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Urinary Proteomics Identifies Cathepsin D as a Biomarker of Rapid eGFR Decline in Type 1 Diabetes

Kidney Precision Medicine Project

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1 A targeted multi-omics approach to identify biomarkers associated with rapid eGFR decline
2 in type 1 diabetes

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1 ABSTRACT

2 *Background*

3 Individuals with type 1 diabetes (T1D) demonstrate varied trajectories of estimated glomerular
4 filtration rate (eGFR) decline. The molecular pathways underlying rapid eGFR decline in T1D are
5 poorly understood, and individual-level risk of rapid eGFR decline is difficult to predict.

6 *Methods*

7 We designed a case-control study with multiple exposure measurements nested within four well-
8 characterized T1D cohorts (FinnDiane, Steno, EDC, CACTI) to identify biomarkers associated
9 with rapid eGFR decline. Here, we report the rationale for and design of these studies as well as
10 results of models testing associations of clinical characteristics with rapid eGFR decline in the
11 study population, upon which “omics” studies will be built. Cases (n = 535) and controls (n = 895)
12 were defined as having an annual eGFR decline of ≥ 3 ml/min/1.73m² and < 1 ml/min/1.73m²,
13 respectively. Associations of demographic and clinical variables with rapid eGFR decline were
14 tested using logistic regression, and prediction was evaluated using area under the curve (AUC)
15 statistics. Targeted metabolomics, lipidomics, and proteomics are being performed using high-
16 resolution mass-spectrometry techniques.

17 *Results*

18 At baseline, mean age was 43 years, diabetes duration was 27 years, eGFR was 94 ml/min/1.73m²,
19 and 62% of participants were normoalbuminuric. Over 7.6 years median follow-up, the mean
20 annual change in eGFR in cases and controls was -5.7 ml/min/1.73m² and 0.6 ml/min/1.73m²,
21 respectively. Younger age, longer diabetes duration, and higher baseline HbA1c, urine albumin-
22 creatinine ratio, and eGFR were significantly associated with rapid eGFR decline. The cross-

1 validated AUC for the predictive model incorporating these variables plus sex and mean arterial
2 blood pressure was 0.74 (95% CI 0.68, 0.79; $p < 0.001$).

3 *Conclusion*

4 Known risk factors provide moderate discrimination of rapid eGFR decline. Identification of blood
5 and urine biomarkers associated with rapid eGFR decline in T1D using targeted omics strategies
6 may provide insight into disease mechanisms and improve upon clinical predictive models using
7 traditional risk factors.

8

9 INTRODUCTION

10 Approximately 25-40% of individuals with type 1 diabetes (T1D) develop diabetic kidney
11 disease (DKD), defined as a reduction in estimated glomerular filtration rate (eGFR) or onset of
12 albuminuria [1,2]. In this population, the reported incidence of kidney failure ranges from 2-35%
13 over 30 years of T1D duration, and up to 60% over 50 years of T1D duration [3,4].

14 Progressive eGFR decline is largely a monotonic process that occurs early in the course of
15 T1 DKD [5]. The annual rate of decline varies across affected individuals, with more rapid rates
16 conferring higher risk of kidney failure [6]. Additionally, eGFR decline may precede the onset of
17 albuminuria, suggesting the presence of disease activity before clinical signs are apparent [5,7].
18 While various pathophysiological processes including hyperglycemia, microvascular dysfunction,
19 inflammation, and fibrosis, are implicated in DKD, the mechanisms underlying the observed
20 differential decline in kidney function across individuals remain poorly understood [8,9]. Insight
21 into the mechanisms of eGFR decline in T1D is important for early identification of individuals at
22 risk for DKD progression and for development of new therapies.

1 The search is ongoing for novel biomarkers that can parse through the heterogeneous nature
2 of DKD pathophysiology and improve the predictive and prognostic abilities of existing clinical
3 models for DKD progression [10]. A number of candidate biomarkers have been associated with
4 putative pathogenic pathways (including inflammation, endothelial dysfunction, and fibrosis) and
5 functional kidney outcomes (such as eGFR decline, albuminuria, and kidney failure) in DKD [11].
6 However, their widespread use is limited by lack of validation and diagnostic precision [12-14].

7 The development of “omic” approaches has facilitated biomarker identification in DKD
8 via quantification of low-molecular weight proteins, metabolites, and lipids in blood and urine
9 using refined mass spectrometry techniques [15,16]. A new multidimensional urinary proteome
10 classifier (CKD273) has identified new peptide markers of interest and demonstrated promise in
11 detecting individuals with diabetes who are at risk for progression of DKD, though studies have
12 primarily focused on individuals with type 2 diabetes (T2D) [17,18]. Recently, 125 plasma amino
13 acid, triglyceride, and lipid metabolites were cross-sectionally associated with eGFR in a large
14 T2D meta-analyses [19]. Among individuals with T1D, use of omic technologies may allow for
15 better characterization of the molecular pathways responsible for eGFR decline and enable
16 application of this insight to the development of predictive models.

17 Recently, an international consortium funded by JDRF was established to identify
18 metabolite, lipid, and protein markers of eGFR decline in individuals with T1D using novel multi-
19 omics techniques. The aims of the JDRF consortium are (1) to discover and validate a set of
20 biomarkers associated with rapid eGFR decline in T1D using novel omics platforms which may
21 provide insight into disease mechanisms, and (2) to use resulting omics data to develop predictive
22 models for rapid eGFR decline in T1D. In this paper, we describe the rationale, design, cohorts,
23 and methods of the JDRF Biomarkers Consortium, and examine associations of clinical variables

1 with rapid eGFR loss as well as prediction of eGFR loss by clinical variables to establish common,
2 appropriate models upon which to add omics measurements.

3

4 MATERIALS AND METHODS

5 *Study design*

6 We designed a case-control study with multiple exposure measurements to test associations
7 of blood and urine biomarkers, measured at baseline, with subsequent rapid eGFR decline in T1D.
8 The case-control study was nested within four T1D cohorts. Cases were defined as having an
9 annual decline in eGFR of ≥ 3 ml/min/1.73m² and controls were defined as having an annual
10 decline in eGFR of < 1 ml/min/1.73m². Blood and urine samples obtained at baseline are being
11 applied to metabolomics, lipidomics, and/or proteomics platforms for measurement of pre-
12 specified biomarkers. A discovery-validation approach will be taken to test associations of
13 biomarkers with eGFR decline.

14

15 *Study population*

16 Our study sample is composed of 1,430 participants (535 cases and 895 controls) from four
17 well-characterized T1D cohorts: the Finnish Diabetic Nephropathy study (FinnDiane), the Steno
18 Diabetes Center Copenhagen study (Steno), the Epidemiology of Diabetes Complications study
19 (EDC) and the Coronary Artery Calcification in Type 1 Diabetes study (CACTI) (shown in Fig.
20 1) [20-23]. Subjects were included based on the following criteria: eGFR ≥ 30 ml/min/1.73m² at
21 baseline, follow up of at least 2 years, ≥ 3 longitudinal eGFR measurements, and baseline urine
22 and blood sample availability. In FinnDiane, cases and controls were frequency-matched by
23 albuminuria strata (normoalbuminuria, microalbuminuria, macroalbuminuria). In the three

1 remaining cohorts, all participants who met the definition of case or control and met the stated
2 criteria were included. To maintain consistency across cohorts given their extended durations, only
3 participants examined between 1995 and 2011 were included.

4 Details on protocols and data collection for each cohort have been previously published
5 and are summarized in the Supplementary Text. Briefly, the FinnDiane cohort includes adults with
6 T1D from healthcare centers across Finland who were evaluated regularly [20]. The Steno cohort
7 includes adults with T1D who attended the Steno Diabetes Center Copenhagen and were followed
8 for a median of 4.7 years [21]. The EDC cohort includes subjects with childhood-onset T1D
9 diagnosed or seen within one year of diagnosis at Children’s Hospital of Pittsburgh between 1950-
10 1980 who were examined in 1986-88, then biennially for 10 years with additional examinations at
11 18 and 25 years [22]. The CACTI cohort includes adults with T1D without a history of
12 cardiovascular disease at enrollment who were assessed at a baseline examination, then 3 and 6
13 years later [23].

14

15 *Outcome*

16 Glomerular filtration rate was estimated using the CKD-EPI creatinine equation in all
17 cohorts [24]. Laboratory methods for serum creatinine measurements differed by study cohort and
18 are detailed in the Supplementary Text. Annual rate of eGFR decline was calculated by fitting
19 regression lines to serial eGFR values (FinnDiane, Steno) or by dividing absolute eGFR change
20 from baseline to last study visit by the number of years between these (EDC, CACTI).

21

22 *Measurement of biological samples*

1 Baseline timed urine and fasting plasma samples were obtained from each cohort.
2 Coordinated shipping and storage efforts were undertaken to preserve biosample integrity and
3 reduce the number of freeze-thaws. Frozen biosamples were stored in a central laboratory at
4 University of California San Diego (UCSD), where they were entered into a sample-tracking
5 database. Sample aliquots were transferred to platform sites for omics analysis and remain stored
6 at -80°C.

7 Targeted metabolomic, lipidomic, and proteomic measurements and analyses are currently
8 being performed concurrently at UT Health Science Center San Antonio/UCSD, University of
9 Michigan, and University of Washington, respectively. Biomarkers measured in each platform are
10 pre-specified based on existing scientific evidence of an association with DKD (Supplementary
11 Table 1). Biomarkers are being quantified using targeted mass-spectrometry with inclusion of
12 stable isotope-labeled standards to enhance accuracy and reduce variation across measurements.
13 Strict quality control methods are employed to monitor instrument accuracy and batch-to-batch
14 variation. The same techniques are being used to measure biomarkers in discovery and validation
15 sets. Biomarkers that differ significantly between cases and controls will be considered for more
16 precise quantification using orthogonal high throughput, quantitative assays.

17

18 Metabolomics

19 Previously, 13 out of 94 urine metabolites were found to differ between DKD and diabetes
20 alone in a cross-sectional study of 108 participants [25]. These 13 metabolites, along with select
21 organic acids, amino acids, purines, and pyrimidines identified by collaborators as being associated
22 with DKD, are being measured in baseline urine and plasma samples using targeted gas

1 chromatography-mass spectrometry (GC-MS) and tandem liquid chromatography-mass
2 spectrometry (LC-MS) techniques [26,27].

3

4 Lipidomics

5 Earlier works identified plasma lipid alterations associated with CKD and T2 DKD
6 progression [28-30]. A targeted LC-MS/MS assay was developed to quantify free fatty acids,
7 acylcarnitines, and other members of complex lipid classes hypothesized to have an association
8 with rapid eGFR decline in T1D. This targeted platform is being applied to baseline plasma
9 samples.

10

11 Proteomics

12 Urine proteins were selected for measurement following a comprehensive literature review
13 which identified 179 candidate proteins from 12 signaling pathways involved in DKD across
14 animal and human studies. Thirty-eight tryptic peptides derived from 20 of these proteins could be
15 reliably measured using protein precipitation and proteolysis-LC-MS/MS with adequate
16 sensitivity and specificity. These peptides are being quantified using LC-MS/MS in baseline urine
17 samples.

18

19 *Statistical analyses*

20 Clinical variable association and prediction studies

21 We developed and evaluated models of eGFR decline using clinical covariates to
22 understand these associations and provide a foundation for biomarker analyses.

1 We first summarized the distribution and central tendencies of clinical variables, then fit
2 clinical covariate data in logistic regression models to test associations of clinical variables with
3 case versus control status. We developed three nested models: Model 1 included demographics
4 (age at entry and sex); Model 2 included demographics, diabetes duration, and baseline HbA1c
5 levels, urine albumin-creatinine ratio (UACR), and mean arterial pressure (MAP, defined as
6 [(systolic blood pressure + (2 × diastolic blood pressure))/3]); Model 3 added baseline eGFR level
7 to Model 2. Odds ratios with 95% CIs were calculated for each variable.

8 Area under the Receiver Operating Characteristic curve (AUC) values were calculated to
9 evaluate model discrimination, and DeLong’s test was used to compare nested model AUCs [31].
10 To accurately estimate model performance in future samples, we used repeated random sub-
11 sampling validation, training models on a random 4/5th of the sample then testing these on the
12 held-out 1/5th. AUCs were calculated on the 1/5th sample. This training-testing process was
13 repeated 500 times providing a distribution of test AUC estimates, from which a median and 95%
14 CI were estimated.

15 Next, we compared model parameters according to four baseline eGFR strata:
16 $30 \leq \text{eGFR} \leq 60$ ml/min/1.73m²; $60 < \text{eGFR} \leq 90$ ml/min/1.73m²; $90 < \text{eGFR} \leq 120$ ml/min/1.73m²; and
17 $120 < \text{eGFR} \leq 150$ ml/min/1.73m². Interaction terms between this categorical eGFR variable and
18 covariates were tested via likelihood ratio tests. If model fit improved significantly at 5%
19 significance, separate models were fit for each stratum and cross-validated AUCs were calculated
20 for stratified models. Finally, to test if the stratum-specific models improved discrimination, we
21 used bootstrap resampling to compare AUCs of stratified and full models [32]. AUCs were
22 calculated on each of 500 bootstrap samples. The difference between the full sample and stratum-
23 specific AUCs were recorded. The 500 bootstrapped differences were used to calculate percentile

1 intervals and test if stratum-specific AUCs differed from the full sample AUC. If the interval
2 excluded zero, we inferred that the AUCs were statistically different. This analysis was repeated
3 for baseline albuminuria strata defined by normoalbuminuria (UACR <30mg/g),
4 microalbuminuria ($30 \leq \text{UACR} < 300$ mg/g), and macroalbuminuria (UACR ≥ 300 mg/g). When
5 sample size was sufficient, we examined combined albuminuria and eGFR subgroups.

6

7 RESULTS

8 *Participant characteristics*

9 Our sample comprised 1430 subjects from the four T1D cohorts, including 535 cases and
10 895 controls (Table 1). Subjects' mean age was 43 years, 50% were female, and mean (SD)
11 diabetes duration was 26.8 (12.6) years. The mean (SD) HbA1c and eGFR were 8.5 (1.3)% and
12 94 (24) ml/min/1.73m², respectively, and median UACR (25th, 75th %ile) was 12 (5, 64) mg/g.
13 Subjects were followed for a median (25th, 75th %ile) of 7.6 (4.9, 11.7) years.

14 Compared with control participants, cases were on average younger, had higher average
15 baseline levels of HbA1c, blood pressure, eGFR, and UACR compared to controls (Table 1). The
16 mean (SD) annual eGFR slope was -5.65 (4.53) ml/min/1.73m² in cases, versus 0.57 (1.87)
17 ml/min/1.73m² in controls.

18

19 *Characteristics associated with eGFR decline*

20 Younger age at study entry was associated with greater risk of rapid eGFR decline in the
21 demographics-only model (Model 1), which had an AUC of 0.61 (Table 2). In Model 2, younger
22 age, higher HbA1c, and higher UACR were associated with rapid eGFR decline. Compared to the
23 demographics-only model, Model 2 had a significantly higher AUC of 0.69 ($p = 0.0015$). In the

1 fully-adjusted model (Model 3) comprising demographic and clinical variables, younger age,
2 longer diabetes duration, and higher HbA1c, UACR, and eGFR conferred greater risk of rapid
3 eGFR decline. Specifically, every 10 more years of age was associated with a 24% lower odds of
4 rapid eGFR decline, every 10 more years of diabetes duration was associated with a 20% higher
5 odds of rapid eGFR decline, each 1% higher HbA1c was associated with a 15% higher odds of
6 rapid eGFR decline, each two-fold greater UACR was associated with a 30% higher odds of rapid
7 eGFR decline, and every 10 ml/min/1.73m² greater baseline eGFR was associated with a 31%
8 higher odds of rapid eGFR decline. Compared to the demographics-only model, Model 3 had a
9 significantly higher AUC of 0.74 (p < 0.001).

10

11 *Consideration of additional risk factors*

12 When added to Model 3, the presence of hypertension (as a binary indicator) was
13 significantly associated with rapid eGFR decline (OR 1.46; 95% CI 1.09, 1.96). Neither
14 angiotensin-converting enzyme inhibitor (ACEi) or angiotensin receptor blocker (ARB) use,
15 presence of retinopathy, nor smoking history (past, current, never smoker) were significantly
16 associated with rapid eGFR decline. When added to Model 3, neither the presence of hypertension,
17 ACEi or ARB use, the presence of retinopathy, nor smoking history improved model
18 discrimination. Thus, we conducted all subsequent subgroup analyses based on Model 3 to
19 minimize the impact of missing information (Supplementary Table 2).

20

21 *Subgroup analysis*

22 Addition of interactions between baseline eGFR or UACR strata and clinical variables
23 improved the fit of the logistic model significantly (likelihood ratio p-value < 0.001). Hence, we

1 applied Model 3 to eGFR, UACR, and combined eGFR and UACR strata with sufficient sample-
2 sizes (Supplementary Table 2). The directions of association for the predictors with rapid eGFR
3 decline in the stratified models were similar to those in the full cohort models. Notably, the
4 magnitude of the association of UACR with rapid eGFR decline was greater in the eGFR 60-
5 90ml/min/1.73m² group (OR 1.39; 95% CI 1.27, 1.53) compared to groups with higher eGFRs
6 (OR 1.10-1.24). Additionally, while higher HbA1c was significantly associated with rapid eGFR
7 decline in groups with microalbuminuria (OR 1.36; 95% CI 1.14, 1.65) and macroalbuminuria
8 (OR 1.66; 1.24, 2.29), this association was not observed in the normoalbuminuria group.

9 Figure 2 depicts the median (95% CIs) cross-validated AUCs. There was substantial
10 variability in discrimination across baseline kidney function categories compared to the full cohort
11 AUC, with cross-validated AUCs ranging from 0.59 to 0.76. The lowest AUCs were observed in
12 the eGFR 120-150 ml/min/1.73m² group (AUC 0.59), the macroalbuminuria group (AUC 0.65),
13 and the combined normoalbuminuria and eGFR 90-120 ml/min/1.73m² group (AUC 0.65).

14

15 DISCUSSION/CONCLUSION

16 We have assembled a collection of well-defined T1D cohorts to identify biomarkers
17 associated with rapid eGFR decline. While demographic and clinical variables were significantly
18 associated with rapid eGFR decline in these cohorts, these do not predict rapid eGFR decline with
19 sufficient precision. Our expectation is that omics-derived urine and plasma biomarkers will be
20 associated with rapid eGFR decline, independent of demography and clinical variables, and
21 enhance the predictive ability of our models.

22 Our use of four large, multi-national, rigorously-derived T1D cohorts leaves us well-
23 positioned to identify biomarkers associated with rapid eGFR decline. These T1D cohorts have

1 large sample sizes, prolonged follow-up, and excellent participant retention. Additionally, each
2 cohort has used standardized methods for participant recruitment, data collection, and biosample
3 preservation. Furthermore, availability of timed urine and fasting plasma samples in all cohorts
4 increases the precision of biomarker measurements and facilitates comparisons across cohorts. The
5 clinical models developed here will be used as the base for discovery and validation omics
6 analyses. Biomarkers associated with rapid eGFR decline will be incorporated into predictive
7 models made using clinical variables and AUC values for these will be calculated to assess model
8 discrimination.

9 We found that younger age, longer diabetes duration, and higher baseline HbA1c, UACR,
10 and eGFR were associated with rapid eGFR decline in our study population. Overall, these
11 findings are consistent those observed in other T1D and T2D populations [33-36]. Notably, a two-
12 fold higher UACR was associated with 30% greater odds of rapid eGFR decline in our fully-
13 adjusted model. However, this association is difficult to interpret and may be biased towards the
14 null because of matching of case-control status by albuminuria strata in one of our study cohorts.
15 To build a foundation for assessing the predictive ability of measured biomarkers, we derived
16 prognostic models using these clinical variables. We found that overall model discrimination was
17 moderate (AUC = 0.74) using age, sex, diabetes duration, HbA1c, blood pressure, albuminuria,
18 and baseline eGFR as predictors of rapid eGFR decline. Similarly-derived models focusing on
19 incident DKD or major kidney-related events as outcomes have had greater predictive success [14,
20 37].

21 The relatively low discriminatory potential of our model could be explained by our focus
22 on an outcome which occurs early in the course of DKD as well as by the high average baseline
23 eGFR in our cohort [33]. Interaction testing confirmed that associations between covariates and

1 rapid eGFR decline differed according to baseline kidney function. Subgroup analyses revealed
2 that model discrimination varied by strata of baseline eGFR and albuminuria. Specifically, the
3 model demonstrated poor predictive ability among those with baseline eGFR 120-
4 150ml/min/1.73m² (AUC = 0.59), baseline macroalbuminuria (AUC = 0.65), and baseline eGFR
5 90-120ml/min/1.73m² and normoalbuminuria (AUC = 0.65). The difficulty in predicting eGFR
6 decline in these subgroups may reflect variable biologic underpinnings of eGFR decline, though
7 regression to the mean may also contribute to these findings. Overall, weak-to-moderate AUCs,
8 especially when eGFR and albuminuria are in the normal range, highlight the need for prognostic
9 biomarkers capable of early identification of high-risk individuals.

10 Using novel, targeted omics strategies, we plan to identify plasma and urine biomarkers
11 associated with rapid eGFR decline which elucidate mechanisms of T1 DKD and build on clinical
12 predictive models. Numerous biomarkers belonging to pathways implicated in DKD, including
13 inflammation, endothelial dysfunction, and fibrosis, have been associated with DKD-related
14 outcomes in cross-sectional and longitudinal studies [11]. At the same time, questions remain
15 regarding the specific molecules comprising these pathogenic pathways and how they contribute
16 to eGFR decline in DKD. This is partly because of heterogeneity across existing studies in clinical
17 outcomes, chosen biomarkers, and methods of biomarker quantification, which has made
18 biomarker validation challenging [11].

19 There has been increasing interest in the association of biomarkers with eGFR decline in
20 DKD [38, 39]. Our panel of 13 urine metabolites was recently found to correlate with eGFR slope
21 in 1,001 subjects with T2D in the Chronic Renal Insufficiency Cohort study [40]. Notably, this
22 panel was responsive to therapy with dapagliflozin and atrasentan, suggesting potential as a
23 surrogate indicator for mitochondrial dysfunction in T2D [41,42]. Members of our group have also

1 identified 125 plasma metabolites associated with eGFR in a meta-analysis including 3,089
2 samples from participants of five independent Dutch T2D cohort studies [19]. Similar biomarker
3 advances have also been made in T1D, albeit in relatively smaller study populations. Serum
4 lipidomic measurements of 669 individuals with T1D from Steno identified cross-sectional
5 associations between phosphatidylcholine and sphingomyelin species and eGFR and albuminuria,
6 out of which 13 lipids were longitudinally associated with eGFR or albuminuria slope [15]. Serum
7 metabolomic measurements in this cohort additionally revealed ribonic acid and myo-inositol to
8 be inversely associated with $\geq 30\%$ eGFR decline [16] Also, in a study of 465 individuals with
9 T1D from CACTI, a panel of 4 out of 252 urine peptides identified in a label-free discovery
10 analysis improved prediction of annual eGFR decline $\geq 3.3\%$ and/or development of albuminuria
11 when added to DKD risk factors (increase in AUC from 0.84 to 0.89) [43].

12 These promising results underscore the need to further study eGFR decline in T1D using
13 large cohorts and refined, combined multi-omic assays. In our study we propose to use these
14 strategies to develop a robust set of biomarkers which are specific to this population, internally
15 and externally validated, and have multi-national applicability. Additionally, our use of a
16 hypothesis-driven, targeted omics approach that will yield highly precise, quantitative, and
17 reproducible results is conducive to our goals of deciphering mechanisms of eGFR decline and
18 improving prediction of this outcome.

19 Our work may serve as a foundation on which future omics research can build. Added
20 insight into pathological pathways may encourage generation of new diagnostic tests and therapies.
21 Following extensive validation and assay optimization, identified biomarkers may be able to act
22 as surrogates for risk of eGFR decline in T1D. This could facilitate recruitment and increase
23 efficiency in clinical trials, as those at increased risk for poor outcomes are more likely to benefit

1 from therapeutic interventions. Ultimately, we hope to integrate metabolomic, lipidomic, and
2 proteomic biomarker data with kidney tissue-derived genetic and transcriptional network data
3 using systems biology and computational bioinformatic techniques with the goal of
4 mechanistically defining pathologic DKD subgroups. By then associating molecularly defined
5 subgroups with clinical characteristics and kidney outcomes, we aim to develop a functional
6 framework for rapid eGFR decline in T1D.

7 Our study has several limitations. Our population is primarily white, limiting applicability
8 of results to wider ranges of races and ethnicities. We defined case-control status using a linear
9 estimation of eGFR decline, though eGFR trajectories may exhibit non-linear patterns. However,
10 existing evidence suggests that non-linear eGFR decline occurs only in a minority [33]. A
11 substantial proportion of our study population has a baseline eGFR >90 ml/min/1.73m², a range in
12 which changes in eGFR are difficult to ascertain. The observation that cases had higher baseline
13 eGFR than controls is likely due to participant selection, as subjects with higher baseline eGFR
14 have more capacity to reach the threshold for case definition. In this group, a “therapeutic” decline
15 in eGFR resulting from reduced “hyperfiltration” is difficult to distinguish from a “pathologic”
16 one. We have defined rapid versus slow decline based on extremes of the eGFR slope distribution
17 and have determined slopes over prolonged follow-up periods, which should reduce
18 misclassification. At the same time, since models were developed using extremes of the eGFR
19 slope distribution, the discriminatory ability of these models would be attenuated by application
20 to a broader cohort with a wide range of eGFR slopes. Also, as mentioned above, the association
21 of UACR with rapid eGFR decline should be interpreted with caution due to matching of case-
22 control status by albuminuria strata in one of our study cohorts. With respect to our biomarker
23 analyses, prolonged storage and inconsistencies in biosample collections across cohorts could

1 influence the accuracy and sensitivity of measurements. Additionally, though biomarkers present
2 in small quantities may be difficult to detect, our targeted approach represents the optimal strategy
3 for increasing measurement precision.

4 In conclusion, we have assembled a large, multinational T1D cohort for determining
5 metabolic, lipid, and protein biomarkers associated with rapid eGFR decline. In this cohort, clinical
6 factors alone are insufficient in predicting rapid eGFR decline, especially among those with normal
7 baseline kidney function. Our application of novel, targeted omics approaches may help improve
8 understanding of the mechanisms underlying rapid eGFR decline and may facilitate identification
9 of those at risk for this outcome.

10

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16

17 STATEMENT OF ETHICS

18 Research protocols for FinnDiane, Steno, EDC, and CACTI were approved by each center's
19 respective ethics committee or institutional review board. All participants provided informed
20 consent.

21

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4

5 AUTHOR CONTRIBUTIONS

6 CPL and LN drafted the manuscript. EV and JZ conducted statistical analyses. ANH, IHdB, KS,
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8 JKS-B, P-HG, PR, and TJO serve as primary investigators of the four studied cohorts and
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10 the final manuscript.

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1 FIGURE LEGENDS

2

3 Fig. 1. Study design of JDRF Biomarkers Consortium, a case-control study nested in four T1D
4 cohorts.

5

6 Fig. 2. Performance of demographic and clinical variables in the prediction of rapid eGFR loss
7 according to baseline eGFR and urine albumin-creatinine ratio. Presented values are cross-
8 validated area under the curve (AUC; median 95% CI).

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Supplementary Online Content

Supplementary Text. Description of the four study cohorts

Supplementary Table 1. Biomarkers measured using targeted omics assays in the JDRF Biomarkers Consortium

Supplementary Table 2. Associations of clinical characteristics with rapid eGFR decline stratified by baseline eGFR and UACR in the JDRF Biomarkers Consortium

Supplementary Text: Description of the four study cohorts

The Finnish Diabetic Nephropathy (FinnDiane) study cohort

The FinnDiane study is a prospective nationwide multicenter study of more than 8,400 adults with T1D from 21 university and central hospitals, 33 district hospitals, and 26 primary health care centers across Finland and is the largest natural history study for T1D and its complications [1]. Patients are followed on a yearly basis and kidney function is measured via urine albumin excretion rates and eGFR measurements. The current study comprises 578 cases and controls studied between 1998 and 2011. Patients participated in the study during a regular visit to their attending physician during which detailed demographic and medical history data were collected with standardized questionnaires. Blood pressure was measured two times with 2-min intervals in the sitting position after an initial 10-min rest and the mean values were used for analysis. Hypertension was defined as $> 130/85$ mmHg over two readings or use of antihypertensive medication. HbA1c was measured with standard methods at the local centers; eGFR was estimated from serum creatinine values by the CKD-EPI equation [2]. Annual rate of eGFR change was calculated by fitting regression lines to serial eGFR measures for each patient. A participant was classified as having retinopathy if he/she received laser treatment. In a subset of 1,346 FinnDiane patients, that had been ETDRS graded from fundus photographs and ophthalmic records, we observed that 81.1% of laser-treated patients eventually were diagnosed with proliferative diabetic retinopathy [3]. This suggests that laser treatment could be used as a surrogate for severe retinopathy in FinnDiane. As the training of the Finnish ophthalmologists is uniform (one main instructor in the country the last 35 years) the indications for laser treatment at different centers are consistent.

All participants gave written consent prior to participation, and the study protocol was approved by the local ethics committees of each participating center. The study is performed in accordance with the Declaration of Helsinki as revised in the year 2000.

The Steno study cohort

The Steno Diabetes Center Copenhagen (SDCC) is specialized in the treatment, research and prevention of diabetes and in the education of healthcare professionals [4]. It is Scandinavia's largest diabetes clinic (www.sdcc.dk).

The current study cohort is comprised of Caucasian adults (age ≥ 18 years) with T1D attending the SDCC out-patient clinic. Their follow up time ranges from 4 to 10 years (median 4.65 years). The SDCC T1D sample may be sub-grouped on the basis of their baseline examination dates. The first set of T1D cases was enrolled between May 1995 to April 1996 while participating in the Low Protein Diet (LPD) study with a T1D onset of at least 10 years [5]. The second set of T1D cases were enrolled in 2004 for a study of biomarkers related to nephropathy, while the last set of T1D cases were enrolled between 2009-2011 and participated in a study of biomarkers of nephropathy and arterial stiffness [6,7].

Patient demographics, biochemical measurements, and medical history data were collected at each follow-up visit. Blood pressure measures were recorded with either a validated tonometric device (BPro; HealthStats, Singapore), or using the Hawksley Random Zero Sphygmomanometer, with a mean of at least two measures [5,7]. Hypertension was defined as use of antihypertensive medication. High-performance liquid chromatography (Bio Rad Laboratories, Munich, Germany) was used to estimate HbA1c (normal range: 4.1-6.4%) and an enzymatic method (Hitachi 912; Roche Diagnostics, Mannheim, Germany) was used to measure serum creatinine concentrations [7]. Urinary albumin excretion ratio (UAER) was measured in 24-h sterile urine collections by enzyme immunoassay; eGFR was calculated using the CKD-EPI creatinine equation. The rate of decline in kidney function was analyzed with regression lines for eGFR over the follow-up period using all measurements of eGFR during the study period. Retinopathy was assessed via retinal photographs taken during regular ophthalmologic examinations. All participants gave written informed consent, and the study was approved by the Danish National Ethics committee. A total of 362 Steno cases and controls are included in the current analysis.

Epidemiology of Diabetes Complications (EDC) study cohort

The EDC is a prospective historical cohort study based on incident cases of childhood onset (<17 years) T1D, diagnosed or seen within one year of diagnosis (1950-80) at Children's Hospital of Pittsburgh [8]. The cohort, which has been shown to be epidemiologically representative of the Allegheny County, Pennsylvania, T1D population, was first assessed for the EDC study between 1986 and 1988 (mean participant age and diabetes duration were 28 and 19 years, respectively) [9]. Subsequently, biennial examinations were conducted for 10 years, with a further detailed examination at 18 and 25 years from enrollment. All EDC study participants provided informed consent, and all study procedures were approved by the University of Pittsburgh Institutional Review Board (IRB).

Demographic, health, self-care and medical history data were collected at each follow-up. Blood pressure was measured with a random zero sphygmomanometer, after a five-minute rest and hypertension was defined as $\geq 140/90$ mmHg or use of antihypertensive medication. Automated high-performance liquid chromatography (Diamat; Bio-Rad, Hercules, CA) was performed for Hemoglobin A1c (HbA1c) [10]. Glomerular filtration rate was estimated by the CKD-EPI creatinine equation. Retinopathy was classified according to the modified Airlie House classification; the methodology has been described in detail elsewhere [11]. Proliferative retinopathy was defined as a grade of ≥ 60 in at least one eye or a history of panretinal photocoagulation for proliferative diabetic retinopathy. To achieve reasonable temporal comparability to the other cohorts, the current study used the years 1996-98 to define the baseline measure; 146 such participants (cases and controls) met the criteria and are included in the present study. Annual rate of eGFR change was calculated using the difference between the first (1996-98 exam) and last available eGFR (2001-03 or 2004-06 exam) and dividing by the number of years between the two measures.

Coronary Artery Calcification in Type 1 Diabetes (CACTI) study cohort

The CACTI study enrolled 652 adults ranging in age from 19-56 years between 2000-2002 into a prospective cohort study designed to examine cardiovascular and related complications of T1D [12]. All participants were free of diagnosed cardiovascular disease, and did not have a cardiovascular event (myocardial infarction, angioplasty or coronary artery bypass graft). Participants were either diagnosed with T1D before the age of 30, or if diagnosed over age 30, they had positive autoantibodies or a clinical course consistent with T1D. All were on insulin within a year of diagnosis and at the time of enrollment. Participants have been followed longitudinally at three and six years for complications, including diabetic nephropathy. All study participants provided informed consent, and the study was approved by the Colorado Multiple IRB.

Demographic, health, self-care and medical history data were collected at baseline and at three subsequent follow-up examinations. Blood pressure was measured three times via random zero sphygmomanometer, after a five-minute rest, and the second and third measurements were averaged. Hypertension was defined as $\geq 140/90$ mmHg or use of antihypertensive medication. HbA1c was assayed by high performance liquid chromatography (BioRad Variant). A participant was classified as having retinopathy if he/she received laser treatment. Glomerular filtration rate was estimated by the CKD-EPI creatinine equation. Average annual decline in eGFR was calculated as absolute change in eGFR from the baseline exam to the last visit in the study divided by the number of years between these two visits. The current analysis is based on 344 patients (with fast (cases) or slow (controls) kidney function decline) from the CACT1 cohort.

Supplementary Table 1: Biomarkers measured using targeted omics assays in the JDRF Biomarkers Consortium

Project	Measured biomarkers	
Metabolomics	2-Methyl acetoacetate	Citric acid
	2-Hydroxybutyrate	Ethylmalonic acid
	2-Hydroxyglutaric acid	Fumaric acid
	2-Hydroxyisovaleric acid	Glycolic acid
	2-Ketoadipic acid	Glyoxylic acid
	2-Ketoglutaric acid	Hippuric acid
	2-Ethyl-3-hydroxypropionate	Homovanillic acid
	3-Hydroxyisobutyrate	Hydrocinnamic acid
	3-Hydroxyisovalerate	Isocitric acid
	3-Hydroxypropionate	Lactic acid
	3-Hydroxyadipic acid	Malic acid
	3-Hydroxybutyric acid	Methylsuccinic acid
	3-Hydroxyglutaric acid	N-Acetylaspartate
	3-Methyladipic acid	N-Acetyl-L-tyrosine
	3-Methylcrotonylglycine	Palmitic acid
	3-Methylglutaconic acid	Pyruvic acid
	4-Aminobutyric acid	Stearic acid
	4-Hydroxyhippurate	Succinic acid
	4-Hydroxyphenyllactic acid	Tiglylglycine
	Acetoacetic acid	Uracil
Aconitic acid	Uric acid	
Adipic Acid		
Lipidomics*	Free fatty acids	Diacylglycerols
	Lysophosphatidylcholines	Cholesteryl esters
	Sphingomyelins	Phosphatidylethanolamines
	Phosphatidylcholines	L-carnitine
	Triglycerols	Acylcarnitines
Proteomics	Podocalyxin	Insulin-like growth factor binding protein 2
	Renin receptor	Insulin-like growth factor binding protein 3
	Urokinase-type plasminogen activator	Insulin-like growth factor binding protein 7
	Epidermal growth factor	Vascular cell adhesion protein 1
	Collagen alpha-1(I) chain	Connective tissue growth factor
	Collagen alpha-1(III) chain	Syndecan-4
	Intracellular adhesion molecule 1	Aquaporin-2
	Cathepsin D	Selenoprotein P
Matrix metalloproteinase 7	Urokinase receptor	
Cytosolic non-specific dipeptidase 2	Endothelial cell-selective adhesion molecule	

*326 lipid species measured across the listed lipid classes.

Supplementary Table 2: Associations of clinical characteristics with rapid eGFR decline stratified by baseline eGFR and UACR in the JDRF Biomarkers Consortium

	Baseline eGFR			Baseline UACR			Combination of 90 < eGFR ≤ 120 and normoalbuminuria* N (cases) = 434 (140)
	60 < eGFR ≤ 90 ml/min/1.73m ² N (cases) = 511 (129)	90 < eGFR ≤ 120 ml/min/1.73m ² N (cases) = 638 (236)	120 < eGFR ≤ 150 ml/min/1.73m ² N (cases) = 166 (117)	Macro: ≥ 300 mg/g N (cases) = 143 (99)	Micro: >30, < 300 mg/g N (cases) = 323 (134)	Normo: ≤ 30 mg/g N (cases) = 881 (269)	
Age (per 10 years)	0.99 (0.71, 1.14)	0.82 (0.66, 1.03)	0.80 (0.44, 1.45)	1.12 (0.68, 1.89)	0.72 (0.55, 0.93)	0.91 (0.74, 1.11)	0.79 (0.60, 1.04)
Sex (Ref: female)	1.04 (0.64, 1.68)	0.81 (0.56, 1.15)	0.47 (0.21, 1.02)	1.63 (0.71, 3.81) ^c	1.25 (0.77, 2.05)	0.58 (0.41, 0.81)	0.68 (0.44, 1.05)
Diabetes duration (per 10 years)	1.16 (0.93, 1.47)	1.31 (1.08, 1.60)	1.48 (0.82, 2.73)	0.93 (0.53, 1.61)	1.25 (0.95, 1.66)	1.34 (1.13, 1.61)	1.30 (1.04, 1.64)
HbA1c (per 1%)	1.21 (0.97, 1.53)	1.26 (1.10, 1.45)	0.98 (0.77, 1.25)	1.66 (1.24, 2.29)	1.36 (1.14, 1.65)	1.08 (0.94, 1.24)	1.22 (1.02, 1.46)
Mean arterial pressure (per 10 mmHg)	1.20 (0.96, 1.49)	1.07 (0.90, 1.27)	0.98 (0.65, 1.49)	1.05 (0.74, 1.49)	1.14 (0.91, 1.45)	1.07 (0.91, 1.26)	1.10 (0.89, 1.36)
UACR (per doubling)	1.39 (1.27, 1.53)	1.14 (1.04, 1.24)	1.10 (0.91, 1.36)	1.60 (1.07, 2.46)	1.32 (1.03, 1.70)	0.98 (0.83, 1.16) ^d	1.06 (0.85, 1.32)
eGFR (per 10 ml/min/1.73m ²)	0.79 (0.61, 1.03)	1.75 (1.38, 2.24)	1.97 (1.09, 3.79)	1.15 (0.99, 1.35)	1.11 (0.99, 1.24)	1.80 (1.60, 2.04)	1.83 (1.37, 2.47)

Cell contents are odds ratios (95% CI) adjusted for age, sex, diabetes duration, and baseline HbA1c, MAP, log₂(UACR), and eGFR.

*Other combinations of UACR and eGFR strata had small cell-sizes, and hence were not evaluated.

The following interactions were significant at a p < 0.10:

- eGFR(90,120]*covariate interaction significant for log₂(UACR) (p=0.002), baseline eGFR (P < 0.001); Reference category: eGFR[60,90]
- eGFR(120,150]*covariate interaction marginally significant for sex (p=0.09); significant for log₂(UACR) (p=0.04), baseline eGFR (P < 0.01); Reference category: eGFR(60,90)
- Macroalbuminuria*covariate interaction significant for sex (p=0.02), HbA1c (p=0.01), log₂(UACR) (p=0.03), baseline eGFR (p < 0.001); Reference category: Normoalbuminuria group
- Microalbuminuria*covariate interaction significant for sex (p=0.01), HbA1c (p=0.047), log₂(UACR) (p = 0.048), baseline eGFR (p < 0.001)

Additional References for Supplementary Material

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Table 1: Baseline characteristics of participants in the JDRF Biomarkers Consortium by cohort and case-control status

	Overall n = 1430	By cohort				By case-control status	
		FinnDiane n = 578	Steno n = 362	EDC n = 146	CACTI n = 344	Case* n = 535	Control** n = 895
<i>Cases</i>	535 (37)	299 (52)	53 (15)	66 (45)	117 (34)	-	-
<i>Demographics</i>							
Female sex	721 (50)	300 (52)	154 (43)	75 (51)	192 (56)	277 (52)	444 (50)
Age (years)	42.8 (12.7)	41.3 (12.4)	52.8 (12.0)	37.3 (7.9)	37.2 (9.1)	39.6 (12.6)	44.7 (12.4)
Race & ethnicity							
White	1400 (98)	578 (100)	362 (100)	140 (96)	320 (93)	518 (97)	882 (99)
Black	11 (1)	0 (0)	0 (0)	6 (4)	5 (1)	6 (1)	5 (1)
Hispanic	10 (1)	0 (0)	0 (0)	0 (0)	10 (3)	8 (1)	2 (0)
Smoking history							
Never	579 (40)	266 (46)	0 (0)	91 (62)	222 (65)	245 (46)	334 (37)
Past	238 (17)	131 (23)	0 (0)	33 (23)	74 (22)	103 (19)	135 (15)
Current	213 (15)	153 (26)	0 (0)	16 (11)	44 (13)	114 (21)	99 (11)
<i>Medical history and clinical characteristics</i>							
Diabetes duration (years)	26.8 (12.6)	25.6 (12.3)	30.9 (16.0)	29.3 (7.6)	23.5 (9.0)	25.8 (12.2)	27.4 (12.8)
Retinopathy status							
Present	658 (46)	263 (46)	228 (63)	71 (49)	96 (28)	235 (44)	423 (47)
Not present	709 (50)	309 (53)	77 (21)	75 (51)	248 (72)	278 (52)	431 (48)
Mean Arterial Pressure (mmHg)	94.6 (11.3)	98.6 (11.1)	96.4 (10.2)	85.3 (9.4)	90.2 (9.5)	95.8 (11.7)	94.0 (11.0)
Systolic Blood Pressure (mmHg)	129.9 (19.5)	137.0 (18.7)	136.5 (17.0)	116.3 (14.9)	116.8 (14.5)	131.3 (20.6)	129.1 (18.7)
Diastolic Blood Pressure (mmHg)	77.0 (10.2)	79.3 (10.2)	76.4 (10.4)	69.8 (9.7)	76.8 (8.8)	78.0 (10.6)	76.4 (10.0)
ACEi or ARB use							
Yes	440 (31)	290 (50)	230 (64)	33 (23)	117 (34)	246 (46)	424 (47)
No	624 (44)	284 (49)	132 (36)	113 (77)	227 (66)	286 (53)	470 (53)
Hypertension diagnosis							
Yes	643 (45)	395 (68)	74 (20)	25 (17)	149 (43)	283 (53)	360 (40)
No	725 (51)	179 (31)	231 (64)	121 (83)	194 (56)	230 (43)	495 (55)
<i>Laboratory data at baseline</i>							
HbA1c (%)	8.5 (1.3)	8.7 (1.6)	8.7 (0.7)	8.4 (1.4)	8.1 (1.3)	8.7 (1.6)	8.3 (1.1)
eGFR (ml/min/1.73m ²)	94 (24)	94 (25)	87 (15)	102 (17)	97 (30)	100 (27)	90 (21)
UACR (mg/g), median (IQR)	12 (5.2-63.5)	23.4 (6.4-123.5)	13.0 (6.0-41.0)	13.6 (6.8-47.8)	6.3 (4.2-16.6)	20.8 (6.4-196.6)	9.3 (5.0-35.3)
UACR group (mg/g)							
Macro: ≥ 300 mg/g	143 (10)	80 (14)	24 (7)	13 (9)	26 (8)	99 (19)	44 (5)
Micro: >30, < 300 mg/g	323 (23)	159 (28)	91 (25)	34 (23)	39 (11)	134 (25)	189 (21)
Normal ≤ 30 mg/g	881 (62)	286 (49)	246 (68)	99 (68)	250 (73)	269 (50)	612 (68)
eGFR slope (ml/min/1.73m ² /y)	-1.8 (4.4)	-3.0 (4.0)	-0.2 (5.0)	-2.5 (2.7)	-1.1 (4.2)	-5.7 (4.5)	0.6 (1.9)

* Case: eGFR slope/yr \leq -3 ml/min/1.73m²

**Control: eGFR slope/yr $>$ -1 ml/min/1.73m²

Entries are mean (SD) for continuous variables and N (%) for categorical variables, unless otherwise indicated. Percentages are calculated as percent of total values.

Number (%) of missing values for each variable in the overall study population: race & ethnicity 9 (1), smoking history 400 (28), diabetes duration 1 (<1), retinopathy status 63 (4), MAP 7 (<1), SBP 7 (<1), DBP 7 (<1), antihypertensive medication 4 (<1), hypertension diagnosis 62 (4), HbA1c 8 (1), baseline UACR 83 (6).

JDRF = Juvenile Diabetes Research Foundation; CACTI = Coronary Artery Calcification in Type 1 diabetes study; EDC = Pittsburgh Epidemiology of Diabetes Complications study; FinnDiane = Finnish Diabetic Nephropathy Study; Steno = Steno Diabetes Center Study; ACEi = angiotensin-converting enzyme inhibitor; ARB = angiotensin receptor blocker

Table 2: Associations of clinical characteristics with rapid eGFR decline among participants from the JDRF Biomarkers Consortium

	Model 1 OR (95% CI)	Model 2 OR (95% CI)	Model 3 OR (95% CI)
Age (per 10 years)	0.72 (0.66, 0.79)*	0.63 (0.56, 0.72)*	0.76 (0.66, 0.87)*
Sex (ref: female)	0.99 (0.80, 1.24)	0.89 (0.70, 1.13)	0.82 (0.64, 1.05)
Diabetes duration (per 10 years)		1.13 (0.99, 1.29)	1.20 (1.05, 1.37)*
HbA1c (per 1%)		1.17 (1.07, 1.29)*	1.15 (1.05, 1.27)*
Mean Arterial Pressure (per 10 mmHg)		1.08 (0.96, 1.21)	1.11 (0.99, 1.25)
UACR (per doubling)		1.19 (1.13, 1.25)*	1.30 (1.23, 1.38)*
eGFR (per 10 ml/min/1.73m ²)			1.31 (1.23, 1.41)*
AUC median (95% CI)	0.61 (0.56, 0.67)	0.69 (0.63, 0.75)	0.74 (0.68, 0.79)

Full Cohort: N = 1430; N (cases) = 535. Cell contents are odds ratios (OR) and 95% CI from logistic regression models, as well as cross-validated area under the curve (AUC; median 95% CI).

*p < 0.05

Fig. 1. Study design of JDRF Biomarkers Consortium, a case-control study nested in four T1D cohorts.

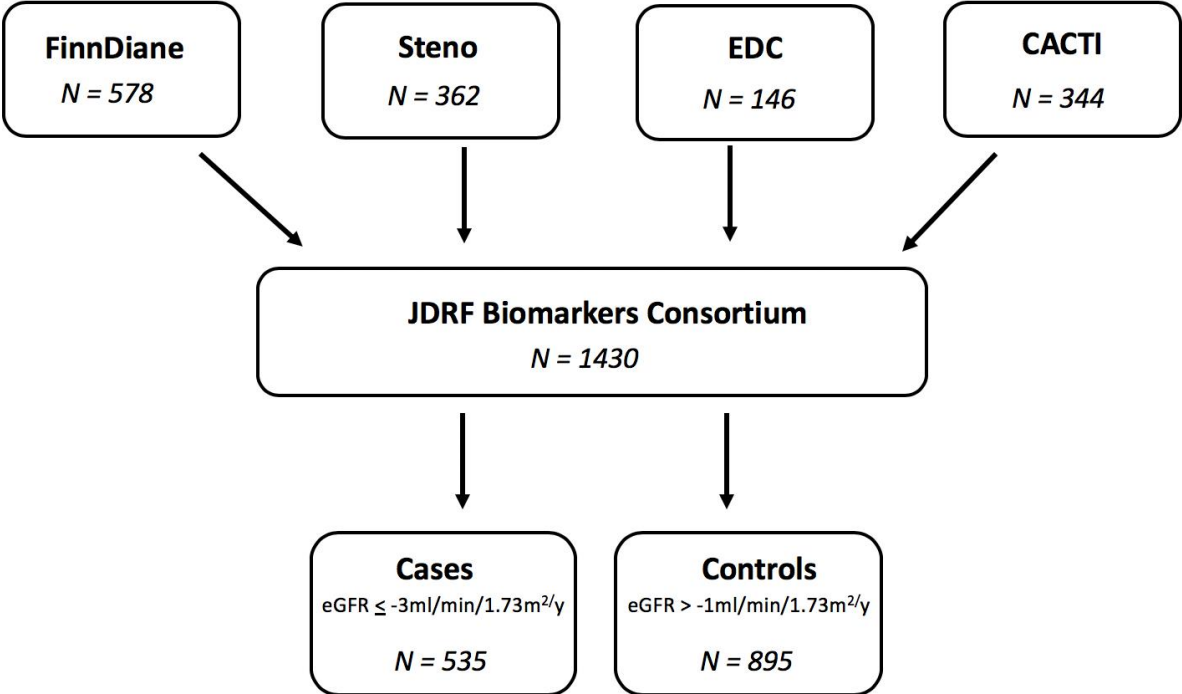
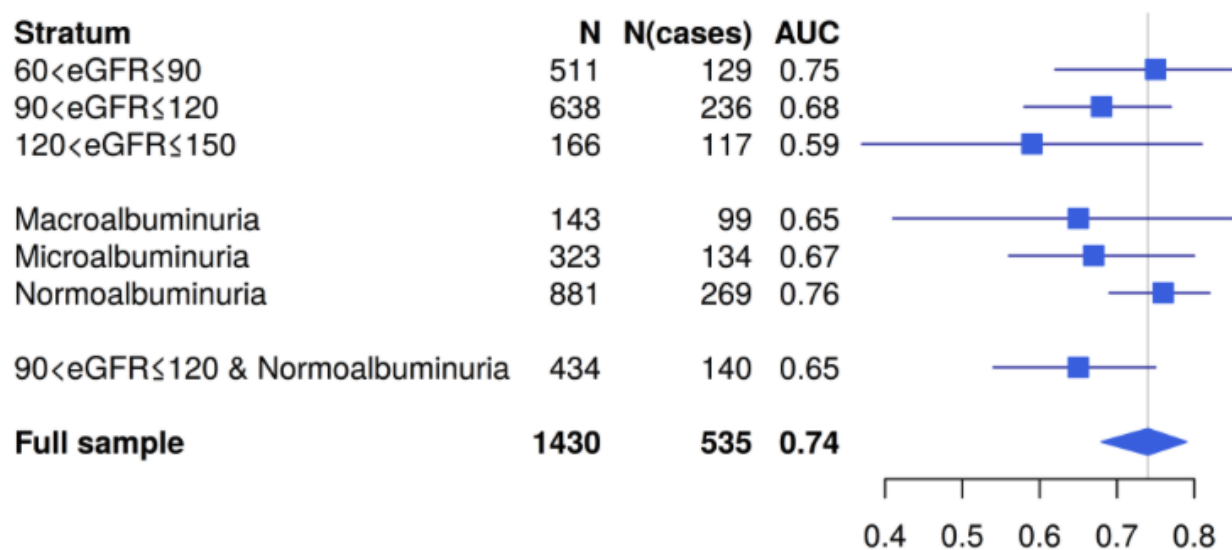


Fig. 2. Performance of demographic and clinical variables in the prediction of rapid eGFR loss according to baseline eGFR and urine albumin-creatinine ratio.



Presented values are cross-validated area under the curve (AUC; median 95% CI).