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Reduced Levels Of Anti-Ageing Hormone Klotho Predict Renal Function Decline In Type 2 Diabetes

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Context and Objective: Soluble Klotho (sKlotho) is a circulating hormone with cardiovascular-renal protective effects. Whether sKlotho predicts estimated glomerular filtration rate (eGFR) decline in type 2 diabetes mellitus (T2DM) patients with relatively preserved renal function is unknown.

Design, setting, participants and measurements: Single-centre observational follow-up study of 101 patients with T2DM and eGFR >45 ml/min, [91% on renin angiotensin system (RAS) blockade] followed for a median (range) of 9 (2-13) years.

Main Outcome: Primary outcome was a >50% decline in eGFR. sKlotho, serum phosphorus, serum calcium, fibroblast growth factor-23 (FGF-23) levels were measured from stored samples collected at baseline. Patients were followed up with standardized clinical and biochemical measurements.

Results: Patients with residual microalbuminuria (MA) despite RAS blockade (n=53) had significantly lower levels of sKlotho median, interquartile range (IQR), 184.7 (130.5-271.8) pg/ml compared to patients without MA (n=39) pg/ml 235.2 (172.0-289.4) p=0.03.
Of the cohort, 21% reached the primary outcome. A 10% higher sKlotho level reduced the incidence of the primary outcome by 12% (Hazard ratio 0.27, 95% CI 0.15-0.52, p<0.001) independent of traditional risk factors in a competing risk analysis. Patients with sKlotho level below the median of 204.4 pg/ml had nearly a 4 fold higher cumulative incidence of the primary outcome compared to those above the median (24% vs 6.2%, p=0.01).

Conclusions: In T2DM patients with relatively preserved eGFR, reduced levels of sKlotho predict renal function decline independent of traditional risk-markers. sKlotho is a novel bio-marker of renal dysfunction and potential treatment target for renoprotection in T2DM.

We report in a 9 year single centre prospective study that low levels of soluble Klotho are an independent predictor of renal function decline in T2DM patients with preserved renal function. .

Introduction

Klotho is an anti-ageing gene encoding a transmembrane protein that works as an obligate co-receptor for Fibroblast growth factor (FGF)-23 to promote phosphorus excretion (1,2). The cleavage of the extracellular portion of the transmembrane protein produces a circulating hormone, named soluble Klotho (sKlotho), which per se may induce phosphaturia independently of FGF-23 and exert cardio-renal benefits through reduction in oxidative stress and endothelial protection (3). The kidney is the main source of circulating sKlotho (4). In cross-sectional studies sKlotho levels decline with advancing stages of chronic kidney disease (CKD) (5,6). More recent prospective studies suggest that reduced levels of sKlotho predict decline in renal function and cardiovascular mortality in elderly non-diabetic subjects and hemodialysis patients respectively (7). Animal data support the concept that Klotho may be not just a biomarker but a pathogenic factor in CKD progression and cardiovascular complications (8). *In vivo* replacement of sKlotho attenuates renal damage in animal models of kidney disease (9). We have recently described an association between reduced levels of sKlotho and microalbuminuria (MA) in patients with type 1 diabetes (T1DM)(10).

To date there are no long term prospective studies that have evaluated if sKlotho levels predict renal function decline in patients with type 2 diabetes mellitus (T2DM). We therefore studied if sKlotho levels predicted renal function decline in 101 T2DM patients, all with estimated glomerular filtration rate (eGFR) >45 ml/min at baseline, who were followed for a median (range) of 9 years (2-13 years).

Material and Methods

Patients were recruited from the Diabetes Clinic at Guy's and St Thomas' Hospitals (London, UK) from 2004 to 2006. They all provided written informed consent. The study was approved by institutional research ethics committee and undertaken in adherence to the Declaration of Helsinki.

Inclusion criteria were patients with T2DM as per World Health Organization definition, diagnosis and classification of diabetes mellitus criteria (11) and evidence of diabetic kidney disease (history of MA and no evidence of non-diabetic kidney disease) above 40 years of age. History of MA was defined as early morning urine albumin to creatinine ratio >3mg/mmol on at least two occasions. Exclusion criteria were clinical or biochemical evidence of significant renal impairment (eGFR <45 ml/min) or history of non-diabetic kidney disease.

There were no patients on vitamin D or phosphate binders. Patients were followed up with standardized clinical and biochemical measurements as per routine clinical care with annual or bi-annual visits.

Plasma-soluble Klotho (Immuno-Biological-Laboratories, Hamburg, Germany) and plasma C-terminal FGF-23 (Immunotopics Inc., San Clemente, CA) were measured in duplicate by enzyme linked immunoassay (ELISA) from samples stored at -80°C (10,12,13). The intra-assay and interassay coefficients of variation for soluble Klotho and FGF-23 ELISA were 2.7% and 6.5% and 4.4% and 6.1%, respectively. Blood samples were immediately centrifuged at 1500*g* at 4°C for 10 min and the supernatant fractions were stored at -80°C (for <24 months) with no freeze–thaw cycles before analysis. This approach has been shown not to impact on the sensitivity of the assay used in this study (12). Serum phosphate was measured in duplicate by spectrophotometry (Pointe Scientific Inc., Canton, MI)(10). Urine Albumin concentration was measured by immunoturbidimetry using a Cobas Miras Plus analyzer (Roche Diagnostics, Basel, Switzerland) from three timed overnight urine collections and median albumin excretion rate (AER) calculated. Serum total cholesterol (enzymatic colorimetry) and creatinine levels were measured using a Cobas Mira Plus analyser (Roche Diagnostics, Rotkreuz, Switzerland)(14) HbA_{1c} was measured by boronate affinity HPLC (Primus CLC330, Kansas City, MO, USA). eGFR was determined using the Chronic Kidney Disease Epidemiology Collaboration Formula (CKD-EPI) (15).

Statistical Analysis

Descriptive statistics were used for the analysis of demographic and clinical features of the cohort. AER and sKlotho levels were log-transformed before calculation because of their positively skewed distribution. Between groups differences were compared by unpaired t test (for continuous parametrically distributed variables) and Mann-Whitney test (for continuous non-parametrically distributed variables). Chi-square test was used to compare categorical variables between groups. The primary outcome was defined as the time to event of >50% fall in eGFR from baseline. As death precludes us from observing decline in kidney function we considered death from any cause to be a competing event in our cohort and fitted a cause specific Cox proportional hazards model (16) as well as a sub-distribution hazards model to adjust for death as a competing risk event (17-19). By following the sub-distribution hazards

approach we are able to relate the covariate effect directly to and calculate the cumulative incidence function (CIF) for each of the events (17-19). The CIF allows for estimation of the incidence of the occurrence of an event while taking competing risk into account. For each patient a linear regression model of time on eGFR (least-squares method) was created and the slope of the regression line was used to estimate the patient's changes of e GFR over time (20). Statistical analyses were performed using SPSS software (version 24; SPSS Inc., Chicago, IL) and R (version 3.4.2 Vienna, Austria) statistical programming language with cmprsk package (version 2.2-7 Vienna, Austria; https://CRAN.R-project.org/package=cmprsk.) for the sub-distribution hazards analysis.

Results

Baseline clinical and demographic characteristics of the cohort are shown in table 1. The mean age (range) was 60 (40-82) years, ~ 60% were male, the mean duration of diabetes (mean ± SD) was 9.8 ± 6.6 years, with a mean eGFR of 90.4 ± 20.0 ml/min. Median (interquartile range) AER was 24.5 (9.00 to 90.25) mcg/min.

Of the 101 patients, 92 (91%) were on renin angiotensin system (RAS) inhibitors (table 2). Of this group 39 (38.6%) patients were normoalbuminuric (AER <20mcg/min) and 53 (52.5%) had raised AER (>20 mcg/min) despite RAS inhibitor use. There were no significant differences in age, duration of diabetes, FGF-23, serum phosphorus, serum calcium, eGFR and HbA1c between patients with normoalbuminuria on RAS treatment as compared to those with raised AER (table 2). However patients with raised AER had significantly higher SBP levels, a raised body mass index (BMI), more frequent use of statins and number of oral hyperglycaemic agents and lower sKlotho levels, as compared to those with normoalbuminuria (table 2). The significant difference in sKlotho levels between the two groups persisted after adjustment for SBP, BMI, eGFR, statin treatment and the use of oral hypoglycemic agents [Odds ratio 0.02, 95% CI 0.001 to 0.36, p=0.007) (table 3). In further analyses we evaluated the relationship between sKlotho and eGFR and AER. sKlotho levels were significantly, albeit modestly, inversely correlated in univariate analyses with baseline AER (Spearman's correlation coefficient -0.245 p=0.01). In contrast no significant correlation between sKlotho and baseline eGFR was observed.

In our cohort, patients in the lower quartile of sKlotho levels had a faster rate of decline in eGFR as compared to those in the higher quartile [median (interquartile range) of -3.3 (-1.73 to -4.48) ml/min/year vs. -1.43 (0.01 to -2.8) ml/min/year, p=0.01].

Of the 101 patients 21% (n=22) reached the primary outcome of a more than 50% decline in eGFR from baseline and 17.8% (n=18) died before reaching a >50% fall in eGFR. Table 4 shows the characteristics of patients above and below the median baseline sKlotho value of 204.4 pg/ml. As compared to patients above the median sKlotho level, a higher degree of albuminuria and lower HbA1c were observed in patients below the median. There were no significant differences in age, gender, SBP, FGF-23, cholesterol, use of RAS blockers and diabetes duration between patients above and below the median value of sKlotho.

Figure 1 shows the estimates for the cumulative incidence function curves for the primary outcome for patients with baseline sKlotho below the median value of 204.4 pg/ml versus those above the median. The cumulative incidence for those below the median approached 24% and for those above the median baseline sKlotho levels reached 6.2% after 10 years of follow-up.

The end of study mean eGFR for the whole cohort was 68.5±28.5 ml/min. In patients who had a fall in eGFR of >50% from baseline, the mean end of study eGFR was 30.3±13.0 ml/min as compared to 79.1±21.7 ml/min in those without >50% fall in eGFR (p=0.001).

We fitted cause-specific and sub-distribution hazards models for both renal function decline and death before decline in kidney function. For each of the two events we regressed

hazards on baseline covariates (age, gender ,eGFR, SBP, HbA1c, albuminuria, total cholesterol and sKlotho levels). Estimated hazard ratios (HR) and their associated 95% CI are reported in table 5. Baseline sKlotho did not predict risk of death before reaching the primary outcome of renal function decline in both the cause-specific and sub-distribution hazards models. A one unit increase in the log-transformed sKlotho decreased the relative incidence of the event by 72% (HR 0.28, 95%CI, 0.15-0.52, p<0.001) when adjusted for multiple risk factors (table 5). This indicates that a 10% increase in the baseline levels of sKlotho results in a 12% decrease in the relative incidence of the primary outcome.

Discussion

In this prospective study we report the novel finding that sKlotho is an independent predictor of >50% decline in eGFR in T2DM patients with relatively preserved renal function. This effect of sKlotho was independent of FGF-23, calcium and phosphate levels and traditional renal risk factors. We also observed that patients with residual albuminuria despite RAS blockade had significantly lower levels of sKlotho as compared to patients with normoalbuminuria on RAS, a finding which has not been described previously. Moreover patients in the lowest quartile of sKlotho had a 1.8 ml/min/year faster annual rate of eGFR decline as compared to patients with the highest levels of soluble Klotho. We also observed that patients with sKlotho level below the median for the group had a nearly 4 times higher incidence of loss of >50% of their renal function during 10 year follow up.

Numerous cross-sectional studies, in predominantly non-diabetic cohorts, have reported inverse association between sKlotho levels and eGFR. In a study with 87 patients (6 with diabetes) with CKD stages from 1 to 5, sKlotho levels were inversely associated with eGFR (5).

More recently in a prospective study of 2496 elderly subjects (37% with diabetes), with a mean baseline eGFR of 73 ml/min**,** doubling of sKlotho levels was independently associated with reduced risk of fall in renal function (defined as a >30% reduction in eGFR from baseline) (7).

In a study by Lee et al, patients with T2DM and preserved renal function **(**mean eGFR >90 ml/min) had significantly lower levels of sKlotho compared to non-diabetic controls. The authors reported that sKlotho levels were inversely related to degree of albuminuria however none of the patients studied were on RAS inhibition (21). Of this cohort 109 patients were followed up for a median 34 months and the authors observed a negative correlation between sKlotho levels and decline in eGFR (22).

In another cross-sectional study of T2DM patients with CKD stages 1 to 4 an inverse relationship between sKlotho levels and degree of albuminuria was observed (23). However the concurrent use of RAS inhibitors was not reported. Interestingly in the same cohort no correlation was found between sKlotho and FGF-23 or other measures of mineral/bone metabolism (24).

In a recent study we observed that T1DM patients with microalbuminuria (all on RAS inhibitors) had lower levels of sKlotho as compared to normoalbuminuric patients of similar long duration of diabetes who were not on any other anti-hypertensive medications (10). Further the significant difference in sKlotho levels we observed between the two groups was independent of levels of vitamin D and parathyroid hormone .

There are limited prospective studies that have evaluated the role of sKlotho in patients with CKD. In a post-hoc analysis of a prospective study of 243 patients with CKD 1 to 5 (30% with eGFR<30)**,** predominantly due to glomerulonephritis (64%) and with only 12% with diabetic kidney disease, lower levels sKlotho independently predicted the composite outcome (doubling of serum creatinine, onset of renal end stage disease or death) after adjustment for age, diabetes, blood pressure, eGFR, parathyroid hormone and FGF-23 (25).

Of note in this cohort only 69% were on RAS blockade. In a more recent study in 769 haemodialysis patients (31% with diabetes) followed for 2 years patients with raised sKlotho levels had a 14% reduced occurrence of cardiovascular events and cardiovascular death compared with those with lower levels (26).

The potential mechanism by which sKlotho exerts cardio-renal protective effects remains unclear. In rodents, Klotho deficiency is associated with kidney fibrosis and vascular calcification (6, 9, 26). Conversely sKlotho replacement reverses or attenuates the kidney damage, ameliorates endothelial dysfunction and prevents the development of vascular calcification (9). In animal studies overexpression of the Klotho gene reduces oxidative stress, renal cell hypertrophy, inflammation and apoptosis (27,28). Activation of the RAS has been proposed as the main pathological mechanism leading to reduction in Klotho expression through increased oxidative stress (27,28). We have previously demonstrated that RAS blockade increases the levels of sKlotho in patients with T2DM (14).

Our study has several limitations. The cohort of patients we studied was relatively small as we excluded subjects with more severe renal impairment which is known to affect sKlotho levels. Larger studies are required to confirm our findings and our results establish the rationale and for such future studies. We could not measure vitamin D or parathyroid hormone levels as we did not have sufficient volumes in our stored samples for these analyses, however sKlotho can exert its effects independent of these variables and changes in Klotho occur prior to clinically relevant alterations in these markers (29-31). The baseline blood pressure of the cohort was not optimal and reflects the challenging clinical patients referred to a hospital clinical service. Of interest despite similar blood pressure control, patients with residual albuminuria on RAS, had significantly lower levels of sKlotho as compared to those without albuminuria. The exact mechanisms by which sKlotho protects against progression of renal function decline are unclear. Our study was not designed to evaluate putative mechanisms by which sKlotho provides renoprotection. However we can speculate based on data from animal studies that sKlotho, a multi-faceted protein, may have direct renoprotective effects modulated via multiple pathways which may be independent of its traditional role in phosphate balance such as actions on endothelial dysfunction and oxidative stress and enhancing anti-inflammatory pathways that are all relevant to driving the progression of diabetic renal disease (28,32-35).

The strengths of our study are that all the patients had relatively preserved renal function (eGFR >45 ml/min), and were a well characterised cohort attending a single centre for their diabetes care over a long follow up period. Our primary outcome of a >50% decline in eGFR is a robust and validated definition of clinically significant renal function decline (36,37). The final mean eGFR of those with progression of renal disease was ~ 30 ml/min, which makes the observed changes in renal function being due to resolution of hyperfiltration unlikely.

Our results suggest that a 10% higher sKlotho level reduces the incidence of the primary outcome by 12% an effect independent of traditional risk factors. We performed competing risk analyses using recommended methods (38,39), which demonstrated a consistent and significant independent effect of sKlotho on the primary renal outcome. More than 90% of our patients were on RAS blockade and the effects we observed were independent of other markers and predictors of renal disease progression. We did not observe any impact of FGF-23 or serum phosphate levels on progression of renal disease. This would suggest that changes in sKlotho may precede alterations in FGF-23 and phosphate and that sKlotho is the likely primary driver of progression of renal dysfunction. This hypothesis is supported by elegant animal studies which demonstrate that sKlotho may induce phosphaturia by FGF-23 independent mechanisms (30,40). However as sKlotho has a reno-protective influences via multiple biological pathways the effects we observed may be independent of its role in phosphate balance (3,8).

In conclusion we have demonstrated that in a clinic cohort of T2DM patients with relatively preserved renal function lower levels of sKlotho are associated with residual albuminuria and faster progression of renal function decline. Our results complement the animal and in-vitro data that indicate sKlotho may be a potential treatment target to delay progression of renal dysfunction and support the emerging role of sKlotho as a potential novel bio-marker and predictor of renal disease in diabetes.

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Author contributions.

GM, and JK had the original idea and designed the study. GM, NF and JK wrote the manuscript. NF collected and analysed the data. LG reviewed and commented on the manuscript.

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Disclosure.
The authors reports no conflicts of interest in this work.

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Figure 1 Legend. Estimates of the effect of sKlotho on the cumulative incidence curves of risk for primary outcome of >50% eGFR decline from baseline in 101 T2DM patients with relatively preserved renal function at baseline.

Table 1. Baseline demographic, clinical and laboratory characteristics of 101 patients with T2DM and relatively preserved renal function.

Age (years)*	60 (40-82)
Gender (male %)	59
Diabetes duration (years)**	9.0 (4.9-13.0)
RAS inhibitor treatment (%)	91
Statin use (%)	70
Oral antidiabetic medications (%)	88
Insulin treatment (%)	37
eGFR (ml/min)	90.7±20.0
SBP (mmHg)	157.4±11.1
DBP (mmHg)	81.4±9.5
AER**(μg/min)	24.5 (9.0-90.2)
BMI (kg/m^2)	31.1±5.5
HbA1c (%)	7.4±1.2
Serum Calcium**(mg/dl)	9.4(9.2-9.7)
Serum Phosphate**(mg/dL)	3.2(2.8-3.6)
FGF-23** (RU/ml)	16.5 (11.0 -22.9)
sKlotho**(pg/ml)	204.4(156.8-281.6)

Abbreviations : SBP=systolic blood pressure,DBP=diastolic blood pressure, AER=albumin excretion rate,BMI=body mass index , RAS=renin angiotensin/aldosterone system, FGF-23 = fibroblast growth factor 23 *mean (range) **median (inter quartile range)

Table 2. Baseline demographic, clinical and laboratory characteristics of 92 T2DM patients with and without residual albuminuria despite RAS inhibitor treatment.

Variable	Residual Albuminuria (n=53)	Normoalbuminuria (n=39)	p-value
Age(years)	59.7±9.4	61.5±9.3	0.40
Gender (male %)	66	54	0.20
Diabetes duration * (years)	9.0(4.6-11.6)	9.9 (5.0-14.1)	0.3
Statin use (%)	77	56	0.03
Oral antidiabetic agents (%)	96	82	0.03
eGFR (ml/min)	90.2±20.3	88.7±17.3	0.70
SBP (mmHg)	159.7±10.7	155.1±11.8	0.06
DBP (mmHg)	82.4±10.9	79.8±7.3	0.20
BMI (kg/m^2)	32.0±5.2	29.6±5.2	0.04
HbA1c (%)	7.5±1.1	7.3±1.3	0.40
FGF-23** (RU/ml)	12.1 (9.8-24.0)	19.2 (15.4-22.5)	0.16
sKlotho **(pg/ml)	184.7 (130.5-271.8)	235.2 (172.0-289.4)	0.03

Abbreviations : SBP=systolic blood pressure,DBP=diastolic blood pressure, AER=albumin excretion rate,BMI=body mass index , FGF-23 = fibroblast growth factor 23 *mean (range) **median (inter quartile range)

Table 3. Multivariate logistic regression analysis of the relationship between sKlotho and residual microalbuminuria in patients with T2DM on RAS inhibitor treatment

	OR	95% CI	p-value
Unadjusted model	0.07	0.007 to 0.72	0.03
Model adjusted for SBP	0.08	0.008 to 0.84	0.04
Model adjusted for SBP and BMI	0.06	0.005 to 0.75	0.03
Model adjusted for SBP,BMI ,eGFR, statin, and oral antidiabetic medication treatment	0.02	0.001 to 0.36	0.007

Abbreviations: SBP=systolic blood pressure,BMI=body mass index.eGFR= estimated glomerular filtration rate. and sKlotho was log-transformed

Table 4. Comparison of baseline demographic, clinical and biochemical characteristics of 101 T2DM patients above and below median sKlotho level

	Above sKlotho median (n=51)	Below sKlotho median (n=50)	p-value
Age (years)	58.8±9.6	61.9±9.0	0.10
Gender (male %)	63	56	0.49
Diabetes duration (years)	9.5 ()	10.2 ()	0.62
Statin use (%)	60.7%	74%	0.15
eGFR (ml/min)	89.2±16.1	91.79±22.8	0.53
SBP (mmHg)	156.5±10.7	158.3±11.4	0.41
DBP (mmHg)	82.9±10.2	79.9±8.9	0.13
AER** (mcg/min)	19.0(6.0-45.0)	36.5(10.7-120.2)	0.06
BMI (kg/m^2)	31.9±5.3	30.3±5.5	0.13
FGF-23** (RU/ml)	18.5(12.9-23.8)	14.0 (10.4-20.8)	0.12
HbA1c (%)	7.7±1.3	7.2±1.0	0.03

Abbreviations : SBP=systolic blood pressure,DBP=diastolic blood pressure,AER=albumin excretion rate, BMI=body mass index, mean (range) FGF-23 = fibroblast growth factor 23 **median (inter quartile range)

Table 5. Hazard ratios and 95% confidence intervals from cause-specific and sub-distribution hazard models for renal function decline (>50% eGFR decline from baseline) and death before renal function decline in 101 T2DM patients with relatively preserved renal function at baseline.

Competing risk		Covariate	HR	95%CI	p-value
GFR decline	Cause specific model	Age	0.99	0.95-1.05	0.9
		Gender	1.33	0.46-3.81	0.6
		sKlotho	0.20	0.08-0.52	0.001
		SBP	0.98	0.93-1.03	0.43
		Albuminuria	1.55	0.90-2.66	0.10
	Sub-distribution hazard	Age	0.98	0.95-1.02	0.5
		Gender	0.70	0.27-1.83	0.5
		GFR	1.00	0.98-1.02	0.8
		sKlotho	0.28	0.15-0.52	<0.001
		SBP	0.99	0.95-1.03	0.9
		Albuminuria	1.28	0.80-2.05	0.3
Death	Cause specific model	Age	1.09	1.02-1.16	0.008
		Gender	0.62	0.22-1.91	0.4
		GFR	0.99	0.97-1.02	0.8
		sKlotho	0.41	0.11-1.46	0.17
		SBP	0.96	0.91-1.01	0.12
		Albuminuria	1.12	0.65-1.92	0.7
	Sub-distribution hazard	Age	1.08	1.03-1.15	0.03
		Gender	1.71	0.56-5.24	0.3
		GFR	0.99	0.97-1.02	0.8
		sKlotho	0.55	0.17-1.71	0.3
		SBP	0.97	0.91-1.02	0.2
		Albuminuria	1.10	0.61-1.66	0.6

Abbreviations: SBP=systolic blood pressure, eGFR =estimated glomerular filtration rate. sKlotho and albuminuria were log transformed