Earlier detection of microalbuminuria in diabetic patients using a new urinary albumin assay

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Background. Microalbuminuria is regarded as the most important predictor of high risk for the development of diabetic nephropathy. Early detection may allow treatment to prevent progression to persistent albuminuria and renal failure. Recent studies have shown that conventional immunoassays underestimate urinary albumin concentration, as albumin in urine may exist in two forms, immuno-reactive and immuno-unreactive. The present study examines the differential lead-time for the development of microalbuminuria as measured by both conventional radioimmunoassay (RIA; measures immuno-reactive) and high-performance liquid chromatography (HPLC; measures total albumin = immuno-reactive plus immuno-unreactive) analysis in both type 1 and type 2 diabetic patients.

Methods. Analysis was performed on 511 stored urine samples collected over the last 13 years from type 1 diabetic patients who either progressed from normo- to microalbuminuria (progressors, $N = 17$), or who remained normoalbuminuric (nonprogressors, $N = 25$) as defined by RIA, and on 634 urine samples collected from patients with type 2 diabetes defined as either progressors ($N = 24$) or nonprogressors ($N = 25$).

Results. For type 1 progressors, the mean lead-time for the HPLC assay versus the RIA was 3.9 years, with a 95% CI of 2.1 to 5.6 years. For type 2 progressors, the mean lead-time was 2.4 years with a 95% CI of 1.2 to 3.5 years. There was no significant difference between the lead-time analysis between type 1 and type 2 diabetic patients.

Conclusion. These results demonstrate that measurement of total albumin may allow earlier detection of microalbuminuria associated with diabetic nephropathy.

Microalbuminuria, defined as an albumin excretion rate (AER) of between 20 and 200 µg/min (30 to 300 mg/day) as measured by immunochemical methods, is one of the earliest indicators of diabetic nephropathy in 20% to 30% of type 1 and type 2 diabetic patients [1]. Microalbuminuria has been confirmed as the best-documented predictor of high risk for development of diabetic nephropathy (DN) in patients with either type 1 [2] or type 2 diabetes [3]. It is imperative to test for microalbuminuria because early detection and treatment with antihypertensive agents and improvements in glycemic control may slow or even prevent progression to persistent albuminuria and end-stage renal failure (ESRF) [2–5]. In addition to predicting nephropathy, microalbuminuria is also a marker of increased risk of cardiovascular disease, the leading cause of death in diabetic patients [1, 4, 6].

Recent studies have demonstrated that conventional immunoassays may underestimate albumin concentration, particularly in urine from diabetic patients, that is, diabetic patients excrete albumin that is not detected by immunoassays (immuno-unreactive) [7]. A new albumin assay using high-performance liquid chromatography (HPLC) (FDA approved) has been developed which can detect both immuno-reactive albumin plus immuno-unreactive albumin [7]. The exact nature of immuno-unreactive albumin is not known but may involve conformational changes as a result of biochemical modification during renal passage. Conventional antibodies generated against native serum albumin do not recognize the modified urinary albumin [7].

The present study set out to examine the differential lead-time for the development of microalbuminuria (AER >20 µg/min) as measured by both conventional radioimmunoassay (RIA; only measures immuno-reactive albumin) and HPLC (measures total albumin including immuno-reactive albumin plus immuno-unreactive albumin) in both type 1 and type 2 diabetic patients. Patients who did not progress to microalbuminuria were also studied.

METHODS

Selection of study participants

The Endocrine Unit at the Austin & Repatriation Medical Center, a tertiary referral center and teaching hospital of the University of Melbourne, Victoria, Australia, has a database of approximately 2500 diabetic patients. Twenty-four–hour urine samples from these patients have been collected and stored at $-20°C$ for the past 15 years. Patient records were analyzed retrospectively and chronologically to find patients which satisfied our inclusion criteria. Only patients attending the Endocrine Clinic at least every 6 months were eligible for inclusion. Patients who were found to have >12 years normoalbuminuria (<20 μg/min) as determined by immunoturbidimetry were included in the study and defined as nonprogressors. Patients who were found to have normoalbuminuria (before transition to microalbuminuria), where there were at least 3 successive urines >20 μg/min as determined by immunoturbidimetry, were included in the study and defined as progressors. Eligible patients were included chronologically until there was a total of 25 type 1 and 25 type 2 nonprogressors, and 17 type 1 (there were only this many patients of this type in the database) and 24 type 2 progressors. Stored urines from each of these patients were chosen entirely at random, ensuring that there was a sample every 6 to 12 months. A total of 511 and 634 urines were examined for the type 1 and type 2 diabetics, respectively. Immunoturbidimetry was used clinically to determine AER, and these results were used retrospectively to determine those patients eligible for inclusion in the study. There was no normalization of AER for age or glomerular filtration rate (GFR). Subsequently, the urines were reanalyzed by RIA (described below), first to ensure that there was no storage artefact, and second to report finite AER levels to be compared with HPLC analysis because the detection limit of the immunoturbidimetry assay used is 6 mg/L, and the detection limit of the RIA is 16 μg/L.

Clinical evaluation

The following information was recorded in the patient's history and then abstracted for this study: patient's age and gender, duration of diabetes, HbA_{1c}, plasma sodium concentration, blood pressure, use of renin-angiotensin system (RAS) inhibitors, retinopathy status, and smoking history. No pre-microalbuminuric (assessed by conventional RIA) patients were studied while on RAS inhibitors. Blood pressure was measured after 5 minutes of recumbency using the disappearance of the Korotkoff sounds. The examination of the ocular fundi was by direct ophthalmoscopy after pupillary dilatation. Retinal lesions were categorized as either background or proliferative retinopathy.

Measurement of urinary albumin excretion

Total urinary albumin (immuno-reactive plus immuno-unreactive) excretion was measured using a newly developed HPLC assay (AusAm Biotechnologies, Inc., Santa Monica, CA, USA). Aliquots from 24-hour urine collections were stored at $-20°C$ until analysis. Aliquots of untreated urine (25 μL) were injected onto a Zorbax Bio series analytic, GF-250 column (either 9.4 mm inner diameter $\times$ 25 mm or 4.6 mm inner diameter $\times$ 25 mm) (Agilent Technologies, Wilmington, DE, USA) and analyzed using an Agilent 1100 HPLC system as previously described [7]. The mobile phase was phosphate-buffered saline (PBS) run at a flow rate of either 1 mL/min or 0.5 mL/min. The albumin peak in the urine sample was generally identified to within ±2% of the elution time of the monomer albumin standard. Low albumin concentration samples measured by HPLC were expressed as being <2 μg/mL. The intra-assay coefficients of variation (CVs) were 5.6% and 6.0% at concentrations of 44.7 mg/L and 141 mg/L, respectively, and the interassay CV was 2.4% at 95.9 mg/L.

Immunoreactive intact urinary albumin concentration was measured by two methods. The major method was a double antibody RIA with CVs of 9.2% and 4.8% at concentrations of 12.2 mg/L and 33 mg/L, respectively, and detection limit of 16 μg/L using an albumin standard (Fraction V; Sigma, St. Louis, MO, USA) that was made volumetrically. Antihuman albumin antibody was from Dako Corporation (Carpinteria, CA, USA).

Immunoreactive intact urinary albumin concentration was also measured by immunoturbidimetry using a Dade-Behring Turbitimer with reagents and calibrators, supplied by Dade-Behring Marburg GmbH (Marburg, Germany). The CVs were 4.1%, 2.2%, and 4.2% at concentrations of 10.6 mg/L, 43.2 mg/L, and 77.9 mg/L, respectively, and detection limit of 6 mg/L. The RIA and immunoturbidimetry assays have been shown to be equivalent [8, 9].

Laboratory assays

Urine and plasma electrolyses were measured on a Hitachi 911 Automatic Analyzer (Roche Diagnostics, Mannheim, Germany). Glycosylated hemoglobin (HbA_{1c}) was measured by automated HPLC (Biorad Diamat, Richmond, CA, USA) or by HbA_{1c} Analyzer (Primus; Kansas City, MO, USA). Fasting lipids were measured by enzymatic calorimetry.

Statistical analysis

Data are expressed as mean ± standard error, where N represents the number of determinations. Data analysis was performed using Minitab version 12.23 (Minitab,

Inc., State College, PA, USA). Data was analyzed for normality using the Anderson-Darling Normality test [10]. The mean and median difference in time taken to reach microalbuminuria (AER >20 μg/min as determined by the geometric mean of three consecutive urine samples) as measured by HPLC versus RIA were determined including 95% CI. Diagnostic specificity and diagnostic sensitivity were obtained using the Normal approximation to the Bionomial Distribution. Comparison of the clinical characteristics between the study patients were performed using a two sample *t* test using SigmaStat (SPSS, Chicago, IL, USA). *P* < 0.05 was considered statistically significant.

Definitions

Persistent microalbuminuria is defined as being present when the geometric mean of three consecutive urine samples exceeds 20 μg/min by RIA. Nonprogressors are defined as patients with >12 years normoalbuminuria (AER <20 μg/min) as determined by immunoturbidimetry. Progressors are defined as patients who were found to have normoalbuminuria (before transition to microalbuminuria), where there are at least 3 successive urines with AER >20 μg/min as determined by immunoturbidimetry.

RESULTS

Clinical characteristics for the type 1 diabetic progressors (*N* = 17) and nonprogressors (*N* = 25) were ascertained (Table 1). Both progressors and nonprogressors had similar ages and duration of diabetes. One third of progressors and 44% of nonprogressors were female. There was no significant difference in systolic blood pressure between progressors and nonprogressors at the start or at the end of the study. Systolic blood pressure increased in both groups over the study period. Diastolic blood pressure was significantly different between progressors and nonprogressors at the start but not at the end of the study. The mean HbA$_{1c}$ was significantly higher in progressors over the course of the study. There was a significant difference in the final plasma sodium between progressors and nonprogressors. The severity of retinopathy was greater in progressors than nonprogressors. A greater proportion of smokers were progressors.

Clinical characteristics for the type 2 diabetic progressors (*N* = 24) and nonprogressors (*N* = 25) were also ascertained (Table 2). Both progressors and nonprogressors had similar ages and duration of diabetes. There were approximately equal numbers of males and females in the progressor groups, and 71% females in nonprogressor groups. There was no significant difference in systolic blood pressure between progressors and nonprogressors at the start or at the end of the study. Diastolic blood pressure was significantly different between progressors and

Table 1. Clinical characteristics of the type 1 diabetic nonprogressors and progressors (mean ± standard error)

	Non-progressors (*N* = 25)	Progressors (*N* = 17)	Significance
Age at start *years*	37 ± 3	37 ± 3	NS
Sex (F/M)	11/14	6/11	
Duration of diabetes at start *years*	10.9 ± 2.0	12.5 ± 2.4	NS
Mean duration of urine sampling per patient *years*	11.4 ± 0.2	8.5 ± 0.6	*P* < 0.0001
Total number of urines analyzed	305	206	
Average time interval per urine sample *years*	0.9	0.7	
SBP at start *mm Hg*	121 ± 2	124 ± 6	NS
SBP at end *mm Hg*	134 ± 4	143 ± 6	NS
DBP at start *mm Hg*	74 ± 1	79 ± 2	*P* = 0.03
DBP at end *mm Hg*	76 ± 2	80 ± 2	NS
HbA$_{1c}$ at start %	8.2 ± 0.4	9.6 ± 0.5	*P* = 0.03
HbA$_{1c}$ at end %	7.5 ± 0.2	9.4 ± 0.4	*P* < 0.0001
Plasma sodium excretion at end *mmol/24h*	140 ± 0.5	138 ± 1	*P* = 0.03
Retinopathy status at end (N/B/P)	14/8/3	3/8/6	
Antihypertensive therapy at start %	0	0	
Antihypertensive therapy at end %	12	65	
Smoking history at start *Y/N*	3/22	7/10	
Smoking history at end *Y/N*	0/25	4/13	

Abbreviations are: SBP, systolic blood pressure; DBP, diastolic blood pressure; B, background retinopthy; P, proliferative retinopathy; Y, yes; N, no.

nonprogressors at the start but not at the end of the study. HbA$_{1c}$ was not significantly different for progressors and nonprogressors over the study period. There was no difference in the final plasma sodium between progressors and nonprogressors. The severity of retinopathy was not significantly different between progressors and nonprogressors. There were a similar proportion of smokers in both groups.

The urines from patients examined in this study were studied up until February of 2001. We have subsequently done a follow-up study of the patients until August of 2003. There was no follow-up data available for 6/25 and 9/25 of the type 1 and type 2 nonprogressors, respectively, and 8/17 and 8/24 of the type 1 and type 2 progressors, respectively. This was a result of death or discontinued attendance at the clinic. None of the remaining nonprogressors developed microalbuminuria as measured by immunoturbidimetry. Table 3 shows the number of type 1 and type 2 progressors, for whom there was follow-up data available, with normo-, micro-, or macroalbuminuria in February of 2001 compared with August of 2003 as measured by immunoturbidimetry.

Stability of stored samples in relation to immunoassays

The equivalence of the immunoturbidimetry assay and RIA at an albumin concentration >6 mg/L has been

Table 2. Clinical characteristics of the type 2 diabetic nonprogressors and progressors (mean ± standard error)

	Non-progressors (N = 25)	Progressors (N = 24)	Significance
Age at start *years*	59 ± 2	61 ± 2	NS
Sex (F/M)	17/8	13/11	
Duration of diabetes at start *years*	11.7 ± 1.5	9.0 ± 1.4	P = 0.19
Mean duration of urine sampling per patient *years*	10.7 ± 0.4	7.5 ± 0.4	P < 0.0001
Total number of urines analyzed	291	343	
Average time interval per urine sample *years*	0.9	0.5	
SBP at start *mm Hg*	147 ± 5	147 ± 4	NS
SBP at end *mm Hg*	147 ± 4	153 ± 4	NS
DBP at start *mm Hg*	76 ± 2	86 ± 2	P = 0.0013
DBP at end *mm Hg*	80 ± 2	80 ± 1	NS
HbA$_{1c}$ at start *%*	8.3 ± 0.4	8.8 ± 0.4	NS
HbA$_{1c}$ at end *%*	7.9 ± 0.2	8.7 ± 0.6	NS
Plasma sodium excretion at end *mmol/24h*	142 ± 1	140 ± 1	NS
Retinopathy status at end (N/B/P)	18/4/3	16/4/4	
Antihypertensive therapy at start *%*	4	12.5	
Antihypertensive therapy at end *%*	44	50	
Smoking history at start *Y/N*	3/22	4/21	
Smoking history at end *Y/N*	2/23	4/21	

Abbreviations are: SBP, systolic blood pressure; DBP, diastolic blood pressure; B, background retinopthy; P, proliferative retinopathy; Y, yes; N, no.
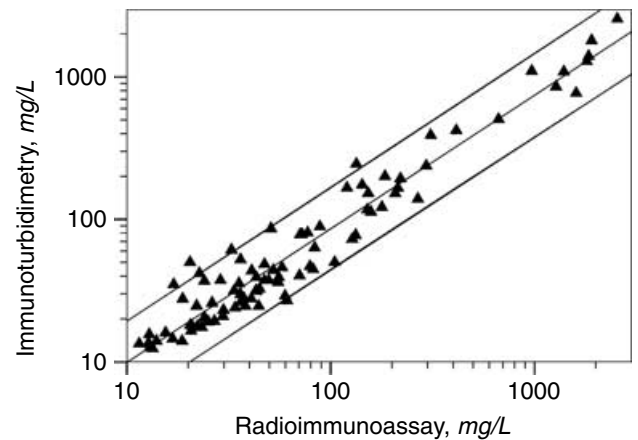
Table 3. Number of type 1 and type 2 progressors, for whom there was follow-up data available, with normo-, micro-, or macroalbuminuria in February 2001 compared with August 2003 as measured by immunoturbidimetry

	Type 1		Type 2	
	Sept. 2001	Aug. 2003	Sept. 2001	Aug. 2003
Normoalbuminuria	0/9	1/9	0/16	1/16
Microalbuminuria	6/9	4/9	13/16	13/16
Macroalbuminuria	3/9	4/9	3/16	2/16

Fig. 1. Comparison of urinary albumin excretion measured by immunoturbidimetry at the time of urine collection (spanning 13 years) and measured by radioimmunoassay (RIA) (range 7–2500 mg/L) in June to September, 2001. y = 0.93x + 0.5 (N = 92, r = 0.97), where x and y represent results obtained by RIA and immunoturbidimetry, respectively. Predicted 95% CIs are also shown.

both RIA and HPLC was examined in both type 1 and type 2 diabetic patients. Among type 1 progressors, the mean lead-time for the HPLC assay versus the RIA was 3.9 years, with a 95% CI of 2.1 to 5.6 years (Fig. 2). For type 2 progressors, the mean lead-time was 2.4 years with a 95% CI of 1.2 to 3.5 years (Fig. 3). In both cases the Anderson-Darling Normality Test [10] showed that the individual lead times were not normally distributed, as would be expected from the non-negative nature of the data; however, the CIs remain valid because of the Central Limit Theorem. There was no significant difference between the lead-time analysis between type 1 and type 2 diabetic patients (two-sample *t* test, P = 0.14).

Diagnostic specificity and sensitivity

CIs and hypothesis tests for the proportions of false positives (diagnostic specificity) and false negatives (diagnostic sensitivity) were obtained using the Normal approximation to the Binomial distribution. For the type 1 nonprogressors there was no significant difference (P = 0.056) between the false positives for the HPLC assay (6/280 urines, 2.1%, CI of 0.8–4.6) and RIA (1/280 urines, 0.4%, CI of 0–2.0). For the type 2 nonprogressors, the false positives for the HPLC assay (28/265 urines, 10.6%, CI of 7.1–14.9) were significantly higher (P < 0.0005) than those for the RIA (0/265 urines, 0%, CI of 0–1.4). For the type 1 progressors, the false negatives for the RIA (67/121 urines, 55.4%, CI of 46.1–64.4) were significantly higher (P < 0.0005) than those for the HPLC (0/121 urines, 0%, CI of 0–3.0). Similarly, for the type 2 progressors the false negatives for the RIA (78/190 urines, 41.1%, CI of 33.9–48.4) were significantly higher (P < 0.0005) than those for the HPLC (5/190 urines, 2.6%, CI of 33.9–48.4).
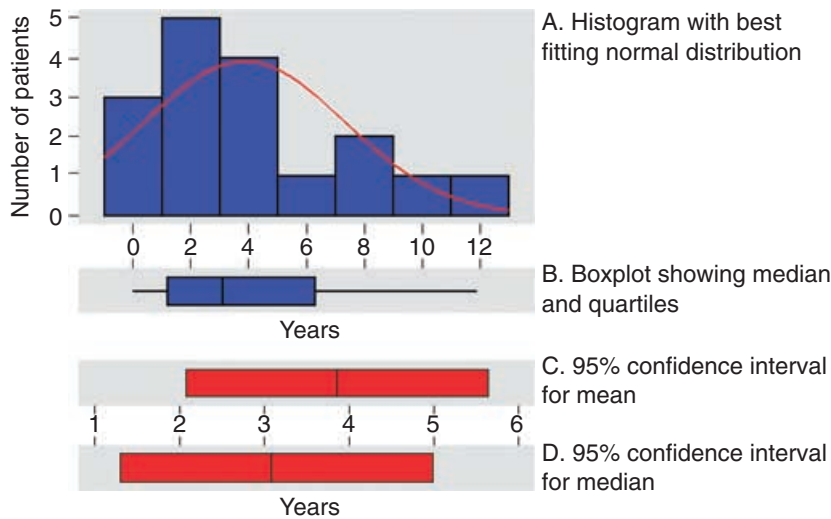
previously reported [8, 9]. In this study, linear regression analysis of stored diabetic urines measured by immunoturbidimetry at the time of collection and by RIA (range 7 to 2500 mg/L) in June through September of 2001 showed good agreement (Fig. 1), demonstrating that there is no storage artefact for urinary albumin; y = 0.93x + 0.5 (N = 92, r = 0.97), where x and y represent results obtained by RIA and immunoturbidimetry, respectively. Immuno-unreactive albumin is also not formed as a result of storage because it is present in fresh diabetic urine [7].

The onset of persistent microalbuminuria as determined by HPLC compared with RIA

The differential lead-time for the development of microalbuminuria (AER >20 μg/min) as determined by

Comper et al: Earlier detection of microalbuminuria in diabetic patients

Fig. 2. **Differential lead-time analysis for the development of microalbuminuria [albumin excretion rate (AER) >20 µg/min as determined by the geometric mean of three consecutive urine samples] as determined by both radioimmunoassay (RIA) and high-performance liquid chromatography (HPLC) for type 1 diabetic patients.** (*A*) Histogram and best fitting Normal distribution showing individual patient data for the time taken (in years) for the detection of microalbuminuira as measured by HPLC versus RIA. (*B*) Boxplot showing median and quartiles for the differential time taken for the detection of microalbuminuria. (*C*) 95% CI for the mean difference in time taken for the detection of microalbuminuria, and (*D*) 95% CI for the median difference in time taken for the detection of microalbuminuria.
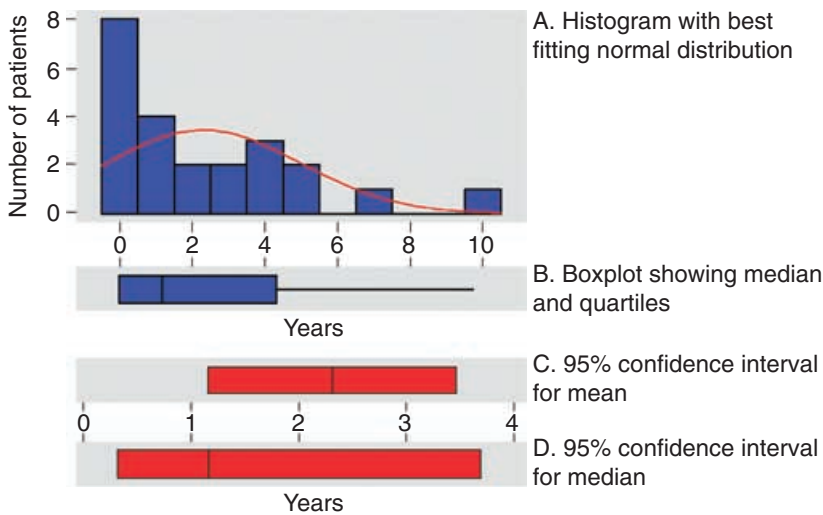

Fig. 3. **Differential lead-time analysis for the development of microalbuminuria [albumin excretion rate (AER) >20 µg/min as determined by the geometric mean of three consecutive urine samples] as determined by both radioimmunoassay (RIA) and high-performance liquid chromatography (HPLC) for type 2 diabetic patients.** (*A*) Histogram and best fitting Normal distribution showing individual patient data for the time taken (in years) for the detection of microalbuminuira as measured by HPLC versus RIA. (*B*) Boxplot showing median and quartiles for the differential time taken for the detection of microalbuminuria. (*C*) 95% CI for the mean difference in time taken for the detection of microalbuminuria, and (*D*) 95% CI for the median difference in time taken for the detection of microalbuminuria.

DISCUSSION

This study demonstrates that the new HPLC assay is able to detect microalbuminuria far earlier than a conventional immunoassay for both type 1 and type 2 diabetic patients. Correspondingly, the proportion of false negatives associated with the conventional immunoassay is profoundly high (range 41% to 55%) compared with the proportion of false positives for the HPLC assay (<11%). Overall, most of the false positives were associated with relatively older patients within each nonprogressor group. This is most probably a result of age-dependent microalbuminuria that appears in the general population [11]. We have also shown in a previous study of a nondiabetic control group that the proportion of false positives for the HPLC is less than 4% [7]. Despite the fact that this is a pilot study with a small number of patients, the results stress the need for an accurate measure-

ment of AER in diabetic patients, and that total albumin may provide a more accurate and earlier detection of microalbuminuria than conventional immunoassay. These findings should be confirmed in a larger study.

Urine consists of a complex mixture of immuno-reactive and immuno-unreactive components, as we have previously described [7, 12–14]. Immunoassays can only detect immuno-reactive intact urinary albumin [7, 12–14] as well as albumin fragments >12 kD and polymer albumin aggregates [15–17]; however, the exact nature by which albumin components are detected by immunoassays remains poorly defined. HPLC analysis of urinary albumin is well defined because this assay is able to detect both intact immuno-reactive albumin, as well as intact immuno-unreactive albumin [7]. Currently, immunoassays and HPLC are unable to quantitatively measure albumin-derived fragments (<10 kD).

CONCLUSION

The results presented in this study suggest that conventional immunochemical-based assays considerably underestimate urinary albumin excretion. The discrepancy between the HPLC assay and immunochemical-based assays demonstrates that the HPLC albumin assay may provide a relatively early diagnosis of incipient kidney disease at a diagnostic threshold of 20 µg/min.

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