV_1, 6-MWD or eGFR, we performed group-based trajectory modelling of FEV_1, 6-MWD or eGFR using the STATA package 'traj'. This approach is based on the SAS PROC TRAJ macro (22), which fits a semiparametric (discrete mixture) model for longitudinal data using maximum likelihood methods. We used the Bayesian information criterion (BIC) to establish the optimal number of groups. Trajectory groups of FEV_1 were termed: group A (increasing FEV_1), group B (stable FEV_1), and group C (declining FEV_1). Trajectory groups of 6-MWD were termed: group A (increasing 6-MWD), group B (declining 6-MWD), and group C (rapidly declining 6-MWD). Trajectory groups of eGFR were termed group A (increasing eGFR), group B (stable eGFR), and group C (declining eGFR). We then performed logistic regression analyses to determine the association between baseline urinary DKK3 and trajectory group C of FEV_1, 6-MWD, or eGFR using group B as reference.

Logistic regression analyses or Cox proportional hazard models were used to assess the association between baseline urinary DKK3 and risk of COPD exacerbation or mortality, respectively. To assess the association between baseline urinary DKK3 and 6-MWD, we used generalized linear models and calculated multivariate adjusted least square means of 6-MWD

according to categories of DKK3. Results from crude and multivariate adjusted models (adjusted for age, sex, GOLD stage, smoking status, body mass index, baseline kidney function, and proteinuria) are presented.

The non-linear association between baseline urinary DKK3 and changes of eGFR after 6 months as well as after 18 months was visualized using restricted cubic splines of urinary DKK3 with three knots placed at 2, 226, and 979 pg/mg creatinine, which corresponds to the 10^{th}, 50^{th}, and 90^{th} percentile of urinary DKK3, respectively. Moreover, we used generalized linear models to estimated multivariate adjusted least square means of the change of eGFR during follow-up according to categories of urinary DKK3. DKK3 was categorized into two groups (≤200 pg/mg and >200 pg/mg creatinine) as described previously (17, 18) to improve the general readability of the manuscript. To exclude any bias derived from definition of DKK3 categories, we assessed the association between log-transformed urinary DKK3 and changes of eGFR in generalized linear models.

Furthermore, we used a machine learning approach to confirm the association between baseline urinary DKK3 and changes of eGFR as well as declining FEV_1 as an unbiased approach. For this purpose, we used kernel-based least squares provided within the STATA package 'krls'. We assessed the predictive value of urinary DKK3 for prediction of declining eGFR or FEV1 in comparison to a model including baseline age, sex, GOLD class, smoking status, body mass index, eGFR, and proteinuria by determining integrated discrimination improvement (IDI) and net reclassification improvement (NRI).

A two-sided *P* value of less than 0.05 was considered statistically significant. Statistical analyses were performed using SPSS version 21.0 and STATA IC 15 with the packages krls, nriidi, postrcspline, and traj.

Results

Urinary DKK3 significantly improves identification of patients with progressive kidney dysfunction

We determined baseline urinary DKK3, eGFR, and proteinuria in 2,314 subjects of the COSYCONET cohort (**Figure 1**). eGFR was quantified after six and 18 months of follow-up. The baseline characteristics are shown in **Table 1**. Mean eGFR at baseline was 82.0+16.4 ml/min/1.73m^2 and proteinuria 5.0 (6.0) mg/g creatinine. At first, we performed group-based trajectory modelling of eGFR (**Figure 2A**). While eGFR remained stable during follow-up in 74.5 % of the participants, declining eGFR was observed in 16.5 %, in whom eGFR decreased by 20 % within 18 months of follow-up. Next, we assessed the association between baseline urinary DKK3, eGFR, and proteinuria and the risk of declining eGFR (i.e. trajectory group C, **Figure 2B**). Patients with elevated baseline urinary DKK3 (i.e. >200 pg/mg creatinine) had a significantly higher risk (OR: 1.55, 95% CI: 1.16-2.08, P=0.003) for declining eGFR during follow-up. Importantly, this association persisted even after adjusting for eGFR and proteinuria (**Supplemental Table S1**) and was independent of a specific DKK3 cut-off (**Supplemental Table S2**) and confirmed by using machine learning (**Supplemental Table S3**). In contrast to DKK3, we did not observe an association between baseline eGFR or proteinuria and changes of eGFR during follow-up (**Figure 2B**, **Supplemental Tables S4-S9**). Adding DKK3 to a clinical model comprising age, sex, GOLD grade, smoking status, and body mass index significantly improved prediction of the risk of declining eGFR (NRI 0.093, 95% CI 0.032-0.154, P=0.0031, IDI 0.005, 95% CI 0.002-0.008, P=0.0029). Contrarily, adding baseline eGFR or proteinuria to this model did not have a significant effect on reclassification (**Figure 2B**). Next, we assessed the association between DKK3 and declining eGFR in a subgroup of patients without any sign of apparent kidney dysfunction (i.e. patients with eGFR >90 ml/min/1.73m^2 and proteinuria <30 mg/g creatinine). Also, in this subgroup, higher baseline DKK3 was associated with a significantly higher risk for declining eGFR during follow-up (OR: 1.92, 95% CI 1.11-3.12, P=0.020). This indicates that DKK3 identifies patients at risk for loss of GFR in the absence of apparent CKD.

To corroborate these findings, we assessed the non-linear association between baseline urinary DKK3 and subsequent loss of eGFR during follow-up using restricted cubic spline plots (**Figure 3A+B**). Higher baseline urinary DKK3 was associated with a larger decline of eGFR after 6 or 18 months. While eGFR remained stable in patients with urinary DKK3 concentrations ≤200 pg/mg creatinine, in patients with urinary DKK3 >200 pg/mg creatinine, eGFR loss was -0.8% (95% CI: -1.6-0.2 %, *P*<0.0001) after 6 months and -1.6% (95 % CI: -2.6–-0.5, *P*=0.005) after 18 months (**Supplemental Tables S11-12**). This association was confirmed in models using DKK3 as continuous log-transformed variable as well as by a machine learning approach (**Supplemental Tables S13-14**).

Urinary DKK3 identifies COPD patients with worsening FEV1

In COSYCONET, FEV_1 and 6-MWD as a measure of physical performance were determined at baseline as well as after six, 18, 36, and 54 months (**Figure 1**). To assess the impact of kidney injury on the subsequent decline of FEV_1, trajectories of FEV_1 were built (**Figure 4A**). This approach identified three groups of COPD patients. While FEV_1 remained stable over time in 58.1 % participants, FEV_1 declined in 39.4 % subjects. In 2.5 % of the patients, FEV_1 improved temporarily. We found that higher baseline urinary DKK3 was associated with a significantly higher risk for declining FEV_1 during follow-up (OR 3.36, 95% CI 2.22-5.08, P<0.001, **Figure 4B**, **Supplemental Table S15**). This association persisted after adjustment for several covariates including age, sex, GOLD class, smoking status, body mass index, baseline eGFR and proteinuria. DKK3 significantly improved risk prediction as compared to a clinical model comprising age, sex, GOLD grade, smoking status and body mass index (IDI P=0.0002, NRI P=0.003). Notably, there was no association between baseline eGFR or proteinuria and declining FEV_1 (**Figure 4B**, **Supplemental Table S16-17**). The association between DKK3 and declining FEV_1 was confirmed using machine learning (**Supplemental Table S18**).

Association between kidney injury, COPD exacerbation, exercise capacity, and mortality

To determine the association between baseline urinary DKK3 and changes of the 6-MWD, as a measure of the exercise capacity, we built trajectories of the 6-MWD (**Figure 4C**). Hereby, we identified three groups of patients. In 90.3 % of the participants, 6-MWD declined during follow-up, whereas 9.0 % of the patients were characterized by rapidly declining 6-MWD. Baseline urinary DKK3 was independently associated with a higher risk for rapidly declining 6-MWD during follow-up (OR 1.56, 95% CI 1.09-2.22, *P*=0.014, **Fig. 4D**, **Supplemental Table S19**). We observed no association between baseline proteinuria or eGFR and declining 6-MWD (**Supplemental Tables S20-21**).

In line with these findings, higher baseline urinary DKK3 was associated with a higher risk of COPD exacerbation during follow-up (OR 1.24, 95% CI 1.03-1.50, *P*=0.026, **Supplemental Table S22**). During a median follow-up of 37.1 months (IQR 33.4 months), 191 participants of COSYCONET died. Higher baseline urinary DKK3 was associated with a higher risk of death during follow-up (HR 1.49, 95% CI 1.08-2.05, *P*=0.015, **Fig. 5**, **Table S8**). These findings demonstrate that subclinical kidney injury (i.e. not detectable by a decrease in GFR or proteinuria), as quantified by the measurement of urinary DKK3, is significantly associated with subsequent worsening pulmonary function and increased mortality in patients with COPD.

Kidney dysfunction aggravates lung injury in a combined organ injury mouse model

To corroborate these clinical findings, we combined established murine models of cigarette smoke (CS)-induced lung injury and adenine diet-induced CKD (CS-CKD model, **Figure 6A**). After three weeks of treatment, bronchoalveolar lavage fluid (BALF) was collected. Whereas CKD without CS had no effect on BALF cell composition, mice treated with CS had significantly higher total cell count, neutrophils, and lymphocytes (**Figure 6B-D**). Interestingly, in CS-CKD mice, total BALF cell numbers, neutrophils, and lymphocytes were significantly higher as compared to mice treated with CS alone. Induction of CKD alone induced accumulation of Ly6B-positive neutrophils and monocytes (**Supplemental Figure 1A**) in lung tissue and enhanced CS-induced lung infiltration with neutrophils/monocytes (Ly6B) and T-lymphocytes (CD3, **Supplemental Figure 1A-B**). In BALF, CS increased concentrations of several pro-

inflammatory cytokines, while no secreted pro-inflammatory cytokines were detected in BALF of CKD mice (**Supplemental Figure 1C** and **Table S24**). Notably, kidney injury induced by the administration of an adenine diet significantly enhanced the concentrations of pro-inflammatory cytokines in BALF of CS-exposed mice as compared to mice treated with CS alone. CS per se did not affect creatinine or urea serum levels in mice with or without adenine diet (**Supplemental Figure 1D-E**). However, CS significantly enhanced kidney fibrosis in CS-CKD mice (**Figure 6E-F**). Moreover, we found that CS alone promoted accumulation of Ly6G-positive neutrophils within the kidney and further aggravated inflammation caused by CKD (**Supplemental Figures 1F-G**).

DKK3 mediates CS-induced lung and kidney injury

To assess the role of DKK3 in the CS-CKD mouse model, we determined the DKK3 expression in the kidney (**Figure 7A-B**). CS per se induced renal tissue DKK3 expression in animals not on adenine diet, whereas no DKK3 was detectable in kidneys from mice subjected to standard diet in the absence of CS. DKK3 expression in CS-treated mice was restricted to the apical area of the tubuli. Adenine diet promoted strong and widespread tubular DKK3 expression, which was further enhanced in the combined CS-CKD model. Here, DKK3 expression was not only apparent in the tubular epithelium but also in the interstitial compartment. Abrogation of *Dkk3* significantly reduced lung inflammation (**Figure 7C-E**) as determined by lower total cell count, neutrophil and lymphocyte count in BALF of CS-CKD mice. Moreover, the concentrations of MIP-2, G-CSF, MMP12, and TNFα in the BALF was significantly lower in *Dkk3*$^{-/-}$ mice as compared to wildtype mice (**Figure 7F-J**). In the kidneys, *Dkk3* deficiency significantly reduced fibrosis as well as serum creatinine and urea levels (**Figure 7K-N**).

Discussion

Here, we identified urinary DKK3 as a marker of tubular stress and tubulointerstitial injury, which allows identification of patients at risk for progressive kidney dysfunction in a cohort of primarily non-CKD patients with COPD. Higher urinary DKK3 was not only associated with declining GFR, but also adverse pulmonary events pointing to a link between both organ systems. Notably, neither baseline eGFR nor proteinuria were associated with any of these events. The pathophysiological role of DKK3 was underscored in animal experiments, in which genetic abrogation of DKK3 attenuated both kidney fibrosis and pulmonary inflammation.

We have recently shown that DKK3 is released from tubular epithelial cells into the urine in a variety of kidney injury models and plays a pivotal role in the development of tubulointerstitial fibrosis (16). Indeed, the tubulointerstitial compartment represents a key player in mediating progressive CKD. The tubular epithelium is highly sensitive to hypoxia, metabolic disturbances, and toxic substances. These conditions initiate a “tubulo-toxic” cascade leading to oxidative stress, cell cycle arrest, pro-inflammatory cell activation, and the expression of profibrotic factors such as transforming growth factor-β1 (TGF-β1). This promotes the generation of myofibroblasts and the deposition of extracellular matrix culminating in irreversible tubulointerstitial fibrosis (23). In our animal models, we found that exposure of mice to CS is sufficient to induce the expression of DKK3 in tubular epithelial cells. While DKK3 is only located in the apical membrane of tubular epithelial cells in CS-treated mice, in adenine-fed mice widespread tubular DKK3 expression could have been observed. In line with our previous studies, there was virtually no DKK3 expression in mice fed with a standard diet exposed to normal air (16, 17). This indicates that DKK3 is only expressed in stressed tubular cells undergoing various types of insults. Therefore, it is plausible that DKK3 released from tubular cells to the surrounding tissue activates fibrogenesis by the regulation of Wnt/β-catenin-dependent signalling pathway (23).

DKK3 belongs to the Dickkopf protein family, which plays an important role in the regulation of Wnt/β-catenin signalling pathway (15). Wnt/β-catenin can be activated in variety of cells such as tubular epithelial cells, fibroblasts, and macrophages in the kidney. Wnt

agonists induce translocation of β-catenin into the nucleus, where it interacts with the transcription factors T cell factor (TCF) and lymphoid enhancer factor (LEF) to drive the expression of a variety of Wnt target genes (24). Thereof, TGF-β (25), SNAIL (26), matrix metalloproteinases (MMP) (27), and plasminogen activator inhibitor 1 (PAI1) (28) are directly involved in the development of kidney fibrosis. Interestingly, we found that genetic abrogation of DKK3 in the combined CS-CKD mouse model significantly reduced the expression of MMP-12 in BALF suggesting that DKK3 might directly regulate the expression of proteases involved in adverse pro-fibrotic remodelling.

The present study has broad clinical relevance. In a well-characterized large-scale non-CKD cohort of patients with COPD, DKK3 but not proteinuria or baseline GFR identified patients with progressively declining kidney function. The cohort mainly comprised patients without apparent CKD, since baseline eGFR was 82.0±16.4 mL/min/1.73m^2 and proteinuria 5.0 (6.0) mg/g creatinine. Nevertheless, in the modelling of eGFR trajectories, we identified a group of patients (i.e. 16.5 %), in whom eGFR declined by 20 % within 18 months. Our findings indicate that urinary DKK3 has the potential to recognize patients with ongoing tubular stress at high risk for deteriorating kidney function, who could not have been detected by currently available methods such as estimation of GFR or proteinuria. Accordingly, baseline urinary DKK3 but not proteinuria or eGFR significantly improved reclassification of patients. These findings are in line with our previous studies, in which urinary DKK3 was associated with short-term risk of eGFR loss in patients with established CKD and the risk of AKI-CKD transition in patients undergoing cardiac surgery (17, 18). The data suggest that DKK3 can be used in a variety of clinical settings to uncover inapparent progressive kidney injury.

Beyond that, the present study highlights an important interaction between the kidney and the lung. Epidemiological studies revealed an association between impaired kidney function and higher mortality as well as increased exacerbation risk in patients with COPD (29, 30). Measurement of urinary DKK3 does not only allow identification of COPD patients with declining eGFR, but also those with decreasing FEV1, physical performance, higher exacerbation risk, and all-cause mortality. Also here, neither baseline eGFR nor proteinuria

were associated with these important clinical events. In our animal models, we found that lung inflammation was significantly attenuated in *Dkk3*$^{-/-}$ mice. These findings suggest that DKK3 as a regulator of the Wnt/β-catenin system is not only relevant for ongoing kidney injury, but also for adverse pulmonary remodelling induced by CS. Indeed, it has been recently shown that the Wnt/β-catenin cascade plays a pivotal role in experimental CS-induced COPD (31). Thereby, DKK3 could serve as a mediator of reciprocal organ injury and fibrosis.

These findings are not only relevant for risk stratification, but also have important therapeutic implications. In particular, patients with elevated urinary DKK3 might benefit from preventive therapeutic strategies such as intensified monitoring of kidney function, optimization of the hydration status, avoidance of nephrotoxic agents, and interdisciplinary patient care. Moreover, it has to be evaluated on whether these patients particularly benefit from early therapeutic interventions such as the inhibition of the sodium-glucose transporter 2 (SGLT2) or endothelin-1 receptor antagonism, which reduce kidney tissue fibrosis in variety of small animal models (32-35).

The study is not without limitations. COSYCONET comprises patients of European ancestry, therefore the generalization of the present findings to other ethnicities has to be determined. We have used recently published cut-offs of urinary DKK3 for the present analyses to improve general readability (17, 18). However, analyses were validated in models including log-transformed DKK3 as a continuous variable and using machine learning methods as an unbiased approach.

In summary, the present study highlights urinary DKK3 as a marker for the early identification of patients at risk for progressive kidney dysfunction in a non-CKD population with preserved GFR and without significant proteinuria. In patients with COPD, urinary DKK3 was not only associated with declining eGFR, but also deteriorating pulmonary function as well as adverse outcomes. Therefore, DKK3 expands the diagnostic spectrum in the field of nephrology and paves the way for early preventive therapeutic strategies.

Disclosures

D.F. is affiliated with DIAREN. All other authors report no competing interests.

References

1. Eckardt KU, Coresh J, Devuyst O, Johnson RJ, Kottgen A, Levey AS, Levin A. Evolving importance of kidney disease: from subspecialty to global health burden. *Lancet* 2013; **382**:158-169.
2. Disease GBD, Injury I, Prevalence C. Global, regional, and national incidence, prevalence, and years lived with disability for 310 diseases and injuries, 1990-2015: a systematic analysis for the Global Burden of Disease Study 2015. *Lancet* 2016; **388**:1545-1602.
3. Chronic Kidney Disease Prognosis C, Matsushita K, van der Velde M, Astor BC, Woodward M, Levey AS, de Jong PE, Coresh J, Gansevoort RT. Association of estimated glomerular filtration rate and albuminuria with all-cause and cardiovascular mortality in general population cohorts: a collaborative meta-analysis. *Lancet* 2010; **375**:2073-2081.
4. Saran R, Robinson B, Abbott KC, Agodoa LYC, Bragg-Gresham J, Balkrishnan R, Bhave N, Dietrich X, Ding Z, Eggers PW, Gaipov A, Gillen D, Gipson D, Gu H, Guro P, Haggerty D, Han Y, He K, Herman W, Heung M, Hirth RA, Hsiung JT, Hutton D, Inoue A, Jacobsen SJ, Jin Y, Kalantar-Zadeh K, Kapke A, Kleine CE, Kovesdy CP, Krueter W, Kurtz V, Li Y, Liu S, Marroquin MV, McCullough K, Molnar MZ, Modi Z, Montez-Rath M, Moradi H, Morgenstern H, Mukhopadhyay P, Nallamothu B, Nguyen DV, Norris KC, O'Hare AM, Obi Y, Park C, Pearson J, Pisoni R, Potukuchi PK, Repeck K, Rhee CM, Schaubel DE, Schrager J, Selewski DT, Shamraj R, Shaw SF, Shi JM, Shieu M, Sim JJ, Soohoo M, Steffick D, Streja E, Sumida K, Kurella Tamura M, Tilea A, Turf M, Wang D, Weng W, Woodside KJ, Wyncott A, Xiang J, Xin X, Yin M, You AS, Zhang X, Zhou H, Shahinian V. US Renal Data System 2018 Annual Data Report: Epidemiology of Kidney Disease in the United States. *Am J Kidney Dis* 2019; **73**:A7-A8.
5. Vistisen D, Andersen GS, Hulman A, Persson F, Rossing P, Jorgensen ME. Progressive Decline in Estimated Glomerular Filtration Rate in Patients With Diabetes After Moderate Loss in Kidney Function-Even Without Albuminuria. *Diabetes Care* 2019; **42**:1886-1894.

6. Tangri N, Stevens LA, Griffith J, Tighiouart H, Djurdjev O, Naimark D, Levin A, Levey AS. A predictive model for progression of chronic kidney disease to kidney failure. *JAMA* 2011; **305**:1553-1559.

7. Tangri N, Grams ME, Levey AS, Coresh J, Appel LJ, Astor BC, Chodick G, Collins AJ, Djurdjev O, Elley CR, Evans M, Garg AX, Hallan SI, Inker LA, Ito S, Jee SH, Kovesdy CP, Kronenberg F, Heerspink HJ, Marks A, Nadkarni GN, Navaneethan SD, Nelson RG, Titze S, Sarnak MJ, Stengel B, Woodward M, Iseki K, Consortium CKDP. Multinational Assessment of Accuracy of Equations for Predicting Risk of Kidney Failure: A Meta-analysis. *JAMA* 2016; **315**:164-174.

8. Porrini E, Ruggenenti P, Mogensen CE, Barlovic DP, Praga M, Cruzado JM, Hojs R, Abbate M, de Vries AP, group E-Edw. Non-proteinuric pathways in loss of renal function in patients with type 2 diabetes. *The lancet Diabetes & endocrinology* 2015; **3**:382-391.

9. Kramer HJ, Nguyen QD, Curhan G, Hsu CY. Renal insufficiency in the absence of albuminuria and retinopathy among adults with type 2 diabetes mellitus. *JAMA* 2003; **289**:3273-3277.

10. Ruggenenti P, Porrini EL, Gaspari F, Motterlini N, Cannata A, Carrara F, Cella C, Ferrari S, Stucchi N, Parvanova A, Iliev I, Dodesini AR, Trevisan R, Bossi A, Zaletel J, Remuzzi G, Investigators GFRS. Glomerular hyperfiltration and renal disease progression in type 2 diabetes. *Diabetes Care* 2012; **35**:2061-2068.

11. Retnakaran R, Cull CA, Thorne KI, Adler AI, Holman RR, Group US. Risk factors for renal dysfunction in type 2 diabetes: U.K. Prospective Diabetes Study 74. *Diabetes* 2006; **55**:1832-1839.

12. Bash LD, Selvin E, Steffes M, Coresh J, Astor BC. Poor glycemic control in diabetes and the risk of incident chronic kidney disease even in the absence of albuminuria and retinopathy: Atherosclerosis Risk in Communities (ARIC) Study. *Archives of internal medicine* 2008; **168**:2440-2447.

13. Afkarian M, Zelnick LR, Hall YN, Heagerty PJ, Tuttle K, Weiss NS, de Boer IH. Clinical Manifestations of Kidney Disease Among US Adults With Diabetes, 1988-2014. *JAMA* 2016; **316**:602-610.

14. Bolignano D, Zoccali C. Non-proteinuric rather than proteinuric renal diseases are the leading cause of end-stage kidney disease. *Nephrol Dial Transplant* 2017; **32**:ii194-ii199.

15. Schunk SJ, Floege J, Fliser D, Speer T. WNT-beta-catenin signalling - a versatile player in kidney injury and repair. *Nat Rev Nephrol* 2020.

16. Federico G, Meister M, Mathow D, Heine GH, Moldenhauer G, Popovic ZV, Nordstrom V, Kopp-Schneider A, Hielscher T, Nelson PJ, Schaefer F, Porubsky S, Fliser D, Arnold B, Grone HJ. Tubular Dickkopf-3 promotes the development of renal atrophy and fibrosis. *JCI Insight* 2016; **1**:e84916.

17. Zewinger S, Rauen T, Rudnicki M, Federico G, Wagner M, Triem S, Schunk SJ, Petrakis I, Schmit D, Wagenpfeil S, Heine G, Mayer G, Floege J, Fliser D, Gröne H-J, Speer T. Dickkopf-3 (DKK3) in Urine Identifies Patients with Short-Term Risk of eGFR Loss. *J Am Soc Nephrol* 2018.

18. Schunk SJ, Zarbock A, Meersch M, Kullmar M, Kellum JA, Schmit D, Wagner M, Triem S, Wagenpfeil S, Grone HJ, Schafers HJ, Fliser D, Speer T, Zewinger S. Association between urinary dickkopf-3, acute kidney injury, and subsequent loss of kidney function in patients undergoing cardiac surgery: an observational cohort study. *Lancet* 2019; **394**:488-496.

19. Karch A, Vogelmeier C, Welte T, Bals R, Kauczor HU, Biederer J, Heinrich J, Schulz H, Glaser S, Holle R, Watz H, Korn S, Adaskina N, Biertz F, Vogel C, Vestbo J, Wouters EF, Rabe KF, Sohler S, Koch A, Jorres RA, Group CS. The German COPD cohort COSYCONET: Aims, methods and descriptive analysis of the study population at baseline. *Respir Med* 2016; **114**:27-37.

20. From the Global Strategy for the Diagnosis, Management and Prevention of COPD, Global Initiative for Chronic Obstructive Lung Disease (GOLD). **http://www.goldcopd.org/.**

21. Inker LA, Schmid CH, Tighiouart H, Eckfeldt JH, Feldman HI, Greene T, Kusek JW, Manzi J, Van Lente F, Zhang YL, Coresh J, Levey AS. Estimating glomerular filtration rate from serum creatinine and cystatin C. *N Engl J Med* 2012; **367**:20-29.

22. Nagin DS, Jones BL, Passos VL, Tremblay RE. Group-based multi-trajectory modeling. *Stat Methods Med Res* 2018; **27**:2015-2023.

23. Ruiz-Ortega M, Rayego-Mateos S, Lamas S, Ortiz A, Rodrigues-Diez RR. Targeting the progression of chronic kidney disease. *Nat Rev Nephrol* 2020; **16**:269-288.

24. Clevers H, Nusse R. Wnt/beta-catenin signaling and disease. *Cell* 2012; **149**:1192-1205.

25. Feng Y, Ren J, Gui Y, Wei W, Shu B, Lu Q, Xue X, Sun X, He W, Yang J, Dai C. Wnt/beta-Catenin-Promoted Macrophage Alternative Activation Contributes to Kidney Fibrosis. *J Am Soc Nephrol* 2018; **29**:182-193.

26. Grande MT, Sanchez-Laorden B, Lopez-Blau C, De Frutos CA, Boutet A, Arevalo M, Rowe RG, Weiss SJ, Lopez-Novoa JM, Nieto MA. Snail1-induced partial epithelial-to-mesenchymal transition drives renal fibrosis in mice and can be targeted to reverse established disease. *Nat Med* 2015; **21**:989-997.

27. Zhou D, Tian Y, Sun L, Zhou L, Xiao L, Tan RJ, Tian J, Fu H, Hou FF, Liu Y. Matrix Metalloproteinase-7 Is a Urinary Biomarker and Pathogenic Mediator of Kidney Fibrosis. *J Am Soc Nephrol* 2017; **28**:598-611.

28. Zhou L, Liu Y. Wnt/beta-catenin signalling and podocyte dysfunction in proteinuric kidney disease. *Nat Rev Nephrol* 2015; **11**:535-545.

29. Trudzinski FC, Alqudrah M, Omlor A, Zewinger S, Fliser D, Speer T, Seiler F, Biertz F, Koch A, Vogelmeier C, Welte T, Watz H, Waschki B, Fahndrich S, Jorres R, Bals R, German Cc. Consequences of chronic kidney disease in chronic obstructive pulmonary disease. *Respir Res* 2019; **20**:151.

30. Trudzinski FC, Kahnert K, Vogelmeier CF, Alter P, Seiler F, Fahndrich S, Watz H, Welte T, Speer T, Zewinger S, Biertz F, Kauczor HU, Jorres RA, Bals R, consortium C. Combined effects of lung function, blood gases and kidney function on the exacerbation

risk in stable COPD: Results from the COSYCONET cohort. *Respir Med* 2019; **154**:18-26.

31. Conlon TM, John-Schuster G, Heide D, Pfister D, Lehmann M, Hu Y, Ertuz Z, Lopez MA, Ansari M, Strunz M, Mayr C, Angelidis I, Ciminieri C, Costa R, Kohlhepp MS, Guillot A, Gunes G, Jeridi A, Funk MC, Beroshvili G, Prokosch S, Hetzer J, Verleden SE, Alsafadi H, Lindner M, Burgstaller G, Becker L, Irmler M, Dudek M, Janzen J, Goffin E, Gosens R, Knolle P, Pirotte B, Stoeger T, Beckers J, Wagner D, Singh I, Theis FJ, de Angelis MH, O'Connor T, Tacke F, Boutros M, Dejardin E, Eickelberg O, Schiller HB, Konigshoff M, Heikenwalder M, Yildirim AO. Inhibition of LTbetaR signalling activates WNT-induced regeneration in lung. *Nature* 2021; **589**:E6.

32. Ali BH, Al Salam S, Al Suleimani Y, Al Za'abi M, Abdelrahman AM, Ashique M, Manoj P, Adham SA, Hartmann C, Schupp N, Nemmar A. Effects of the SGLT-2 Inhibitor Canagliflozin on Adenine-Induced Chronic Kidney Disease in Rats. *Cell Physiol Biochem* 2019; **52**:27-39.

33. Woods TC, Satou R, Miyata K, Katsurada A, Dugas CM, Klingenberg NC, Fonseca VA, Navar LG. Canagliflozin Prevents Intrarenal Angiotensinogen Augmentation and Mitigates Kidney Injury and Hypertension in Mouse Model of Type 2 Diabetes Mellitus. *American journal of nephrology* 2019; **49**:331-342.

34. Kang WL, Xu GS. Atrasentan increased the expression of klotho by mediating miR-199b-5p and prevented renal tubular injury in diabetic nephropathy. *Sci Rep* 2016; **6**:19979.

35. Spires D, Poudel B, Shields CA, Pennington A, Fizer B, Taylor L, McPherson KC, Cornelius DC, Williams JM. Prevention of the progression of renal injury in diabetic rodent models with preexisting renal disease with chronic endothelin A receptor blockade. *Am J Physiol Renal Physiol* 2018; **315**:F977-F985.

Figure legends

Figure 1. Outline of the COSYCONET clinical study.

(A) Outline of the COSYCONET trial.

Figure 2. Urinary DKK3 identifies patients with declining estimated glomerular filtration rate (**eGFR).**

(A) Group-based trajectory modelling of eGFR identified three distinct eGFR trajectories corresponding to patients with increasing eGFR (group A), stable eGFR (group B), and declining eGFR (group C). Shaded areas indicate 95% CIs. **(B)** Association between urinary DKK3, eGFR, and proteinuria at baseline and eGFR trajectory group C (reference: group B, stable eGFR). Shown are the OR and 95% CI. Analyses were adjusted for age, sex, GOLD grade, smoking status, body mass index, eGFR, and proteinuria, where applicable. NRI, net reclassification improvement. IDI, integrated discrimination improvement. **(C)** Association between baseline urinary DKK3 and eGFR trajectory group C in patients with eGFR <90 ml/min/1.73m^2 and/or proteinuria ≥30 mg/g creatinine and patients with eGFR >90 ml/min/1.73m^2 and proteinuria <30 mg/g creatinine.

Figure 3. Baseline urinary DKK3 associates with changes of estimated glomerular filtration rate (**eGFR) during followup.**

(A) and **(B)** Restricted cubic spline plots of the association between baseline urinary DKK3 concentrations and eGFR after 6 or 18 months of follow-up, respectively. The red line indicates the estimated change of eGFR with respective 95% CIs (light grey area). All plots are adjusted for age, sex, GOLD class, smoking status, body mass index, baseline eGFR and proteinuria. Blue spikes show the individual distribution of urinary DKK3 concentrations.

Figure 4. Urinary DKK3 identifies patients with worsening pulmonary function and physical performance.

(A) Group-based trajectory modelling of forced expiratory volume in one second (FEV_1) identified three distinct FEV_1 trajectories corresponding to patients with increasing FEV_1 (group A), stable FEV_1 (group B), and declining FEV_1 (group C). Shaded areas indicate 95% confidence intervals (CI). **(B)** Association between urinary DKK3 concentrations dichotomised at 200 pg/mg creatinine, eGFR, and proteinuria at baseline and FEV_1 trajectory group (reference: group B, stable FEV_1). Results are adjusted for age, sex, GOLD class, smoking status, body mass index, baseline eGFR and proteinuria, where appropriate. **(C)** Group-based trajectory modelling of six-minute walking distance (6-MWD) identified three distinct 6-MWD trajectories corresponding to patients with increasing 6-MWD (group A), declining 6-MWD (group B), and rapidly declining 6-MWD (group C). Shaded areas indicate 95% confidence intervals (CI). **(D)** Association between urinary DKK3 concentrations dichotomised at 200 pg/mg creatinine, eGFR, and proteinuria at baseline and 6-MWD trajectory group (reference: group B, declining 6-MWD). Results are adjusted for age, sex, GOLD class, smoking status, body mass index, baseline eGFR and proteinuria, where appropriate.

Figure 5. Urinary DKK3 is associated with higher mortality.

Survival plot for the association between baseline urinary DKK3 concentrations (dichotomised at 200 pg/mg creatinine) and all-cause mortality in 2,311 participants of the COSYCONET cohort. Results are adjusted for age, sex, GOLD class, smoking status, body mass index, baseline eGFR and proteinuria.

Figure 6. Characterization of a murine CS-CKD model.

(A) Outline of the experimental studies. **(B-D)** Quantification of total cells, neutrophils, and lymphocytes in the bronchoalveolar lavage fluid (BALF) after three weeks in the combined CS-CKD model. **(E)** Quantification of kidney fibrosis and **(F)** representative histological images of Sirius-Red stained kidney samples. Each dot refers to an individual animal.

Figure 7. DKK3 promotes lung inflammation and kidney fibrosis

(A-B) Quantification of kidney tissue DKK3 expression and representative immunofluorescence images. G indicated glomeruli **(C-E)** Quantification of total cells, neutrophils, and lymphocytes in the bronchoalveolar lavage fluid (BALF) after three weeks in the combined CS-CKD model in wildtype (WT) and *Dkk3*$^{-/-}$ mice. **(F-J)** Quantification of different cytokine in the BALF of wildtype and *Dkk3*-/- mice after three weeks in the combined CS-CKD model. **(K)** Quantification of kidney tissue fibrosis and **(L)** representative histological images of Sirius-Red stained kidney samples after three weeks in the combined CS-CKD model. **(M-N)** Serum creatinine and urea concentrations. Each dot refers to an individual animal. MIP-2 = Macrophage Inflammatory Protein 2, MMP-12 = matrixmetalloproteinase-12, IL-12 = interleukin-12, G-CSF = Granulocyte-Colony Stimulating Factor, TNF-α = tumour necrosis factor-α.

Table 1: Baseline characteristics of participants of the COSYCONET cohort divided into categories according to urinary DKK3

	Total cohort (N=2,314)	Urinary DKK3 ≤200 pg/mg creatinine (N=1,048)	Urinary DKK3 >200 pg/mg creatinine (N=1,266)	*P* Value
Age (years)	65.0±8.6	64.2±8.5	65.3±8.7	0.001
Sex (% male)	59.1	60.2	58.2	0.328
Body mass index (kg/m^2)	27.1±5.4	27.5±5.4	26.8±5.4	0.001
GOLD Grade 0 (%)	15.0	16.3	13.9	0.047
GOLD Grade 1 (%)	10.4	10.7	10.1	
GOLD Grade 2 (%)	37.8	36.8	38.7	
GOLD Grade 3 (%)	29.6	27.7	31.1	
GOLD Grade 4 (%)	7.2	8.4	6.1	
FEV1 (% predicted)	55.0 (30.0)	55.0 (32.0)	55.0 (29.0)	0.469
TLCO (% predicted)	53.7 (29.7)	55.2 (29.1)	53.5 (29.5)	0.235
Smoking (%)	92.4	92.6	92.2	0.915
Packyears (-)	42.0 (42.5)	43.0 (41.5)	42.5 (44.3)	0.824
Serum Creatinine (mg/dL)	0.9±0.2	0.9±0.2	0.9±0.2	0.991
eGFR (mL/min/1.73m^2)	82.0±16.4	82.5±16.0	81.3±16.7	0.119
Urinary DKK3 (pg/mg creatinine)	227 (414)	77 (119)	467 (493)	<0.001
Proteinuria (mg/g creatinine)	5.0 (6.0)	4.0 (4.0)	5.0 (7.0)	<0.001

TLCO = Transfer factor of the lung for carbon monoxide. Data are presented as mean ± SD for parametric data, median (interquartile range) for nonparametric data, and n (%) for categorical data.

Figure 1

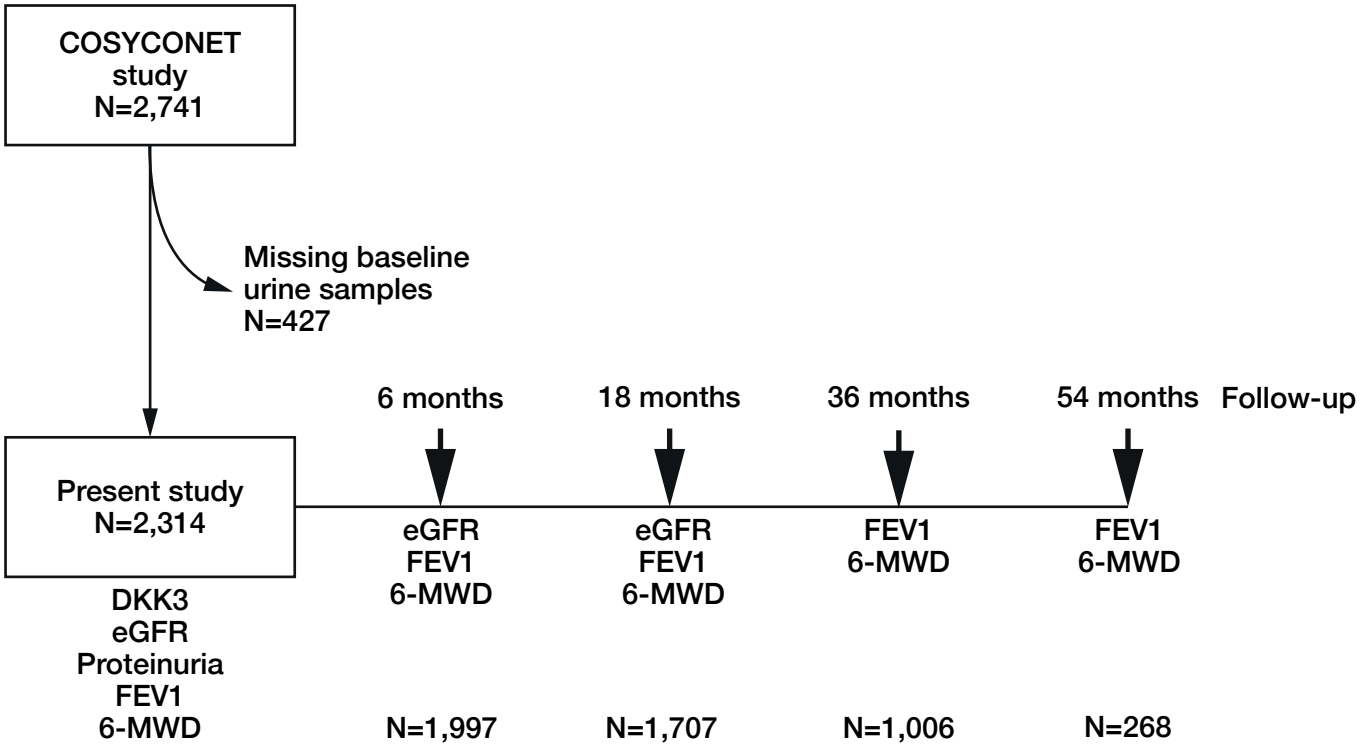
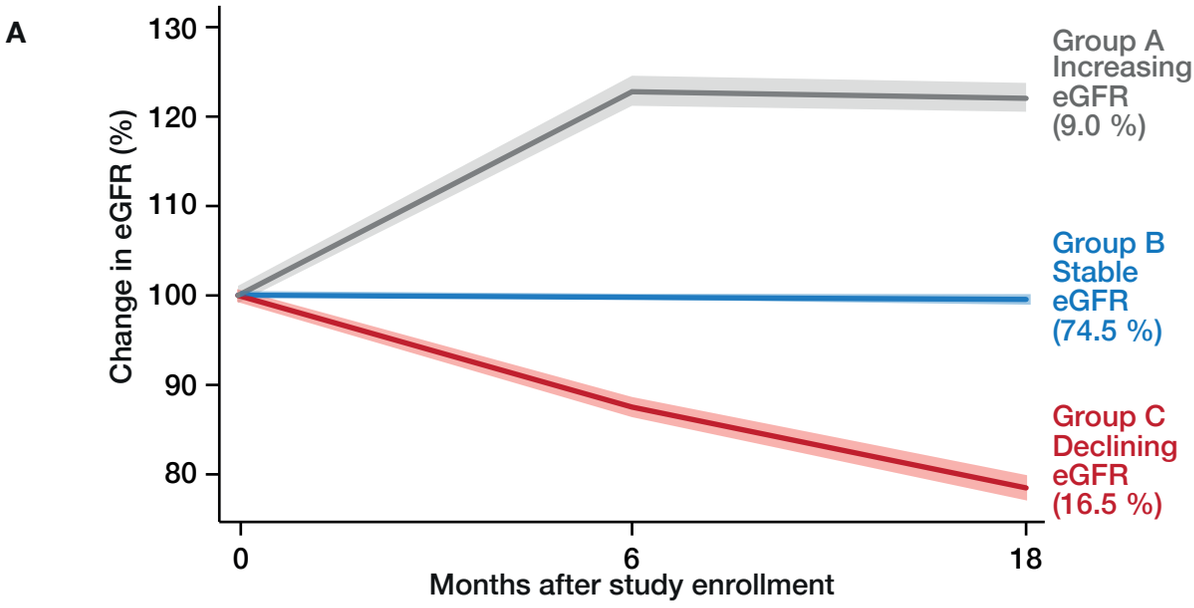
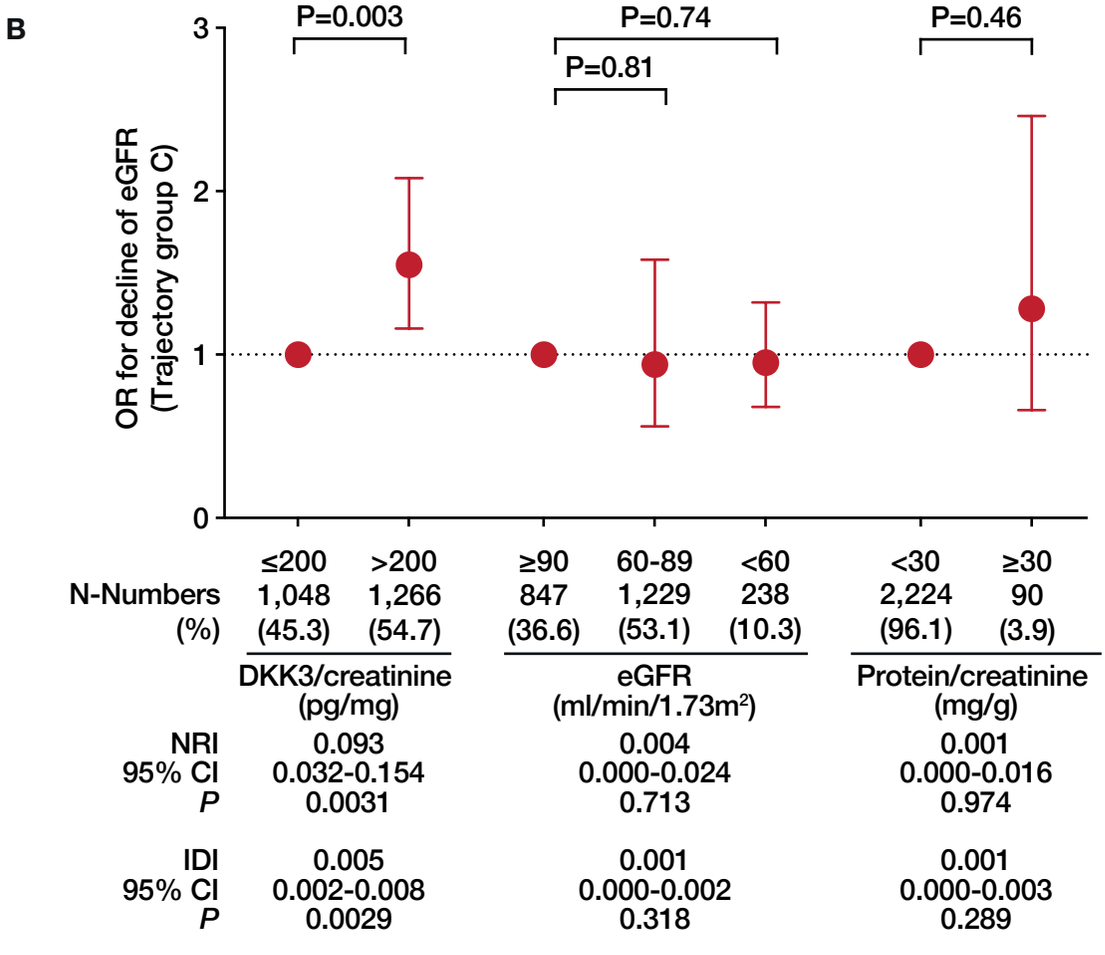
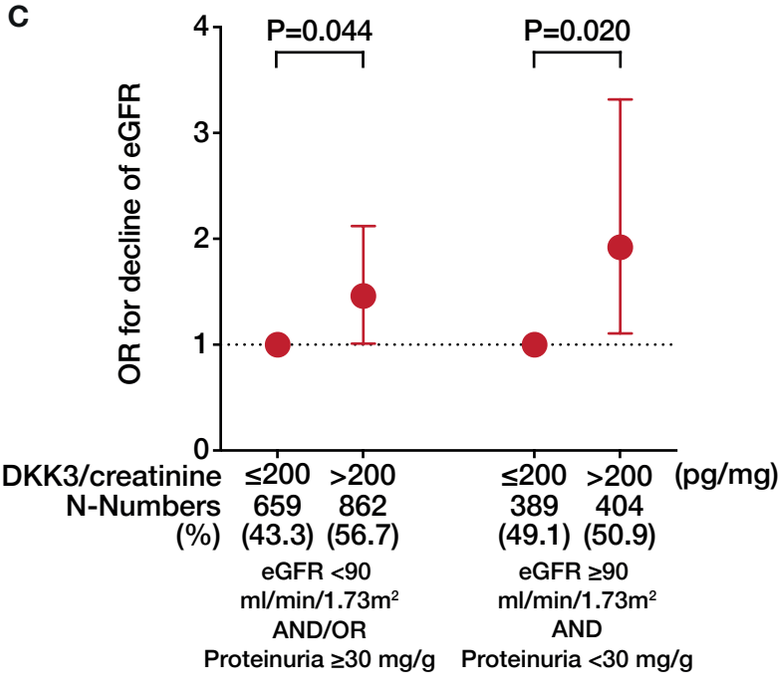

COSYCONET
study
N=2,741
Missing baseline
urine samples
N=427
6 months
18 months
36 months
54 months
Follow-up
Present study
N=2,314
DKK3
eGFR
Proteinuria
FEV1
6-MWD
eGFR
FEV1
6-MWD
N=1,997
eGFR
FEV1
6-MWD
N=1,707
FEV1
6-MWD
N=1,006
FEV1
6-MWD
N=268

Figure 2



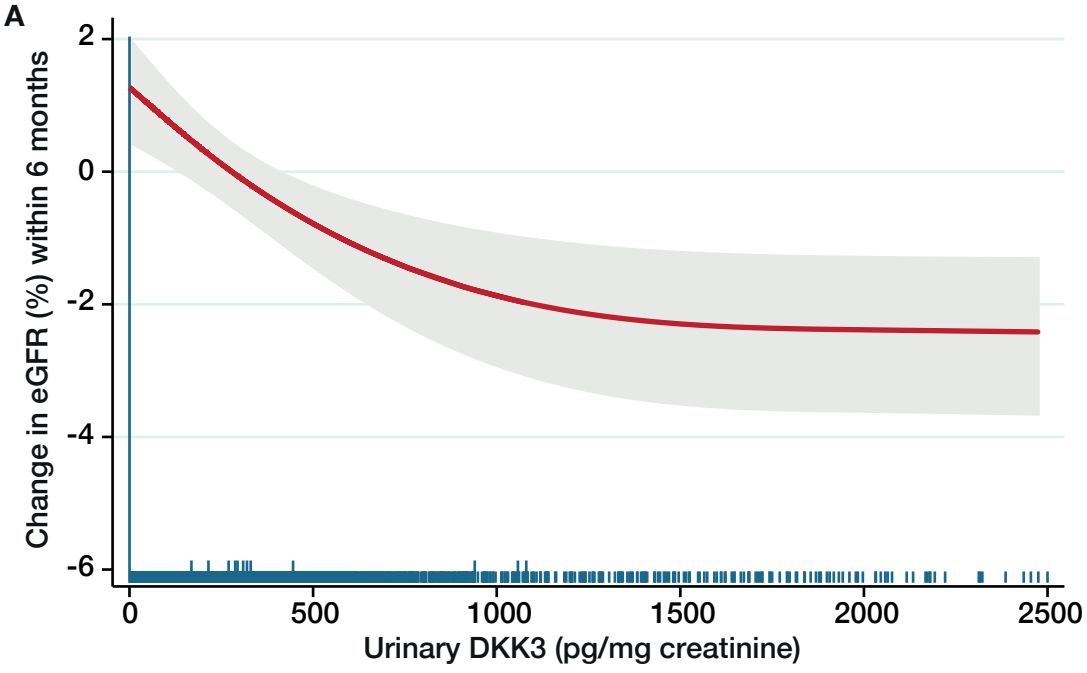


	≤200	>200	≥90	60-89	<60	<30	≥30
N-Numbers	1,048	1,266	847	1,229	238	2,224	90
(%)	(45.3)	(54.7)	(36.6)	(53.1)	(10.3)	(96.1)	(3.9)
	DKK3/creatinine (pg/mg)		eGFR (ml/min/1.73m²)			Protein/creatinine (mg/g)	
NRI	0.093		0.004			0.001	
95% CI	0.032-0.154		0.000-0.024			0.000-0.016	
P	0.0031		0.713			0.974	
IDI	0.005		0.001			0.001	
95% CI	0.002-0.008		0.000-0.002			0.000-0.003	
P	0.0029		0.318			0.289	



DKK3/creatinine (pg/mg)	≤200	>200	≤200	>200
N-Numbers	659	862	389	404
(%)	(43.3)	(56.7)	(49.1)	(50.9)
	eGFR <90 ml/min/1.73m² AND/OR Proteinuria ≥30 mg/g		eGFR ≥90 ml/min/1.73m² AND Proteinuria <30 mg/g	

Figure 3


A
Change in eGFR (%) within 6 months
2
0
-2
-4
-6
0
500
1000
1500
2000
2500
Urinary DKK3 (pg/mg creatinine)

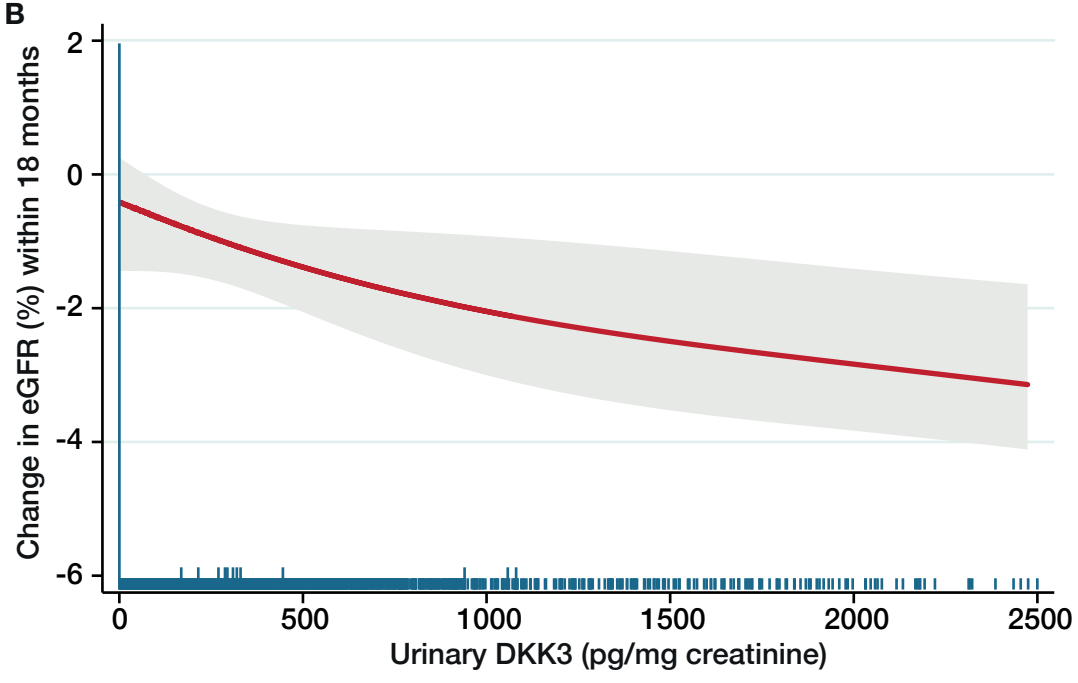
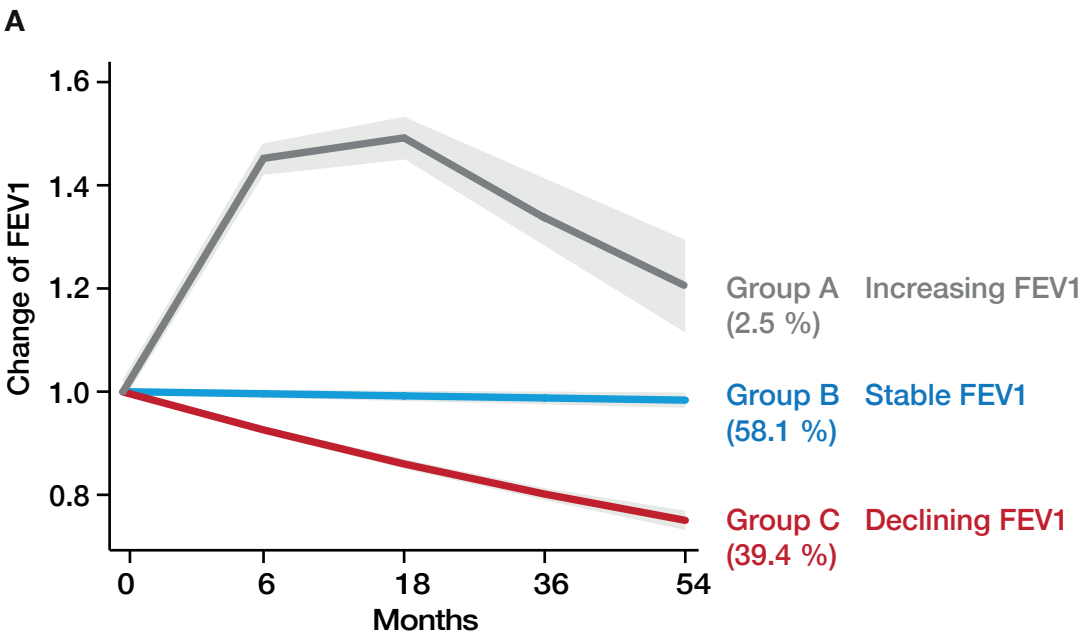
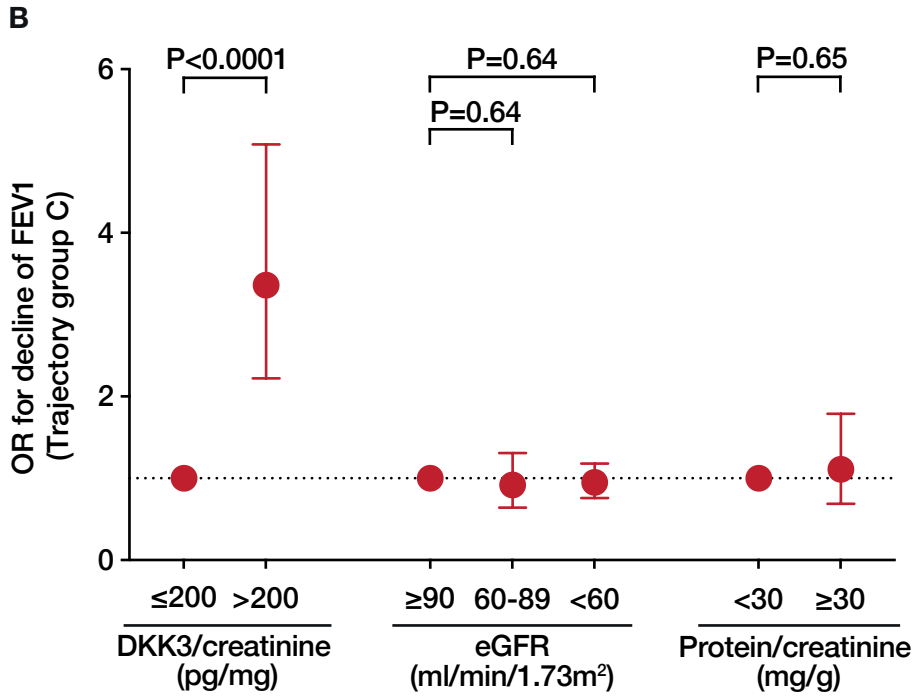
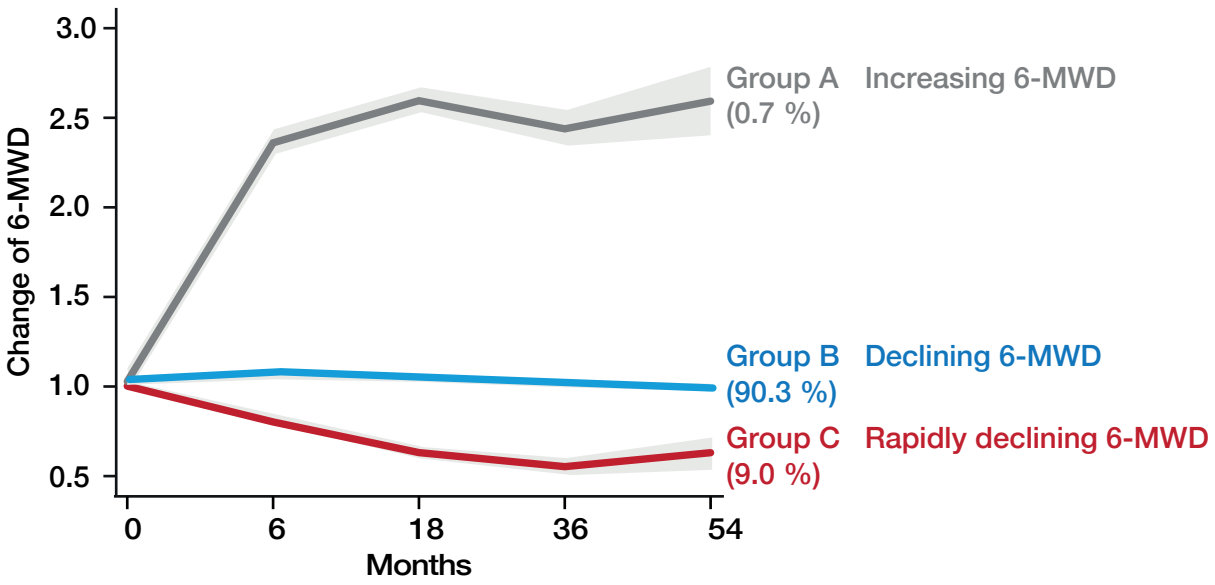

B
Change in eGFR (%) within 18 months
2
0
-2
-4
-6
0
500
1000
1500
2000
2500
Urinary DKK3 (pg/mg creatinine)

Figure 4


A
Change of FEV1
1.6
1.4
1.2
1.0
0.8
0
6
18
36
54
Months
Group A Increasing FEV1
(2.5 %)
Group B Stable FEV1
(58.1 %)
Group C Declining FEV1
(39.4 %)


B
OR for decline of FEV1
(Trajectory group C)
6
4
2
0
P<0.0001
P=0.64
P=0.64
P=0.65
≤200 >200
DKK3/creatinine
(pg/mg)
≥90 60-89 <60
eGFR
(ml/min/1.73m²)
<30 ≥30
Protein/creatinine
(mg/g)


C
Change of 6-MWD
3.0
2.5
2.0
1.5
1.0
0.5
0
6
18
36
54
Months
Group A Increasing 6-MWD
(0.7 %)
Group B Declining 6-MWD
(90.3 %)
Group C Rapidly declining 6-MWD
(9.0 %)

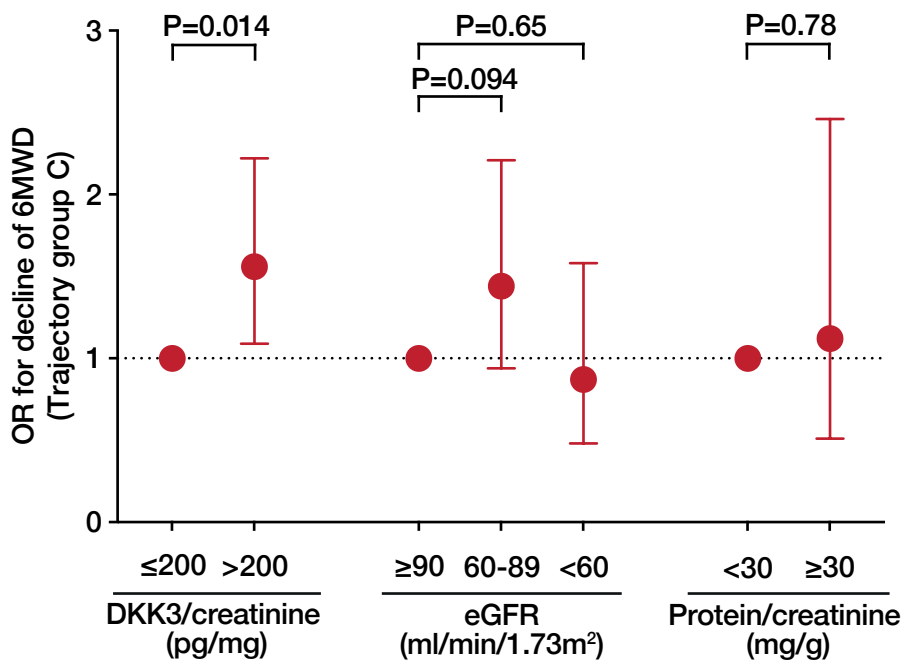
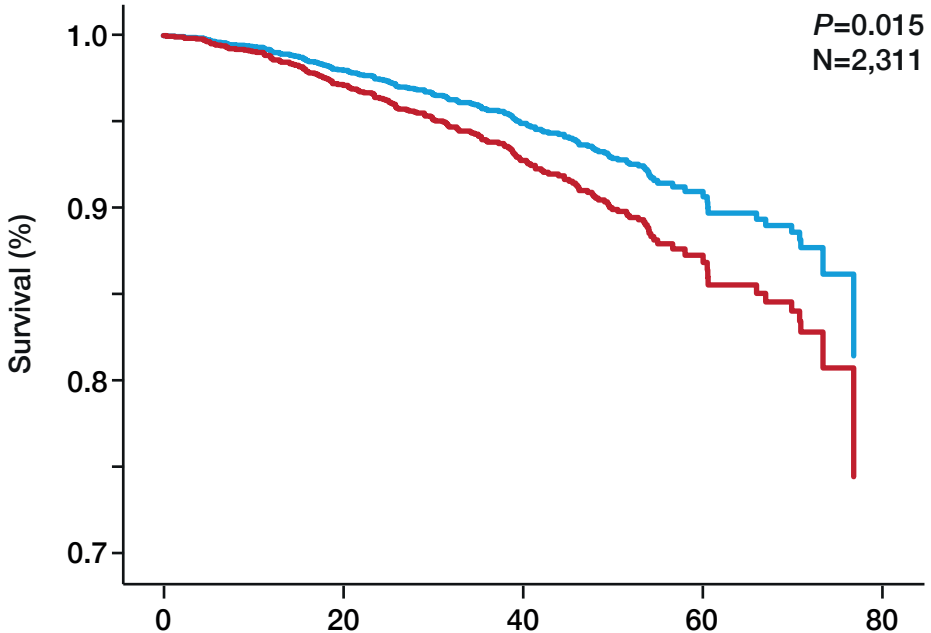

D
OR for decline of 6MWD
(Trajectory group C)
3
2
1
0
P=0.014
P=0.65
P=0.094
P=0.78
≤200 >200
DKK3/creatinine
(pg/mg)
≥90 60-89 <60
eGFR
(ml/min/1.73m²)
<30 ≥30
Protein/creatinine
(mg/g)

Figure 5



No. at risk				
DKK3 ≤200 pg/mL	1,046	791	456	94
DKK3 >200 pg/mL	1,265	971	527	3

DKK3 ≤200 pg/mL
DKK3 >200 pg/mL

Figure 6

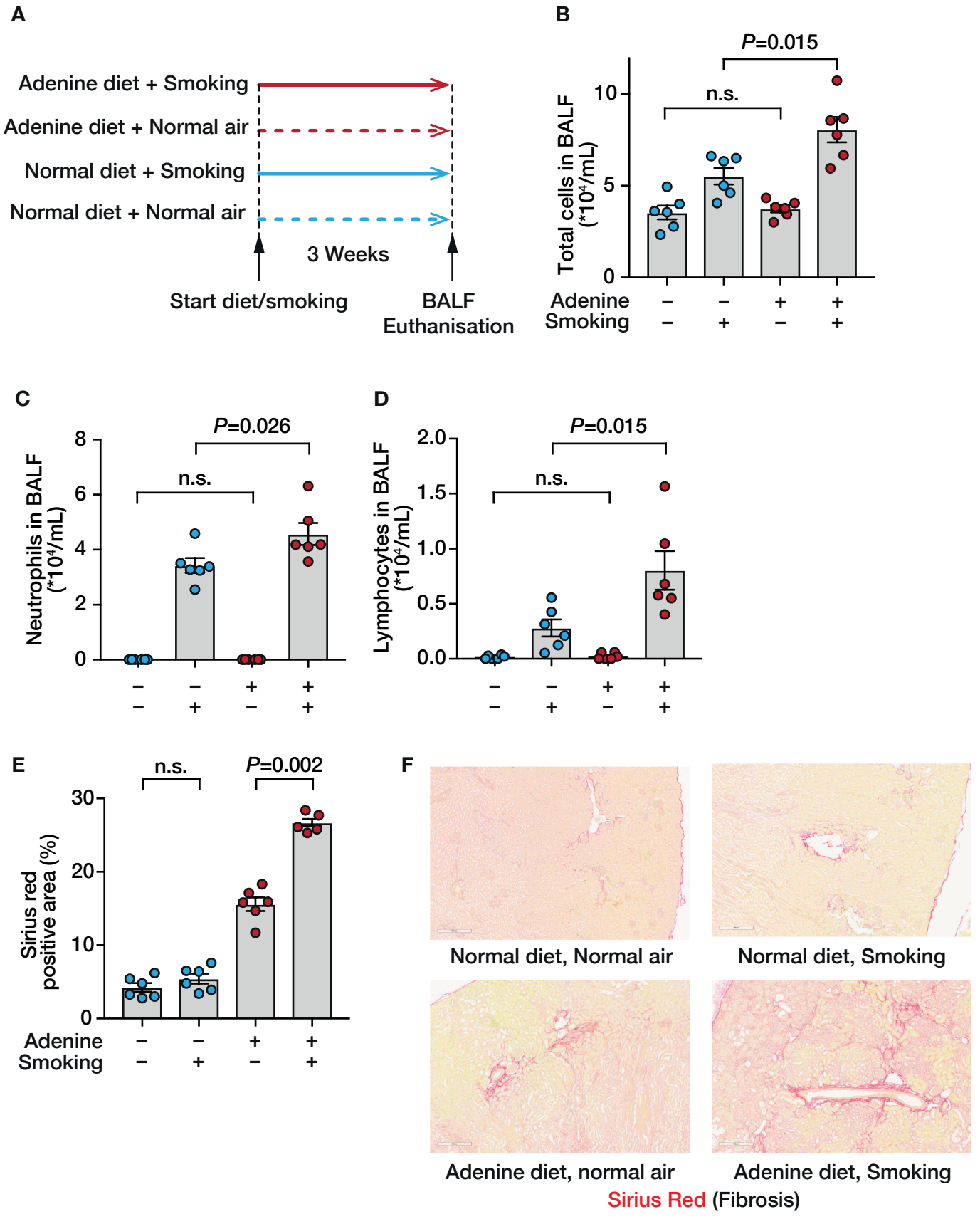
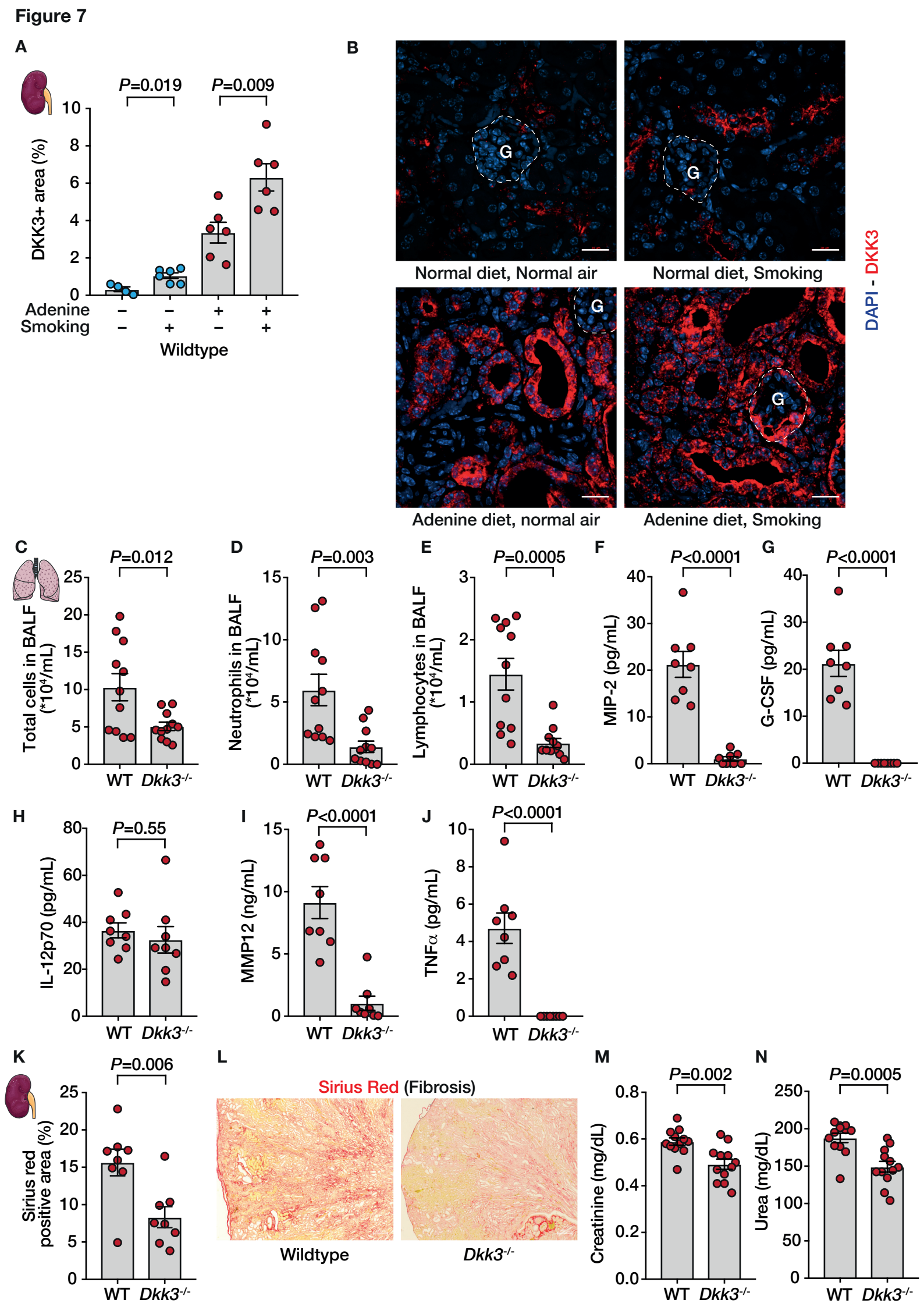

A
Adenine diet + Smoking
Adenine diet + Normal air
Normal diet + Smoking
Normal diet + Normal air
3 Weeks
Start diet/smoking
BALF
Euthanisation
B
P=0.015
n.s.
Total cells in BALF (*10⁴/mL)
10
5
0
Adenine
Smoking
C
P=0.026
n.s.
Neutrophils in BALF (*10⁴/mL)
8
6
4
2
0
D
P=0.015
n.s.
Lymphocytes in BALF (*10⁴/mL)
2.0
1.5
1.0
0.5
0.0
E
n.s.
P=0.002
Sirius red positive area (%)
30
20
10
0
Adenine
Smoking
F
Normal diet, Normal air
Normal diet, Smoking
Adenine diet, normal air
Adenine diet, Smoking
Sirius Red (Fibrosis)

Figure 7


A
P=0.019
P=0.009
DKK3+ area (%)
Adenine
Smoking
Wildtype
B
G
Normal diet, Normal air
Normal diet, Smoking
Adenine diet, normal air
Adenine diet, Smoking
DAPI - DKK3
C
P=0.012
Total cells in BALF (*10⁴/mL)
D
P=0.003
Neutrophils in BALF (*10⁴/mL)
E
P=0.0005
Lymphocytes in BALF (*10⁴/mL)
F
P<0.0001
MIP-2 (pg/mL)
G
P<0.0001
G-CSF (pg/mL)
H
P=0.55
IL-12p70 (pg/mL)
I
P<0.0001
MMP12 (ng/mL)
J
P<0.0001
TNFα (pg/mL)
K
P=0.006
Sirius red positive area (%)
L
Sirius Red (Fibrosis)
Wildtype
Dkk3-/-
M
P=0.002
Creatinine (mg/dL)
N
P=0.0005
Urea (mg/dL)
WT
Dkk3-/-

Supplemental figure 1