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Short title: Variation in GFR decline in type 2 diabetic nephropathy
ABSTRACT

Variation in the decline of GFR in type 2 diabetic Pima Indians with albuminuria

Background. The course of type 2 diabetic nephropathy often includes many years of hyperfiltration followed by a relatively rapid progression to end-stage kidney failure. It is not clear how much variation exists in the rate of progression within a single genetically similar population.

Methods. In an 8-year longitudinal study of Pima Indians with type 2 diabetes and nephropathy, we compared GFR in individuals enrolled with new-onset microalbuminuria who progressed to macroalbuminuria during follow-up (n=13), microalbuminuric subjects who did not progress (n=13), and subjects with new-onset macroalbuminuria at screening (n=22). Patients had urine albumin/creatinine ratio determined every 3-6 months and GFR determined serially by urinary iothalmate clearances. GFR courses were modeled using an adaptation of smoothing and regression cubic B-splines.

Results. GFR profiles of progressors differed significantly from those of non-progressors (P=0.003); average GFR slopes were -9.3 mL/min/y and -4.9 mL/min/y, respectively. There were no significant baseline differences between progressors and non-progressors with respect to any measured clinical parameters. The course of GFR following progression to macroalbuminuria in initially microalbuminuric subjects also differed from that in new-onset macroalbuminuric subjects. The slope in the latter was -16.9 mL/min/y versus -10.5 mL/min/y in the former.

Conclusions. Type 2 diabetic Pima Indians manifest different rates of decline in their GFR depending on their level of albuminuria at screening and its subsequent evolution. These findings suggest that the course of decline of GFR in diabetic Pima Indians, although generally progressive, follows distinct trajectories in definable subgroups.

Keywords: Hotelling's T², splines, iothalmate clearance, nephropathy, progression
INTRODUCTION

The natural history of diabetic nephropathy is characterized by a variable period of hyperfiltration followed by an inexorably progressive decline of GFR once overt proteinuria appears [1]. Variations in the rate of GFR decline in patients with type 2 diabetic nephropathy have been described before in studies with short follow-up periods [2]. The Pima Indians of Arizona have a very high incidence rate of type 2 diabetes mellitus and an inordinately high risk of end-stage kidney failure due to diabetic nephropathy. Considerable heterogeneity in the actual course of diabetic nephropathy, however, is present in this population. For example, we reported earlier that the pattern of progression from microalbuminuria to macroalbuminuria may be quite variable [3].

Differences in the patterns of decline of GFR due to diabetic nephropathy – both between and within defined populations – are probably influenced by a number of different environmental and genetic factors. The level of glycemic control (as reflected in the HbA1c), the prevalence of hypertension and hyperlipidemia, and smoking habits are some of the environmental factors that have been shown to influence the risk of renal failure. Genetic factors may explain familial clustering of nephropathy in diabetic individuals [4]. An association of ACE genotype with progression of diabetic nephropathy was found in some studies, but not in others [5-7]. The influence of ACE genotype on progression risk may be modified by other background genetic factors, as it seems to vary among different racial groups [8]. In addition, differences in glomerular size may also influence the rate of GFR decline during the terminal, rapidly progressive phase of nephropathy, beyond their effects on the rate of development of glomerular sclerosis [9].

The purpose of the present study was to investigate differences in the courses of GFR decline among Pima Indians with type 2 diabetes and incipient or overt nephropathy by examining the GFR courses over 8 years of follow-up in subjects with either microalbuminuria or macroalbuminuria newly detected at screening. Robust statistical techniques were adapted to a quantitative analysis of the varying courses of GFR during the evolution of diabetic nephropathy in this population. We compared the GFR courses of initially microalbuminuric subjects who progressed to macroalbuminuria to subjects who remained persistently microalbuminuric. The course of GFR after progression to macroalbuminuria in initially microalbuminuric subjects was also compared with that in subjects with macroalbuminuria newly detected at screening. Our findings suggest that several distinct patterns exist in the decline of GFR within this population, patterns that depend in large part on the presenting level of albuminuria and its subsequent evolution.
METHODS

Experimental subjects

Subjects were recruited from a larger cohort of Pima Indians with type 2 diabetes whose renal function was studied over 4 years [1]. Of these subjects, 22 individuals with new-onset macroalbuminuria and 26 with new-onset microalbuminuria at the time of screening and enrollment continued to be studied for a total follow-up of at least 8 years. Macroalbuminuria and microalbuminuria were defined based on results of the geometric mean of the albumin/creatinine ratio (ACR) in 3 screening urine samples together with the ACR in 2 subsequent samples following enrollment. Individuals were classified on the basis of having at least two of these three values for urinary ACR within the microalbuminuric (30-299 mcg/mg) or macroalbuminuric (≥ 300 mcg/mg) range. All members of the Pima Indian population in the Gila River Indian Community are invited to undergo ACR screening approximately every two years. Particular attention was paid to examining all available records of quantitative proteinuria or albuminuria of the enrolled subjects so as to exclude any individuals in whom prior evidence of either microalbuminuria or macroalbuminuria was found.

During this extended follow-up, 13 of the initially microalbuminuric subjects progressed to macroalbuminuria, and 13 remained persistently microalbuminuric. The approximate date of progression was determined from the time course of ACR values by the consensus of three observers. This approach was necessitated by the significant variability often seen in the course of ACR in such subjects [3].

The GFR courses after progression to macroalbuminuria were examined in 11 of the initially microalbuminuric subjects who had at least 4 years of follow-up after progression. In addition to these eleven progressors, we studied the post-progression courses in seven microalbuminuric subjects, whose ascertainment with microalbuminuria preceded enrollment in the study, but who progressed to persistent macroalbuminuria under observation. Thus, only the macroalbuminuric stages of these individuals’ GFR courses were included in the present study.

Study Protocol

The original four-year longitudinal study of Pima Indian subjects with microalbuminuria or macroalbuminuria [1] envisaged physiologic studies (GFR, renal plasma flow, etc.) occurring every 6 months. This frequency of sampling was accomplished in about one-half of the patients for the first four years. In the remaining one-half of the subjects the first follow-up study was one year after the baseline studies, with the study frequency increasing to 6 monthly thereafter. Although many of the participants continued to have regular physiologic studies as a part of a long-term extension of the original four-year protocol, many of the microalbuminuric subjects had a hiatus of about 2 years in their physiologic studies before recommencing intensified follow-up for a final 3-4 years. Hence the sampling of physiologic data was irregularly spaced. The resulting difficulties
with tracking raw data were overcome by suitably adapting the technique of smoothing splines (vide infra).

**Laboratory methods**

GFR was estimated by urinary iothalmalate clearance and renal plasma flow (RPF) by PAH clearance. The filtration fraction was calculated as the ratio of GFR to RPF. The plasma oncotic pressure ($\pi_A$) was determined by membrane osmometry, urinary albumin concentration by nephelometry or ELISA, and HbA1c by HPLC, as previously described [3]. In some cases, values for HbA1c were inferred from HbA1 measurements using electrophoresis, by means of a regression equation based on samples from 133 patients for which both methods were used [10]. Serum cholesterol was measured by a colorimetric method.

The iothalmalate and PAH clearance studies consisted of four timed urine collections bracketed by five plasma samples starting one hour after bolus intravenous infusions of iothalmalate (300 mg for subjects up to 100 kg BW, 3 mg/kg for subjects > 100 kg BW) and PAH (8 mg/kg for subjects with serum creatinine concentrations less than 2.0 mg/dL; 4 mg/kg for subjects with serum creatinine concentrations $\geq$ 2.0 mg/gL). The bolus was followed by continuous infusions to maintain plasma levels of 1-2 mg/dL and 1-3 mg/dL for the two clearance markers, respectively. The clearances were conducted during a sustained diuresis brought about by drinking 10 mL/kg BW of water (maximum 900 mL) initially and replacing urinary losses throughout the study. A PAH extraction ratio of 0.85 (for the calculation of effective renal plasma flow) was used for subjects with a GFR $\geq$ 80 mL/min; a ratio of 0.70 was used if GFR was < 80 mL/min [11]. GFR and RPF were not adjusted for body surface area, to avoid changes due only to secular increases in body weight.

**Morphometric Analysis**

Four microalbuminuric progressors and four non-progressors had baseline biopsies as part of an earlier study [12]. Briefly, 14G needle cores were prepared for light microscopic and ultrastructural analysis using routine methods. Cores were embedded in Epon and serially sectioned at 2.5 $\mu$m intervals. Glomeruli in the sections were classified as sclerotic if the sections exhibited a loss of capillary structure with solidification of the tuft. The apparent prevalence of sclerotic glomeruli was corrected for the tendency of these glomeruli, which are smaller than patent glomeruli, to be completely included more often in biopsy cores. Glomerular volumes were estimated using the maximum planar area method. Fractional interstitial area was estimated by point counting. Midsections of three patent glomeruli from each core were re-embedded and thin sectioned for ultrastructural examination at $\times$1600 to $\times$2820 by electron microscopy. The average numbers of epithelial and endocapillary cells per glomerulus were calculated using the method of Weibel [13] on montages of entire glomerular profiles. Estimates of GBM thickness were made using the orthogonal intercept method [14]. GBM thickness and
foot process width were measured on high-power (×9400 to ×11280) micrographs of the same glomeruli.

Statistics and curve estimation.

The courses of GFR were estimated for each patient in a manner to be described, always from iothalamate clearance data (with 4 measurements per study). An average of 11 complete clearance studies were obtained, the range being 6-19. Clearance values of 3 and 7 mL/min were imputed for the earliest time of evaluation when the patient was on dialysis. It happens that when dialysis begins, the GFR is roughly 5mL/min. The fitting for each individual was from study entry either to last measured GFR or to onset of dialysis. For the comparisons of initially microalbuminuric progressors and non-progressors, the fitted curves were evaluated at equally spaced points over 8 years. The courses of progressors, from the points of progression, were compared to those of initially macroalbuminuric subjects by evaluating fitted curves at equally spaced points over 4 years. These are the periods for which complete data were available for patients we studied.

We assume that data for individuals are consistent with a random coefficient model with basis functions consisting of 21 cubic B-splines with knots separated by 18 months on a fixed grid [16]. For any patient, most basis functions are identically 0 over the domain of his or her observations, the domain for which GFR is fitted for the patient. Those basis functions are not involved in the fitting for that fixed patient. Our technology is not at all dependent on the exact cardinality of the basis other than requiring that it be finite. Neither is it dependent on the B-spline functional form. However, the technology is for functions of a priori fixed form and therefore that are not adaptive. For the $i$th individual, the $k$th measurement of variable $y$ (GFR) at time $t_{ij}$ is given by:

$$y_{ijk} = y_i^k(t_{ij}) = \sum_{m=1}^{M} a_{im} B_m(t_{ij}) + \varepsilon_{ijk},$$

where $B_m$ is the $m$th basis function, $a_{im}$ is the $m$th random coefficient for subject $i$, and $\varepsilon_{ijk}$ is a random error with zero mean and variance $\sigma_y^2$. The covariance of observed data was estimated as a sum of two terms. One arises as a diagonal matrix that summarizes variances at the specific distinct times of measurement. The other summarizes variances and covariances of the random coefficients $\{a_{im}\}$. The latter cannot be computed directly, but are inferred from subtraction, as in many approaches to the estimation of variance components [15].

Much of our work was devoted to inferring the covariance structure of the random coefficients $\{a_{im}\}$ [17]. To begin fitting the random coefficient spline model, courses were fitted first by smoothing splines for each individual, where there were two smoothing parameters, one each for initially microalbuminuric and initially macroalbuminuric patients. All subsequent fitting was done separately for the two groups. One of our key assumptions is that the fixed points of time for which GFR is
inferred are weighted equally. The hiatus described in our study protocol entailed
difficulties similar to Gibbs effects for some fitted curves [18]. Our approach helped to
mitigate these difficulties. It was necessary to smooth in multiple stages because of the
above-mentioned erratic (Gibbs) behavior and the fact that the number of observation
times was of the same order as the number of (non-zero) basis functions in the range of
the data. Using regression splines alone would have yielded erratic fitted GFR curves.
Fitting was done using the time from onset of diabetes, while group comparisons were
made on the fitted curves aligned to the start of microalbuminuria or macroalbuminuria.

Fitting that was mentioned and that is now described in some detail was done separately
for initially microalbuminuric and macroalbuminuric subjects. The iterative process of
fitting began without covariances for the initial smoothing spline parameter that is
common to all patients (in each of the two groups). Subsequent iterations used the
empirical covariances on a two-dimensional grid of smoothing (λ) and variance
regularization (θ) parameters, as in Friedman’s approach to classification [19]. The
multiple of the identity that figures in our analogue to Friedman’s shrinkage is the median
of the current estimate of the eigenvalues of the covariance of \( \{a_m\} \). Values for λ and θ
were chosen by leave-one-out cross-validation [20]. Individual smoothing splines were
fitted by minimizing:

\[
(y - \hat{y}_i)^t \hat{\Sigma}_i^{-1} (y - \hat{y}_i) + \lambda \int [\hat{f}''(t)]^2 dt,
\]

where \( y_i \) is the column vector of measured GFR values for subject \( i \); \( \hat{y}_i = \hat{f}(t_i) \) is the
vector of fitted GFR values; \( \hat{\Sigma}_i \) is the estimated covariance of \( y_i \) (incorporating the
parameter θ); \( \lambda \) is the chosen smoothing parameter; and \( \hat{f}_i \) is the smoothing spline for
subject \( i \) (and \( \hat{f}_i'' \) its second derivative). Estimating the covariance of \( \{a_m\} \) began with
an empirical estimate of the covariances of the observed data. Because this approach
uses all available data, and the observations were not over a common set of points, this
estimated covariance generally was not non-negative definite. First the eigenstructure of
this estimated covariance was restricted, and then we chose the two regularization
parameters by cross-validation, as cited [17]. All this yielded the smoothing splines,
from which the subject-by-subject random coefficients were computed by linear
regression from their smoothing spline fits. The fits were done on six month, subject-
specific grids.

Statistical comparisons of the GFR courses (progressors vs non-progressors over 8 years
and the post-progression course of initially microalbuminuric subjects vs subjects
screened as macroalbuminuric over 4 years) were made on 5-component vectors. The
components were five inferred GFR values evenly spaced over the duration of follow-up
for each subject (i.e. either 4 or 8 years). A permutation approach [20] was used to
estimate the P value of the actual distributions of 5-vectors of these inferred values based
on a 2-sample Hotelling’s \( T^2 \) statistic [21]. The computer permutations for the estimate
were based on 1000 runs.
One of the initially microalbuminuric subjects had a GFR course that appeared to deviate significantly from the rest of that cohort. The GFR appeared to increase considerably at the end of follow-up, although there was also a large difference in GFR between the last two measurements. We tested for outlier status of this individual with respect to both the cohort of 13 newly microalbuminuric progressors and the expanded group of 18 microalbuminuric subjects (not all newly detected with microalbuminuria at study entry) who progressed to macroalbuminuria under observation. From the mean and covariance structure of the 5 fitted GFR values of the cohort, a Hotelling’s $T^2$ statistic was developed to compare the apparent outlier with the remainder of the groups [21]. The P values for the comparisons were 0.086 for the group of 13 individuals and 0.055 for the group of 18 individuals. We also varied the numbers of fitted values for which tests of outlier status were done. P-values were very small with few well-chosen points, and much larger for many such choices. Obviously, we might also have considered first and second order derivatives of the random cubic B-spline fits, though that remains a topic for future investigation at this point. In any case, P-values and appearance of the graphs led us to exclude the individual in question from the final analysis. Readers who are interested in Splus code can visit http://www-stat.stanford.edu/~olshen/manuscripts/BoothroydCode.pdf

For ease of clinical interpretation and due to the variable durations of follow-up, the change in GFR was also expressed as a time-averaged rate of change (mL/min/y) in the various groups. These rates were calculated by simple linear regression on the full nine fitted GFR values. Statistical comparisons were not performed on these average values, although approximate confidence intervals are presented.

Differences in baseline values of clinical and demographic parameters (age, gender, duration of diabetes, HbA1c, etc.) were evaluated by either $t$ test or Mann-Whitney test for continuous variables and Fisher’s exact test for categorical variables. ACR differences between groups were assessed by $t$ test on log [ACR]. Data are represented as mean±SD or median [interquartile range].
RESULTS

Glomerular function and structure

The baseline characteristics of the macroalbuminuric and microalbuminuric subjects are reported in Table 1. The groups differed with respect to duration of diabetes, mean arterial pressure (MAP), ACR, filtration fraction and πₐ.

The baseline characteristics of the progressors and non-progressors among the initially microalbuminuric subjects are reported in Table 2. There were no differences in gender mix or age between the microalbuminuric subjects who progressed to macroalbuminuria and those who did not. There were also no significant differences between these groups in baseline values for duration of diabetes, GFR, renal plasma flow, filtration fraction, ACR, MAP, serum cholesterol, or πₐ. There was no significant difference between groups in HbA1c, although the progressors tended to have somewhat higher values (10.8±1.7 vs 9.8±1.6%, P = 0.12). To examine whether subsequent differences in any of these variables may have contributed to the different GFR courses, we also examined the same clinical variables 4 years after study entry. Data were not available for some variables. There were no significant differences between progressors and non-progressors in GFR (149±38 vs 148±51 mL/min) or filtration fraction (0.18±0.03 vs 0.18±0.03) at 48 months. The progressors had higher ACR (median [IQR]: 361 [59-738] vs 47 [32-250] mg/g; P = 0.03), MAP (93±9 vs 86±8 mmHg, P = 0.05), and HbA1c (11.9±1.4 vs 9.6±2.1%, P = 0.005). Five of 13 progressors had developed macroalbuminuria by 48 months.

Morphometric features of the subset of initially microalbuminuric subjects who had a baseline renal biopsy are reported in Table 3. There were no significant differences between the progressors and non-progressors who had biopsies with respect to the frequency of global sclerosis, glomerular tuft volume, fractional interstitial area, GBM thickness, foot process width, or endocapillary cell number per glomerulus. The number of epithelial cells per glomerulus was lower in the progressors, although this difference failed to reach statistical significance (598±193 and 433±81, respectively; P = 0.32).

Clinical characteristics at the time of progression of the initially microalbuminuric subjects who progressed to macroalbuminuria under observation are reported in Table 4. Compared to the screened macroalbuminuric subjects at baseline, the microalbuminuric progressors at the time of progression had a lower median ACR (403 vs 904 mg/g, P = 0.014) and a higher filtration fraction (0.19±0.02 vs 0.16±0.04, P = 0.025). In contrast, there were no differences with respect to the duration of diabetes, age, gender mix, GFR, RPF, MAP, πₐ, HbA1c, or serum cholesterol. No structural comparisons were made between the screened macroalbuminuric subjects and those initially microalbuminuric subjects who progressed under observation, as the biopsies of the microalbuminuric progressors were obtained before they became macroalbuminuric.
Modeling of GFR course

Figure 1 shows three examples of raw GFR data (3-4 clearance periods per study) and the fitted GFR curves made using smoothing and regression B-splines. A nonlinear GFR trend is apparent in each case illustrated. A substantial intra-day variability in GFR among the four collection periods is obvious in some clearance studies, although the overall intra-day variability was virtually identical to that of our historical healthy controls and other groups with renal disease (coefficient of variation ~10%).

The fitted individual GFR curves of the 22 macroalbuminuric and 26 microalbuminuric subjects are shown in Figure 2. It is clear from the figure that almost all of the former had developed kidney failure within 8 years of entering the study, while only 2 of 26 microalbuminuric subjects had gone on to kidney failure over this period. This is despite the fact that the screened macroalbuminuric subjects had only a slightly lower GFR than the microalbuminuric subjects at the time of enrollment (136±56 vs 155±47 mL/min, P = NS).

A comparison of the mean fitted GFR curves over 8 years of follow-up in the initially microalbuminuric subjects shows that those who progressed to persistent macroalbuminuria had a greater rate of decline of GFR than those who remained microalbuminuric (Figure 3). There was an apparent stabilization in the GFR toward the end of follow-up among the persistently microalbuminuric subjects. The GFR curves of these two groups differed significantly as indicated by a comparison of their 5-vectors of fitted values using Hotelling’s $T^2$ test ($P = 0.004$).

We also compared the GFR decline over the first four years from the onset of macroalbuminuria in subjects who were found to be macroalbuminuric at screening with that in subjects who progressed to macroalbuminuria after enrollment with microalbuminuria (Figure 4). The GFR curves over four years in these two groups differed significantly as indicated by comparison of their 5-vectors of fitted values ($P = 0.013$). Inclusion of the apparent outlier would have increased the significance of the difference between the post-progression course of the initially microalbuminuric subjects and the initially macroalbuminuric subjects, but decreased to borderline significance the difference between the microalbuminuric progressors and non-progressors.

We found a non-linear GFR time course in many of our patients (Figure 2). To make our results more easily clinically interpretable, however, we calculated an average rate of change of GFR for the various groups based on a linear regression on the fitted GFR values. The average rate of decline of GFR over 8 years of follow-up in the non-progressors was 4.9 mL/min/y (95% confidence interval 2.7 to 7.1 mL/min/y), while in the initially microalbuminuric progressors it was 9.3 mL/min/y (95% confidence interval 6.9 to 11.8 mL/min/y). The average rate of decline of GFR for four years after progression in the initially microalbuminuric subjects was 10.5 mL/min/y (95% confidence interval 7.7 to 13.4 mL/min/y), compared to 16.9 mL/min/y (95% confidence interval 11.4 to 22.4 mL/min/y) in subjects screened with macroalbuminuria.
DISCUSSION

Diabetic nephropathy is the leading cause of kidney failure worldwide. Establishing a comprehensive characterization of the decline of GFR in patients with type 2 diabetic nephropathy is important both for studies of causal factors and of proposed therapies. In addition, defining quantitative differences in the rates of decline of GFR in different populations may help to elucidate potential genetic and environmental risk factors for disease progression. The Pima Indians of Arizona have a very high incidence of type 2 diabetes mellitus and diabetic nephropathy [1]. Moreover, when compared to European populations, diabetic Pima Indians have an accelerated rate of decline in GFR once they develop overt nephropathy [9]. Thus, differences in the patterns of loss of GFR among nephropathic Pima Indians may in fact be more pronounced than within these comparison populations.

To study changes in GFR early in the course of diabetic nephropathy requires the use of precise methods such as urinary iothalamate clearances. Because the initial glomerular hyperfiltration characteristic of diabetes results in relatively low serum creatinine concentrations, the earliest declines in GFR will be accompanied by undetectable changes in the serum creatinine concentration [22]. Using precise methods, we previously were able to demonstrate relatively small decreases in GFR over just four years in Pima Indians with incipient (microalbuminuric) diabetic nephropathy [23].

Even using precise methods, however, significant variability exists over time in measurements of GFR in diabetic Pima Indians. This variability includes both the usual intra-day coefficient of variation of approximately 10% in iothalamate clearances among the clearance periods, as seen in other populations, and a significant non-linearity in the time course of GFR in many cases (Figures 1 and 2). We have addressed these challenges by developing a method of analyzing data by adapting smoothing and regression cubic B-splines to our purposes, with statistical comparisons among the resulting fitted GFR curves based on the permutation distribution of Hotelling’s $T^2$ statistic [17]. This approach allows us to better incorporate the totality of the individual clearance data as well as to test for differences in GFR trajectories more complex than simple linear trends.

That the smoothing procedures did not significantly distort the underlying data is suggested by the fact that the mean average rate of GFR change based on the raw data did not differ significantly from the mean of the average rate of GFR change based on the fitted data in any of the groups (data not shown). Figures 1 and 2 illustrate that the rate of GFR change is in fact not constant over time, so that simple average rates of GFR change with time often fail to capture the complexity of the GFR time courses. Although statistical methods based on smoothing and regression B-splines utilize the totality of GFR data to yield more informative GFR-vs-time curves, their use may introduce challenges with regard to clinical interpretation, inasmuch as the principal outcome variable is a 5-vector of fitted GFR values rather than a simple rate of change in GFR. In the current study we have addressed this difficulty by also providing an average rate of GFR change as a single number (mL/min/y) based on a linear fit to the data. Statistical
tests were performed only on the complete 5-vector representations, for the reasons outlined above.

Using these novel statistical methods we were able to demonstrate that the time courses of GFR declines in Pima Indians with type 2 diabetes who progress from microalbuminuria to macroalbuminuria during follow-up differ from those of individuals who remain microalbuminuric throughout. Patients with persistent microalbuminuria have a somewhat more benign course, with GFR decreasing by 40 mL/min on average over 8 years and generally remaining within the normal range at the end of follow-up. It is not clear whether the apparent flattening of the group average GFR curve toward the end of follow-up in the persistently microalbuminuric subjects represents true long-term stabilization (Figure 3), as fitted values may show some instability at the boundaries of their ranges. There were no significant differences in the baseline clinical parameters describing the initially microalbuminuric patients who followed these two courses (Table 2), although there were higher values of HbA1c and MAP and disease duration in the subjects who progressed to macroalbuminuria during follow-up, suggesting that these subjects had slightly more severe or advanced disease even at the time of ascertainment than those who did not progress. The baseline average GFR and median ACR values in the two groups, on the other hand, were virtually identical (154±27 and 157±63 mL/min for GFR and 61 mcg/mg and 69 mcg/mg for ACR in progressors and non-progressors, respectively). We had baseline structural data on only four progressors and four non-progressors. Of these data, only the number of visceral epithelial cells per glomerulus showed even a modest difference between groups, with the progressors having a lower number of epithelial cells at their baseline biopsy (433±81 vs 598±193, P = 0.32). A previous study in Pima Indians showed that a lower number of podocytes predicted a more rapid increase in albuminuria over time [24]. A comparison of clinical characteristics among the progressors and non-progressors at 48 months of follow-up showed that for those variables in which there was a statistically non-significant trend for the future progressors to exceed the non-progressors at baseline (MAP, HbA1c), statistically significant differences had emerged at 48 months. Whether these differences represent causal factors accounting for the different outcomes must be addressed by further study.

GFR courses also differed in subjects who were found at screening to have macroalbuminuria compared to the "post-progression" courses of subjects who had microalbuminuria at screening and who developed macroalbuminuria during follow-up. The former had almost twice the rate of GFR decline of the latter. These groups did differ with respect to their ACR and filtration fraction at the time of enrollment or progression, with the screened macroalbuminuric subjects having significantly more albuminuria and lower filtration fractions than those who progressed to macroalbuminuria under observation. The two groups, however, did not otherwise differ in their clinical characteristics (Table 4). That individuals screened with macroalbuminuria probably represent "more severe" rather than simply "more advanced" cases of diabetic nephropathy than those who progressed while under observation is suggested by their trend toward a shorter duration of diabetes at the time of their detection with
macroalbuminuria. Indeed, the subjects who were screened as macroalbuminuric may have had even longer "occult" period of macroalbuminuria than the subjects who progressed to macroalbuminuria under observation. The former may have progressed up to 2 years earlier (between biennial screenings for albuminuria), whereas the initially microalbuminuric progressors had more precisely defined dates of progression as a result of screening for albuminuria every 3-6 months during the course of the study. Thus, the duration of their diabetes at the time of progression to macroalbuminuria may have been up to 2 years less than the 15.2 years at the time of enrollment in the study, compared to 16.5 years in the initially microalbuminuric subjects at the time they progressed. This supports the existence of a more aggressive and rapidly progressive course in those individuals found to have macroalbuminuria on screening than in individuals who are initially detected with microalbuminuria and progress to macroalbuminuria under observation.

The average rates of decline of GFR differed considerably among the three groups: 4.9 mL/min/y in microalbuminuric non-progressors, 9.3 mL/min/y in initially microalbuminuric progressors, and 16.9 mL/min/y in the subjects screened with macroalbuminuria. All three groups had average rates of GFR decline considerably greater than those expected in normal individuals over 40 years old as a result of aging, viz., ~0.5-1 mL/min/y [25;26]. In addition, in the latter two groups of Pima Indians the rate of GFR decline was more than twice that found in studies of European type 2 diabetic patients with macroalbuminuria [2;27].

As stated above, the GFR time courses were nonlinear in many individuals (Figures 1, 2). Three general classes of shapes could be distinguished: an essentially linear decline, a bimodal decline and a pattern of variable deceleration. The pattern of linear decline most likely reflects subjects already on the "slippery slope", in whom compensatory hyperfiltration in the remnant nephrons was already maximal at the time of their entry into the study. In these individuals, all subsequent loss of functioning nephrons was reflected in a loss of GFR. This could occur even in subjects who had measured GFR values greater than 200 mL/min at study entry. Under the same interpretation, those subjects who had a bimodal decline in GFR entered the slippery slope phase during follow-up. Most interesting are those subjects who showed a pattern of deceleration or stabilization of their GFR course during follow-up. A longer period of follow-up will be necessary to determine whether their GFR stability is persistent.

Our interpretation of these GFR time courses is subject to several limitations. Although the initially microalbuminuric subjects were enrolled under consistent entry criteria, those who progressed to macroalbuminuria during follow-up had some clinical characteristics (greater duration of diabetes, higher HbA1c, greater MAP) suggesting more advanced or severe disease at the time of enrollment. These differences were not statistically significant and seem too small to account for the later development of the significant differences in the course of GFR between the two groups. By 48 months of follow-up some of these factors were significantly different between progressors and non-progressors, despite the fact that their GFR values were still nearly identical. The role of these co-variates in the subsequent GFR course will need to be examined in a separate
analysis of the relevant time series. The suggestion that initial podocyte number was less in the initially microalbuminuric subjects who progressed to macroalbuminuria during follow-up rests on a small number of biopsies.

The subjects screened with macroalbuminuria and those who progressed to macroalbuminuria under observation may not have been as comparable as the microalbuminuric progressors and non-progressors at enrollment. In particular, the precision of the estimation of the time of onset of macroalbuminuria was different in these two groups. We reported before on the considerable heterogeneity in time courses of progression of microalbuminuria to macroalbuminuria in diabetic Pima Indians[3]. The combined effects of difficulties in “aligning” the starting points of their macroalbuminuric courses and the non-linear features of their GFR curves tends to exacerbate the difficulties in comparing their subsequent GFR courses. Even if the alignments of comparable “starting points” for the microalbuminuric and macroalbuminuric phases were offset by 2 years, it is clear from Figures 3 and 4 that a simple time shift of that magnitude will not superimpose the different curves.

We have not formally evaluated the contribution of exposure to ACE inhibitors or angiotensin receptor blockers to the differences in GFR courses in the different groups. Although a slightly greater number of initially microalbuminuric subjects (2/18) were receiving ACE inhibitors at the time of their progression to macroalbuminuria than were receiving them when found to be macroalbuminuric at screening (0/22), four of the latter subjects were taking ACE inhibitors at some point during the first 4 years of the study. It is unlikely that these low rates of exposure significantly affected the GFR courses of the groups as a whole. The relatively infrequent use of ACE inhibitors reflects the low incidence of hypertension in the diabetic population of Pima Indians. Only in the last several years has it become a common practice to use ACE inhibitors in normotensive type 2 diabetic individuals.

The lack of association of progression from microalbuminuria to macroalbuminuria during 8 years of follow-up with traditional clinical variables (gender, age, HbA1c, MAP, etc.) determined at baseline suggests that other factors may influence both the progression to macroalbuminuria and the attendant differences in the courses of GFR decline. Of course, significant differences in MAP and HbA1c between progressors and non-progressors that had emerged by 48 months of follow-up may have contributed to the subsequent divergence of their GFR courses. This needs to be addressed in a separate analysis based on a comparison of the time series of these variables. In addition, although we did not have baseline structural information on most of the subjects, it is possible that progression to macroalbuminuria and a more rapid decline in GFR over follow-up was due in part to a lower baseline number of podocytes [24].

Our study did not address the role of genetic factors – such as differing genotypes of ACE, angiotensinogen, bradykinin 2 receptor, nitric oxide synthase – that may influence characteristics of the progression of diabetic nephropathy. As in some other populations, the insertion/deletion polymorphism of the angiotensin-I converting enzyme gene is associated in Pima Indians with differences in plasma ACE activity [28] and thus might
influence the evolution of kidney disease. The ability to precisely quantify changes in GFR over extended periods of time should allow more sensitive investigations of the role of both genetic and environmental factors in disease progression. This may prove particularly valuable in studies conducted within well-characterized and relatively genetically homogenous populations with a high incidence of diabetic nephropathy such as the Pima Indians of Arizona [29]. Characterization of GFR courses in terms of 5-vectors may seem to complicate easy physiologic interpretation of GFR changes. It may however also be amenable to associational study of complex genotype segregation with specific quantitative traits, using modifications of techniques developed for more simple (one-dimensional) quantitative traits. For any quantitative analysis, representation of the course of GFR as a 5-vector captures more information than an average linear rate of GFR change without imposing arbitrary structure on the GFR course.
ACKNOWLEDGEMENTS

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Reference List


Table 1. Baseline Characteristics of Macroalbuminuric and Microalbuminuric Subjects.

<table>
<thead>
<tr>
<th></th>
<th>Microalbuminuric</th>
<th>Macroalbuminuric</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>44±9 SD</td>
<td>45±9</td>
<td>NS</td>
</tr>
<tr>
<td>Duration of Diabetes</td>
<td>12±4</td>
<td>15±4</td>
<td>0.01</td>
</tr>
<tr>
<td>Gender</td>
<td>17♀, 9♂</td>
<td>11♀, 11♂</td>
<td>NS</td>
</tr>
<tr>
<td>ACEi use (%)</td>
<td>0</td>
<td>0</td>
<td>NS</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>91±9</td>
<td>102±11</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ACR (mg/g)*</td>
<td>65 [48-146]</td>
<td>904 [527-1838]</td>
<td>-</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>10.4±1.5</td>
<td>10.4±1.3</td>
<td>NS</td>
</tr>
<tr>
<td>GFR (mL/min)</td>
<td>155±47</td>
<td>136±56</td>
<td>NS</td>
</tr>
<tr>
<td>RPF (mL/min)</td>
<td>841±238</td>
<td>847±252</td>
<td>NS</td>
</tr>
<tr>
<td>Filtration fraction</td>
<td>0.19±0.04</td>
<td>0.16±0.04</td>
<td>0.04</td>
</tr>
<tr>
<td>$\pi_A$ (mmHg)</td>
<td>22.6±1.8</td>
<td>21.1±2.4</td>
<td>0.016</td>
</tr>
<tr>
<td>Cholesterol (mg/dL)</td>
<td>178±26</td>
<td>176±46</td>
<td>NS</td>
</tr>
<tr>
<td>n</td>
<td>26</td>
<td>22</td>
<td></td>
</tr>
</tbody>
</table>

* median [interquartile range]
ACEi, angiotensin converting enzyme inhibitor; MAP, mean arterial pressure; ACR, albumin/creatinine ratio; RPF, renal plasma flow; $\pi_A$, plasma oncotic pressure.
Table 2. Baseline Characteristics of Progressor and Non-Progressor Groups of Initially Microalbuminuric Subjects.

<table>
<thead>
<tr>
<th></th>
<th>Progressors</th>
<th>Non-Progressors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>45±8</td>
<td>44±10</td>
</tr>
<tr>
<td>Gender (♂, ♀)</td>
<td>4/9</td>
<td>5/8</td>
</tr>
<tr>
<td>Duration of diabetes (y)</td>
<td>13±3</td>
<td>11±4</td>
</tr>
<tr>
<td>ACEi use (%)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cholesterol (mg/dL)</td>
<td>179±26</td>
<td>177±27</td>
</tr>
<tr>
<td>ACR (mg/g)*</td>
<td>61 [50-85]</td>
<td>69 [41-159]</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>94±10</td>
<td>89±7</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>10.8±1.7</td>
<td>9.8±1.6</td>
</tr>
<tr>
<td>GFR (mL/min)</td>
<td>154±27</td>
<td>157±63</td>
</tr>
<tr>
<td>RPF (mL/min)</td>
<td>854±175</td>
<td>830±291</td>
</tr>
<tr>
<td>Filtration fraction</td>
<td>0.18±0.02</td>
<td>0.19±0.04</td>
</tr>
<tr>
<td>$\pi_A$ (mmHg)</td>
<td>23.1±2.1</td>
<td>22.1±1.3</td>
</tr>
<tr>
<td>n</td>
<td>13</td>
<td>13</td>
</tr>
</tbody>
</table>

*Median [IQR].
Table 3. Baseline structural characteristics of progressor and non-progressor microalbuminuric subjects.

<table>
<thead>
<tr>
<th></th>
<th>Progressor</th>
<th>Non-Progressor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glomerular volume (μm³ ×10⁶)</td>
<td>7.04±2.42</td>
<td>7.87±0.78</td>
</tr>
<tr>
<td>Global sclerosis (%)</td>
<td>6±6</td>
<td>5±3</td>
</tr>
<tr>
<td>Fractional Interstitial Area (%)</td>
<td>23.8±3.2</td>
<td>22.2±4.8</td>
</tr>
<tr>
<td>GBM thickness (nm)</td>
<td>487±52</td>
<td>441±109</td>
</tr>
<tr>
<td>Foot process width (nm)</td>
<td>634±46</td>
<td>623±120</td>
</tr>
<tr>
<td>Epithelial cell #/glomerulus</td>
<td>433±81</td>
<td>598±193</td>
</tr>
<tr>
<td>Endocapillary cell #/glomerulus</td>
<td>3663±1840</td>
<td>3471±518</td>
</tr>
<tr>
<td>n</td>
<td>4</td>
<td>4</td>
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</tbody>
</table>
Table 4. Clinical Characteristics at Time of Progression in Initially Microalbuminuric Subjects Compared with Macroalbuminuric Subjects at Screening.

<table>
<thead>
<tr>
<th></th>
<th>Progressors</th>
<th>Macroalbuminuric</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>47±8</td>
<td>45±9</td>
<td>NS</td>
</tr>
<tr>
<td>Gender (♂/♀)</td>
<td>6/12</td>
<td>11/11</td>
<td>NS</td>
</tr>
<tr>
<td>Duration of diabetes (y)</td>
<td>16.5±4.0</td>
<td>15.2±4.4</td>
<td>NS</td>
</tr>
<tr>
<td>ACEi use (%)</td>
<td>11</td>
<td>0</td>
<td>NS</td>
</tr>
<tr>
<td>ACR (mg/g)*</td>
<td>403 [311-645]</td>
<td>904 [527-1838]</td>
<td>0.014</td>
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<tr>
<td>HbA1c (%)</td>
<td>10.9±1.7</td>
<td>10.6±1.6</td>
<td>NS</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>98±7</td>
<td>103±11</td>
<td>0.10</td>
</tr>
<tr>
<td>GFR (mL/min)</td>
<td>135±36</td>
<td>136±56</td>
<td>NS</td>
</tr>
<tr>
<td>RPF (mL/min)</td>
<td>752±156</td>
<td>847±252</td>
<td>NS</td>
</tr>
<tr>
<td>Filtration Fraction</td>
<td>0.19±0.02</td>
<td>0.16±0.04</td>
<td>0.02</td>
</tr>
<tr>
<td>Cholesterol (mg/dL)</td>
<td>180±44</td>
<td>176±46</td>
<td>NS</td>
</tr>
<tr>
<td>ρA (mmHg)</td>
<td>22.3±2.6</td>
<td>21.1±2.4</td>
<td>NS</td>
</tr>
<tr>
<td>n</td>
<td>18</td>
<td>22</td>
<td></td>
</tr>
</tbody>
</table>

*Median [IQR]

Number of progressors with data was 8-10 for RPF, filtration fraction and ρA.
FIGURE LEGENDS

1. Regression and smoothing B-spline fits to the raw GFR data of three subjects: A, initially microalbuminuric non-progressor; B, initially microalbuminuric progressor; C, initially macroalbuminuric subject. Notice the significant intra-day variability in GFR for some of the individual clearance studies.

2. GFR courses of 22 macroalbuminuric (red) and 26 initially microalbuminuric (blue) Pima Indians with type 2 diabetes. The GFR courses are non-linear in many of the subjects.

3. Mean GFR courses (fitted values) of initially microalbuminuric subjects who progressed to persistent macroalbuminuria (n=13, open circles) or remained microalbuminuric (n=13, closed squares) over the course of 8 years of follow-up.

4. Mean GFR courses (fitted values) of subjects with macroalbuminuria at screening (n=22, open circles) and after progression to macroalbuminuria in subjects who were microalbuminuric at screening and later progressed to macroalbuminuria (n=18, closed squares).
Figure 1.
Figure 3
Figure 4