Supplementary Appendix

Supplement to Melsom T et al. Sex differences in age-related loss of kidney function.

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Supplementary Methods

Using age as the time variable - Convergence

This investigation of longitudinal data from RENIS aims to estimate GFR change rates. Because the cohort included persons at different ages at baseline, the observed GFR changes will reflect both cross-sectional age differences and longitudinal age changes, i.e. changes in GFR both within and between persons. The models used in the investigation assume that the cross-sectional age differences and longitudinal age changes converge onto a common trajectory.

Sliwinski et al provide a method for checking if this assumption is met.[1] The method estimates the parameter ω, which is the mixing weight that controls the relative contribution of the cross-sectional and longitudinal age slope (to the estimation of the age slope). The higher the value of ω, the more the age slope would reflect the cross-sectional information about between-person age differences, and the lower the value of ω the more the age slope would reflect the longitudinal information about age changes. A cross-sectional study corresponds to $\omega = 1$ and an age-homogenous longitudinal study to $\omega = 0$. $\omega < 0.20$ indicates that the estimated age slope primarily reflects longitudinal information.

With Sliwinski et al's equations,[1] we estimated the convergence age slope in the RENIS cohort at -1.06 mL/min/1.73 m^2/year (95% CI -1.11 to -1.00 mL/min/1.73 m^2/year), the between-person age difference at -0.95 mL/min/1.73 m^2/year (95% CI -1.07 to -0.83 mL/min/1.73 m^2/year) and the within-person change rate at -1.08 mL/min/1.73 m^2/year (95% CI -1.14 -1.02 mL/min/1.73 m^2/year). The difference between the two last estimates was 0.13 mL/min/1.73 m^2/year (95% CI -0.00 to 0.26 mL/min/1.73 m^2/year), i.e. not statistically different from zero.

Based on these estimates, we calculated ω in the present study at 0.18, which means that the GFR change rates in models with chronological age as the time variable primarily reflects the within-person GFR change rate. We included chronological age at baseline as the independent variable in all models to adjust for the between-person age differences in GFR and make the estimated GFR change rates reflect within-person changes, as recommended by Sliwinski et al.[1]

Power calculations

Software for power calculations of GAMMs is not readily available. Accordingly, a power calculation for the hypothesis that sex has a statistically significant effect on the GFR decline rate was explored by simulation of 2000 iterations in a linear mixed model without non-linear functions. The parameters for the simulation were taken from the results of a linear mixed model of RENIS baseline and follow-up data. Variance parameters for a model including the most important predictors of GFR decline rate were used to assess the possibility of a new dichotomous predictor to detect an effect on the remaining inter-individual variation of the GFR decline rate with an expected sample size of 1550 persons. The power of detecting an effect of -0.13 mL/min/year or lower of the predictor with negligible correlation with the other independent variables was calculated. It was found to be 0.88, assuming $\alpha=0.05$. Compared to the mean GFR change rate, the power of the study to detect clinically significant effects was judged sufficient. Since the final number of included persons (N=1384) was lower than expected, the actual power was somewhat lower than in this simulation.

Calibration of the HPLC and LC-MS/MS analyses of serum iohexol

In RENIS-T6 and RENIS-FU, serum iohexol was analyzed by HPLC as described in previous publications.[2,3] In 2017, the Department of Medical Biochemistry at the University Hospital of North Norway replaced HPLC with LC-MS/MS as its standard assay for analyzing serum iohexol, which was subsequently used in RENIS-3 (the method is described below). This made it necessary to establish a calibration equation for the conversion of results between the two methods. Serum samples from the single sample iohexol clearance studies of all the 1324 participants in the Renal Iohexol Clearance Survey Follow-Up (RENIS-FU) in 2013 – 2015 had been stored at -80 °C. A random sample of this material was thawed and reanalyzed with LC/MS concurrently with samples from RENIS-3.

Calculation of sample size for calibration

To calculate the necessary sample size to calibrate between HPLC and LC/MS with Deming regression, we followed the method of Linnet.[4] Although this method was published before Martin developed his as iteratively reweighted general Deming regression, we assume that it is valid for this form of Deming regression as well.[5]

We assumed that the standard deviation (SD) for both the new and old iohexol-analyses were proportional to the values, i.e. that the coefficient of variation (CV) was constant. In our laboratory, the analytical CV during the study periods was 3% in RENIS-T6 and 3.1% in RENIS-FU.[3] The range of the iohexol values in both studies combined was 24 to 165 mg/L. However, the distribution was skewed, and 98% of the observations were located in the interval of 32 to 96 mg/L.

Basing our calibration on a sample from this interval, we obtained a range ratio (maximum divided by minimum observed original iohexol measurement) of 3. A higher range ratio requires a lower sample size to detect a deviation with the same power. We wanted to detect an intercept less than 2.0 and an absolute slope difference of greater than 0.025 (arbitrarily chosen low values). The midpoint of the interval of interest was 64 mg/L. This gives a delta-intercept of (2.0/(0.03 x 64))=1.04; and a delta-slope of (0.025/0.03)= 0.83. Interpolating in Linnet's Table 2 with these parameters gave a necessary sample size of roughly 200. Because the distribution was normal and not uniform, this was multiplied by a factor of 1.3 to 1.5 to obtain the correct sample size for the slope, giving a total of 260 to 300. Because a precise estimate of the parameters was essential, we chose a sample size of 300. These were sampled randomly among all RENIS-FU-samples with iohexol-values in the interval 32 to 96.

Calibration equation

Sufficient serum for analysis with LC/MS was found for 287 of the 300 randomly selected participants. A scatterplot of HPLC vs. LC/MS results identified four extreme outliers which were excluded from the analysis.

Iohexol measured in RENIS-FU with HPLC was used as the dependent variable and iohexol measured in RENIS-3 was used as the independent variable in Deming regression in STATA 15. Log-transformation of the variables was found to give the best fit. The ratio of measurement error variances for the two variables was set at 1. The result of the Deming regression was:

Deming regression   Number of obs = 283

	Mean	Std. Dev.
liohexol_f~1	3.9208	.19928
liohe~202004	3.8201	.19599

Variance ratio = 1
Root MSE = .0327593

	Coefficient	Jackknife std. err.	t	P>|t|	[95% conf. interval]	
liohexol202004	1.017258	.015119	67.28	0.000	.9874976	1.047018
_cons	.0348231	.0582042	0.60	0.550	-.0797468	.149393

Accordingly, the calibration equation was:

$$iohexol_{RENIS-FU} = e^{0.035} \times iohexol_{RENIS-3}{}^{1.017}$$

This equation was used to calibrate serum iohexol measured in RENIS-3 to RENIS-FU. We have previously performed a similar calibration of iohexol measured at baseline in RENIS-T6 to RENIS-FU.[3] This makes iohexol measured across all three rounds of RENIS comparable.

LC-MS/MS measurement of iohexol

Chemicals and solutions

Iohexol, Iohexol-d5, and iohexol for quality controls (QCs) were obtained from Toronto Research Chemicals Inc. (Ontario, Canada) and TCI Chemicals (Tokyo, Japan). LC-MS grade methanol was purchased from Honeywell™ Riedel-de Häen™ (Seelze, Germany) and LC-MS grade formic acid from Fluka (Sigma-Aldrich, St. Louis, MO). Ultrapure water (18.2 MΩ) was obtained from a Millipore Advantage Milli-Q system (Millipore SAS, Molsheim, France).

Determination of iohexol in human serum

We prepared two stock solutions of iohexol in methanol and stored them at -30 °C. A 6-point calibration curve and two QCs for iohexol were constructed in drug-free serum (1-240 mg/l) and a Tecan Freedom Evo 200 (Männedorf, Switzerland) liquid handling workstation was used for sample preparation. We prepared the calibrators, QCs, and samples (50 µL) by adding 50 µl internal standard (aqueous iohexol-d5, 3.3mg/L) in a 96-well MegaBlock® 1.2 mL, PP, (Sarstedt, Germany). 0.5 mL of ice-cold methanol was added to each of the wells. The plate was mixed on a Bioshake (Quantifoil Instruments, Jena, Germany) at 1500 rpm for 3 min and centrifuged at 240 x g for 8 min (Hettich Rotina 320R, Tuttlingen, Germany). Then, 100 µl of the supernatant was transferred to a 96-well collection plate (Waters, Milford, MA). After sealing of the plate, 0.1 µl of the supernatant was analyzed by LC-MS/MS using a Waters Acquity UPLC I-Class FTN system with an autosampler and a binary solvent delivery system (Waters, Milford, MA) interfaced to Waters Xevo TQ-S benchtop tandem quadrupole mass spectrometer (Waters, Manchester, UK). The chromatography was performed on a 2.1 x 100 mm Waters Acquity Cortecs® T3, 1.6 µm column. Eluent A consisted of 0.1% formic acid in water and eluent B of 0.1% formic acid in methanol. Gradient elution was performed with 2% B at the start and had a linear increase to 60% B until 0.6 min, a linear increase to 98% B until 1.5 min, and re-equilibration until 2.7 min with 1% B. The flow rate was 0.3 mL/min and the column temperature was maintained at 50 °C. The mass spectrometer was run in positive electrospray ion mode and the spray voltage was set to 0.9 kV. The system was controlled by MassLynx version 4.1 software. The desolvation gas temperature was 500 °C, the source temperature was 150 °C, desolvation gas flow was 1000 L/h, the cone gas flow was 150 L/h, and the collision gas pressure was 4 x 10-3 mBar. For quantitative analysis of iohexol we used the following multiple reaction monitoring (MRM) transitions (bold

transitions are qualifiers): m/z 821.9->803.8/**602.4** and 826.9->808.8/**607.5** (iohexol and iohexol-d5).

Precision and accuracy

The method was validated and found to be linear from 1.5 to at least 240 mg/L ($r2 > 0.999$). The lower limit of quantification was 0.5 mg/L (0.1 µl injection volume). The coefficient of variation (CV) for intraday precision was 2.8 % calculated by assaying three samples (low, medium, and high concentration) six times on the same day. Accuracy for recovery test was 91.1-107.9 % (9 levels, $n = 3$ for each). Between-day CV for iohexol was 5.4% on four consecutive days. The quality is assured through the Equalis external quality assessment program for iohexol four times a year.

Table S1. Characteristics of all persons invited to the Renal Iohexol Clearance Survey (RENIS) and of persons actually included in each of its three waves as registered in the main part of the sixth Tromsø Study (before RENIS baseline).

	RENIS-T6			RENIS-FU			RENIS-3			All invited persons	
			P-value			P-value			P-value		
N	1627	(100)		1324	(100)		1384	(100)		2825	(100)
Age (SD), years	57.8	(3.8)	<0.001	57.7	(3.9)	<0.001	57.9	(3.9)	0.08	58.0	(3.9)
Sex, men	801	(49)	<0.001	657	(50)	<0.001	640	(46)	0.4	1283	(45)
Body mass index (SD), kg/m2	26.9	(4.0)	0,002	26.9	(3.9)	0.2	26.8	(3.9)	0.3	26.7	(4.1)
Current smoking, n (%)	345	(21)	0.6	255	(19)	0.01	265	(19)	0.002	609	(22)
Systolic BP (SD), mmHg	134.5	(20.4)	0.1	134.1	(20.0)	0.7	133.6	(20.0)	0.4	134.0	(20.1)
Diastolic BP (SD), mmHg	79.7	(10.6)	<0.001	79.6	(10.6)	0.006	79.1	(10.7)	0.9	79.1	(10.6)
Use of antihypertensive medication, n (%)	261	(16)	0.08	206	(16)	0.4	208	(15)	0.9	424	(15)
LDL cholesterol (SD), mmol/L	3.8	(0.9)	0.03	3.7	(0.9)	0.02	3.8	(0.9)	0.1	3.8	(0.9)
HDL cholesterol (SD), mmol/L	1.5	(0.4)	0.01	1.6	(0.4)	0.09	1.6	(0.4)	0.5	1.6	(0.4)
Lipid-lowering medication, n (%)	110	(7)	0.2	90	(7)	0.3	90	(7)	0.6	177	(6)
Hemoglobin A1c (SD), mmol/mol	5.6	(0.4)	0.4	5.5	(0.4)	0.02	5.5	(0.4)	0.01	5.6	(0.4)
GFRcrea[a] (SD), mL/min/1.73m2	94.4	(9.8)	0.002	94.3	(9.7)	0.05	93.8	(9.9)	0.6	93.9	(9.9)
GFRcys[a] (SD), mL/min/1.73m2	101.4	(12.6)	0.002	101.6	(12.5)	0.001	101.3	(12.6)	0.02	100.8	(12.6)
GFRcreacys[a] (SD), mL/min/1.73m2	100.0	(11.2)	<0.001	100.0	(11.0)	0.003	99.6	(11.2)	0.2	99.3	(11.2)
Albuminuria (ACR>3.4 mg/mmol)[b], n (%)	24	(1.5)	0.8	19	(1.4)	0.7	13	(0.9)	0.01	43	(1.5)

Values are given as n (%) or mean (standard deviation). P-values are given for tests of difference between included and not included persons in each wave of RENIS. Tests were performed with ANOVA or chi square-test for continuous and dichotomous variables as appropriate. Variables presented in this table were registered in the main part of the sixth Tromsø Study, conducted 5.2 (IQR; 3.0-6.2) months before RENIS-T6.

[a]GFR is estimated based on the CKD-EPI (2012) Equations from creatinine, cystatin C, or both.

[b]ACR> 30 mg/g

Table S2. The relationship between age, sex, health status, and GFR in the generalized additive mixed model.

	Model 3		
	β	(95 % CI)	P value
Linear effects on baseline GFR.			
Intercept, mL/min/1.73 m2	86.0	(84.1 to 87.9)	
Male sex	2.46	(-0.36 to 5.28)	0.09
Being healthy[a]	-3.47	(-5.24 to -1.70)	<0.001
Linear effects on GFR change rate, mL/min/1.73 m^2/year			
Being healthy[a]	0.22	(0.10 to 0.35)	<0.001
Nonlinear effects	Effective degrees of freedom[b]		
Age (time variable), y	2.09		<0.001
Interaction between age and male sex	2.82		<0.001
Aikaike Information Criterion[c]	33929		

All models were adjusted for sex-specific baseline age.

[a]Healthy is defined as having no cardiovascular disease, cancer, diabetes, hypertension, smoking, lipid-lowering medication, or digoxin, as well as a BMI <30 kg/m^2 and urinary ACR < 3.4 mg/mmol (<30mg/g).

[b]Effective degrees of freedom is related to the complexity of the smoothness of a given variable and can be a decimal number. Higher degrees correspond to a wigglier curve; a degree of 1 corresponds to a linear relationship.

[c]The Akaike Information Criterion (AIC) measures the trade-off between the goodness of fit and the simplicity of a model. Lower values correspond to a better trade-off.

Table S3. The relationship between age, sex, health status, and GFR in a generalized additive mixed models.

	Model 4[a]		
	β	(95 % CI)	P value
Linear effects on baseline GFR			
Intercept, mL/min/1.73 m2	77.6	(72.4 to 82.8)	
Male sex	2.03	(-0.78 to 4.82)	0.16
Being healthy[b]	-3.46	(-5.35 to -1.56)	<0.001
Linear effects on the GFR change rate, mL/min/1.73 m²/year			
Being healthy	0.24	(0.10 to 0.38)	<0.001
Nonlinear effects	Effective degrees of freedom[b]		
Age (time variable), y	2.40		0.03
Interaction between age and male sex	2.54		<0.001
Aikaike Information Criterion[d]	33838		

[a]Adjusted for sex-specific baseline age, and body-mass index, fasting glucose, and systolic BP as time-dependent continuous variables (including an interaction-term with time for each of them)
[b]Healthy is defined as no cardiovascular disease, cancer, diabetes, hypertension, smoking, lipid-lowering medication or digoxin, BMI <30 kg/m² and urinary ACR < 3.4 mg/mmol (<30 mg/g).
[c]Effective degrees of freedom is related to the complexity of the smoothness of a given variable and can be a decimal number. Higher degrees correspond to a wigglier curve; a degree of 1 corresponds to a linear relationship.
[d]The Akaike Information Criterion (AIC) measures the trade-off between the goodness of fit and the simplicity of a model. Lower values correspond to a better trade-off.

Table S4. The relationship between age, sex, health status and absolute GFR in mL/min in generalized additive mixed models.

	β	(95 % CI)	P-value
Linear effects on the baseline GFR			
Intercept, mL/min	92.6	(92.6 to 94.8)	
Male sex	7.17	(3.80 to 10.54)	<4e-5
Being healthy[a]	-3.22	(-5.20 to -1.24)	<0.01
Linear effects on GFR change rate, mL/min/year			
Being healthy[a]	0.18	(0.03 to 0.32)	0.01
Nonlinear effects	Effective degrees of freedom[b]		
Age (time variable), y	1.00		<0.001
Interaction between age and male sex	3.28		<3e-16

The model was adjusted for sex-specific baseline age and time-dependent variables body weight and height and their interaction with time (effect on the slope).
[a]Healthy is defined as no cardiovascular disease, cancer, diabetes, hypertension, smoking, lipid-lowering medication or digoxin, BMI <30 kg/m^2 and ACR >3.4 mg/mmol (<30mg/g).
[b]Effective degrees of freedom are related to the complexity of the smoothness of a given variable and can be a decimal number. Higher degrees correspond to a wigglier curve; a degree of 1 corresponds to a linear relationship.

Table S5. The relationship between age, sex, health status and eGFR in generalized additive mixed models.

	Model 3 eGFRcrea			Model 3 eGFRcys			Model 3 eGFRcyscrea		
	β	(95 % CI)	P value	β	(95 % CI)	P value	β	(95 % CI)	P value
Linear effects on eGFR at baseline									
Intercept, mL/min/1.73 m^2	87.0	(85.6 to 88.4)		85.3	(83.6 to 87.1)		87.4	(84.1 to 87.9)	
Male sex	-0.26	(-2.29 to 1.77)	0.8	3.34	(0.80 to 5.89)	0.01	1.38	(-1.01 to 3.77)	0.3
Being healthy[a]	-1.42	(-2.57 to -0.27)	0.02	0.53	(-0.91 to 1.97)	0.5	-0.63	(-1.92 to 0.64)	0.3
Linear effects on eGFR change rate, mL/min/1.73 m^2/year									
Being healthy[a]	0.11	(0.03 to 0.19)	0.01	0.07	(-0.04 to 0.18)	0.2	0.10	(0.00 to 0.19)	0.04
Nonlinear effects	Effective degrees of freedom[b]			Effective degrees of freedom[b]			Effective degrees of freedom[b]		
Age (time variable), y	1.00		<2e-16	7.20		<2e-16	5.64		<2e-16
Interaction between age and male sex	1.54		0.7	1.00		<0.001	1.00		0.2

All models were adjusted for sex-specific baseline age.

[a]Healthy is defined as no cardiovascular disease, cancer, diabetes, hypertension, smoking, lipid-lowering medication or digoxin, as well as a BMI <30 kg/m^2 and urinary ACR < 3.4 mg/mmol (<30 mg/g). [b]Effective degrees of freedom is related to the complexity of the smooth of a given variable and can be a decimal number. Higher degrees correspond to a wigglier curve; a degree of 1 corresponds to a linear relationship.

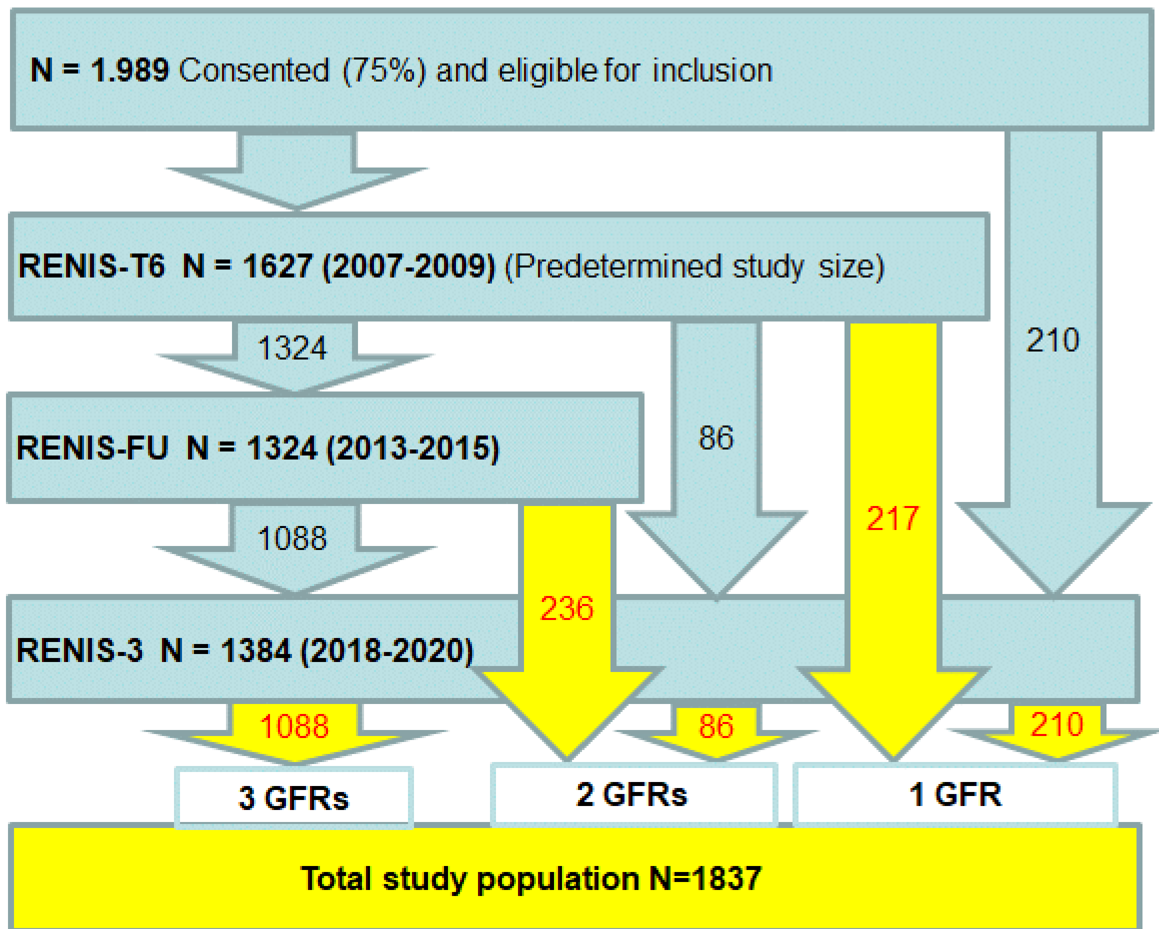

Figure S1. The total study population with at least one GFR measurement in the Renal Iohexol Clearance Survey (RENIS)

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