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Research: Complications

Impact of tubulointerstitial lesions on anaemia in patients with biopsy-proven diabetic nephropathy

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Abstract

Aims To investigate the relationship between the progression of anaemia and renal pathological findings in patients with diabetic nephropathy.

Methods A total of 223 patients with diabetes underwent renal biopsy from 1985 to 2010 and were confirmed to have pure diabetic nephropathy according to the recent classification, of whom 113 (baseline haemoglobin ≥ 11 g/dl) were enrolled in the study. Linear regression analysis was used to estimate the changes in haemoglobin levels during the follow-up period.

Results In a multivariate model adjusted for clinical and histopathological variables, higher interstitial fibrosis and tubular atrophy scores were more strongly associated with a decrease in haemoglobin levels than were lower scores. Compared with an interstitial fibrosis and tubular atrophy score of 0, the standardized coefficients for interstitial fibrosis and tubular atrophy scores of 1, 2 and 3 were 0.20 (95% CI −0.31 to 0.93), 0.34 (95% CI −0.22 to 1.34) and 0.47 (95% CI 0.07 to 1.96), respectively, whereas a higher glomerular class, a higher vascular lesion score and the presence of exudative lesions were not strongly correlated with the decrease in haemoglobin.

Conclusions Tubulointerstitial lesions that are more advanced are significantly associated with the progression of anaemia in patients with diabetic nephropathy after adjustment for numerous covariates. This finding suggests that tubulointerstitial lesions may be a useful prognostic indicator for anaemia in patients with diabetic nephropathy, and that decreased erythropoietin production attributable to the progression of tubulointerstitial lesions is a major cause of anaemia in these patients.

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Introduction

In patients with diabetes, anaemia is not only an important indicator of renal prognosis but also of cardiovascular morbidity and mortality [1,2]. Recent studies have clearly shown that anaemia occurs more frequently in patients with chronic kidney disease who have diabetes, and occurs at an earlier stage of chronic kidney disease in patients with diabetes [1,3]. El-Achkar *et al.* [4] showed that the prevalence of anaemia was higher in patients with diabetes than in patients without diabetes, even in those with an estimated glomerular filtration rate (eGFR) of 60–89 ml/min per 1.73 m^2. In addition, New *et al.* [5] reported that the relationship between haemoglobin and eGFR was approximately linear at eGFR values of < 83 ml/min per 1.73 m^2.

The major causes of anaemia in patients with diabetes and chronic kidney disease are iron deficiency, erythropoietin (EPO) deficiency, and impaired responsiveness to the actions of EPO [1]. One of the reasons why anaemia is more common and occurs earlier in patients with diabetes is that EPO deficiency develops at an earlier stage of chronic kidney disease in diabetic nephropathy than in other renal diseases [6]. Investigation of the pathophysiology of renal anaemia has shown *in vivo* that this anaemia is mediated via reduced production of EPO by myofibroblasts and that renal interstitial fibrosis involves accumulation of myofibroblasts, suggesting a relationship between interstitial fibrosis and renal anaemia [7]; however, the association of histopathological findings, including interstitial fibrosis, with renal

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What's new?

- The relationship between progression of anaemia and renal pathological findings was investigated in patients with biopsy-proven diabetic nephropathy.
- Tubulointerstitial lesions that were more advanced were significantly associated with the decrease of haemoglobin after adjustment for numerous covariates.
- This finding suggests that tubulointerstitial lesions may be a useful prognostic indicator for anaemia in patients with diabetic nephropathy, and that decreased erythropoietin production as a result of progression of tubulointerstitial lesions is a major cause of anaemia in these patients.

anaemia remains unclear in patients with chronic kidney disease, especially those with diabetic nephropathy. Accordingly, the aim of the present study was to investigate the association between the progression of anaemia and renal pathological findings in patients with chronic kidney disease and biopsy-proven diabetic nephropathy.

Materials and methods

Study design

Of 223 patients with diabetes mellitus who underwent renal biopsy at our hospital from March 1985 to January 2010 and were confirmed to have pure diabetic nephropathy, which was defined as diabetic nephropathy without other coexisting renal diseases (except for nephrosclerosis) or kidney transplantation, 113 patients were considered to be eligible and were enrolled in the study. Diabetic nephropathy was diagnosed as in our previous study [8]. Exclusion criteria were haemoglobin < 11 g/dl, eGFR < 15 ml/min/1.73 m^2, and treatment with erythropoiesis-stimulating agents (ESA) at the time of renal biopsy, as well as follow-up for less than < 12 months. The haemoglobin cut-off level threshold for Hb of 11 g/dl was based on the results of a similar previous study and the recommendation of the Japanese Society for Dialysis Therapy (JSDT) [9,10]. According to the guidelines for renal anaemia in patients with chronic kidney disease, the target range for haemoglobin during treatment with erythropoiesis-stimulating agents is 11—12 g/dl [9]. The protocol of the present study was reviewed and approved by the ethics committee of Toranomon Hospital in February 2014. This study was registered with the University Hospital Medical Information Network (UMIN) in January 2014. The University Hospital Medical Information NetworkUMIN identification number of this study is UMIN000012847.

Follow-up

The follow-up period was defined as the period from the time of renal biopsy to the last time of investigating clinical variables, the time of commencing treatment with erythropoiesis-stimulating agents, the time of commencing dialysis because of end-stage renal disease, or a few months before death, whichever came first. None of the patients received kidney transplantation during follow-up.

Clinical and laboratory variables

Haemoglobin and serum creatinine levels were measured more than once a year and at final follow-up (at least three times in total) in each patient. The average of the first two haemoglobin values obtained after the renal biopsy was used as the baseline haemoglobin level, in consideration of the physiological variation of haemoglobin levels. This was done to avoid the influence of regression-to-the-mean, as well as to conform to the method used in the study on eGFR by Yokoyama *et al* [11]. The eGFR was calculated using the Japanese coefficient-modified Chronic Kidney Disease Epidemiology Collaboration equation [12], while baseline urinary protein excretion was measured from a 24-h urine sample. HbA_{1c} levels were presented as National Glycohemoglobin Standardization Program values according to the recommendations of the Japanese Diabetes Society and International Federation of Clinical Chemistry values [13]. Haematuria was defined and the average annual values such as urinary protein excretion, systolic and diastolic blood pressure and HbA_{1c} during follow-up were calculated as described in our previous study [8]. Treatment with an angiotensin-converting enzyme inhibitor or angiotensin II receptor blocker was defined as administration for more than half of the follow-up period. Iron deficiency anaemia was defined according to the Japanese Society for Dialysis Therapy criteria as haemoglobin < 13.5 g/dl in men and < 11.5 g/dl in women, transferrin saturation < 20%, and serum ferritin < 100 ng/ml [9].

Renal biopsy and pathological classification

The indications for renal biopsy were proteinuria > 0.5 g/day or atypical diabetic nephropathy, as described previously [8]. Tissue was obtained by needle biopsy and the specimens were processed for light microscopy and immunofluorescence, as well as for electron microscopy. Specimens for light microscopy were stained with haematoxylin and eosin, periodic acid Schiff, Weigert's elastica-van Gieson, Masson trichrome or periodic acid methanamine silver stain according to routine methods. The mean ± SD (range) number of glomeruli examined was 15.1 ± 9.5 (5–46). Classification of diabetic nephropathy and histological scoring were carried out according to the criteria defined by Tervaert *et al*. [14]. Exudative lesions were also evaluated, as in our previous study [8].

Statistical analyses

Data are summarized as percentages, mean ± SD values, or medians with interquartile ranges, as appropriate. Categorical

variables were analysed using the chi-squared test or Fisher's exact test, while continuous variables were compared using the *t*-test, Mann–Whitney *U*-test, Kruskal–Wallis H-test, or ANOVA. For each patient, a linear regression model of time vs haemoglobin was created, and the slope of the regression line was used to estimate the changes in haemoglobin over time. The haemoglobin slope was also expressed as an annual percentage (the slope divided by the baseline haemoglobin level). Patients were categorized into tertiles according to the rate of haemoglobin decrease: the first tertile (non-decliners) had an annual haemoglobin change of < 0.14 g/dl, the second tertile (moderate decliners) had an annual haemoglobin decrease of 0.14 to 0.51 g/dl, and the third tertile (rapid decliners) had an annual haemoglobin decrease of > 0.51 g/dl. A linear regression model of time vs eGFR was also created, and the slope of the regression line was used to estimate the changes in eGFR over time. As was done for haemoglobin, the eGFR decrease was calculated as an annual percentage (slope divided by the baseline eGFR). Multivariate linear regression analysis was performed to explore the association of annual haemoglobin decrease with baseline clinical and histopathological covariates. In adjusted regression model 1, the dependent variable was the annual decrease in haemoglobin level (which was included as a continuous variable), while age, sex, type of diabetes, BMI, systolic blood pressure, diabetic retinopathy, eGFR, urinary protein excretion, HbA_{1c}, baseline haemoglobin, current or previous smoking habit, and use of angiotensin-converting enzyme inhibitors or angiotensin II receptor blockers at the time of renal biopsy were included as covariates. In adjusted regression model 2, the dependent variable was the annual decrease in haemoglobin (included as a continuous variable), while only the more significant clinical covariates were used and the following renal pathological findings were also included as categorical covariates: glomerular class; interstitial fibrosis and tubular atrophy (IFTA) score; interstitial inflammation score; arteriolar hyalinosis score; arteriosclerosis score; and the presence of exudative lesions. Standardized coefficients for glomerular classes IIA, IIB, III and IV were calculated compared with the standardized coefficient for glomerular class I. To calculate standardized coefficients for the IFTA score, interstitial inflammation score, arteriolar hyalinosis score and arteriosclerosis score, the reference values were an IFTA score of 0, an interstitial inflammation score of 0, an arteriolar hyalinosis score of 0, and an arteriosclerosis score of 0, respectively.

Results

Of the 223 patients screened, 113 met the inclusion criteria and were enrolled in the study to investigate the predictors of haemoglobin decrease. The median (interquartile range) follow-up period was 5.4 (2.5–11.4) years.

The characteristics of all patients and of the three tertiles of decreases in haemoglobin level are shown in Table 1. Of the 113 patients, 89 were men. The patients' mean ± SD age at the time of renal biopsy was 55 ± 13 years. A total of 104 patients (92.0%) had Type 2 diabetes, and the remaining nine patients (8.0%) had Type 1 diabetes. The mean ± SD baseline haemoglobin was 13.3 ± 1.8 g/dl, and mean ± SD eGFR was 58.3 ± 20.6 ml/min per 1.73 m^2. The mean ± SD urinary protein excretion rate was 2.3 ± 2.6 g/day, and 94 patients (83.2%) had macroalbuminuria. Older age, higher systolic blood pressure, serum creatinine level, urinary protein excretion rate and number of anti-hypertensive agents, lower BMI, baseline haemoglobin level, eGFR, creatinine clearance rate and serum albumin level were significantly associated with the tertiles with the more rapid decreases in haemoglobin level. In all patients, the mean haemoglobin decrease was 0.5 ± 0.8 g/dl per year and 4.7 ± 6.7% per year. In the non-decliner, moderate decliner and rapid decliner groups, the mean ± SD decreases in haemoglobin were −0.03 ± 0.2 g/dl per year, 0.3 ± 0.1 g/dl per year, and 1.4 ± 0.8 g/dl per year, respectively.

Comparison of histopathological findings among the haemoglobin decrease tertiles showed that glomerular class, IFTA score, interstitial inflammation score, arteriolar hyalinosis score and arteriosclerosis score were all higher in the tertiles with more rapid decreases in haemoglobin, and more patients had exudative lesions than in the tertiles with the slower decreases in haemoglobin (Table 1).

The clinical variables measured during the follow-up period and at final follow-up are shown in Table 2. During follow-up, the mean ± SD decrease in eGFR for all patients was 6.3 ± 6.8 ml/min/1.73 m^2 per year, while the mean ± SD urinary protein excretion rate was 2.9 ± 2.7 g/day (or g/g creatine). The annual decrease in eGFR, mean urinary protein excretion rate, mean systolic blood pressure and final number of anti-hypertensive agents were significantly larger in the tertiles with more rapid decreases in haemoglobin than in the tertiles with slower decreases in haemoglobin.

The correlation between the rate of decrease in haemoglobin level and rate of decrease in eGFR during follow-up is shown in Fig. 1. There was a significant positive correlation between the two rates ($r^2 = 0.57$; $P < 0.001$). There were also weak correlations between the rate of haemoglobin decrease and the mean urinary excretion rate, mean systolic blood pressure or mean diastolic blood pressure ($r^2 = 0.33$, 0.080 and 0.059, respectively).

The results of multivariate linear regression analysis are shown in Table 3. In model 1, a higher urinary protein excretion rate and a lower BMI were significant predictors of the annual decrease in haemoglobin level after adjustment for clinical confounders [standardized coefficient: 0.28 ($P = 0.007$) and 0.27 ($P = 0.008$), respectively]. In a multivariate linear regression model adjusted for the clinical confounders used in model 1 and histopathological variables, diabetic retinopathy, HbA_{1c} and eGFR were the variables with the smallest relation to the decrease in haemoglobin level ($P = 0.85$, 0.79 and 0.74, respectively); therefore, model 2 was devised by excluding diabetic retinopathy,

Table 1 Baseline clinical variables and histopathological findings for all patients and for groups stratified by tertile of decrease in haemoglobin level

Clinical variables	All patients (N = 113)	Haemoglobin decrease tertile: Non-decliner <0.14 g/dl per year (n = 38)	Moderate decliner 0.14 to 0.51 g/dl per year (n = 38)	Rapid decliner > 0.51 g/dl per year (n = 37)	P
Male, %	79	79	79	78	1.0
Age, years	55 ± 13	50 ± 15	58 ± 11	58 ± 11	0.019
BMI, kg/m^2	24.5 ± 4.0	25.4 ± 4.6	25.0 ± 3.7	23.2 ± 3.5	0.049
Type 2 diabetes, %	92.0	84.2	97.4	94.6	0.12
Duration of diabetes, years	14.2 ± 8.1	12.7 ± 8.1	14.5 ± 8.3	15.5 ± 7.9	0.40
Systolic blood pressure, mmHg	143.2 ± 18.4	137.5 ± 19.5	143.6 ± 15.1	148.8 ± 19.2	0.028
Diastolic blood pressure, mmHg	81.6 ± 11.5	80.3 ± 12.1	81.7 ± 11.0	82.8 ± 11.5	0.64
Retinopathy, %	60.2	44.7	65.8	70.3	0.054
Current or previous smoking habit, %	61.9	60.5	55.3	70.3	0.40
Baseline haemoglobin*, g/dl	13.3 ± 1.8	14.0 ± 1.6	13.3 ± 1.5	12.7 ± 1.9	<0.01
Haemoglobin decrease†, g/dl per year	0.5 ± 0.8	−0.03 ± 0.2	0.3 ± 0.2	1.4 ± 0.8	<0.01
Haemoglobin decrease, % per year	4.7 ± 6.7	−0.2 ± 1.2	2.4 ± 0.9	12.1 ± 7.1	<0.01
Serum creatinine, mg/dl	1.2 ± 0.5	1.0 ± 0.2	1.2 ± 0.6	1.5 ± 0.6	<0.01
Creatinine clearance rate, ml/min	62.3 ± 27.1	78.1 ± 28.3	59.2 ± 20.4	49.8 ± 23.3	<0.01
eGFR, ml/min/1.73 m^2	58.1 ± 20.9	68.9 ± 15.4	59.5 ± 21.0	46.1 ± 18.8	<0.01
Urinary protein excretion rate, g/day	2.3 ± 2.6	1.3 ± 2.0	1.9 ± 1.9	3.7 ± 3.1	<0.01
Normoalbuminuria/ microalbuminuria/ macroalbuminuria, %	1.8/15.0/83.2	5.3/34.2/60.5	0/10.5/89.5	0/0/100	
Serum albumin, g/dl	3.4 ± 0.7	3.8 ± 0.6	3.5 ± 0.6	3.0 ± 0.7	<0.01
HbA_{1c}					
mmol/mol	64 ± 21	64 ± 23	65 ± 18	62 ± 23	0.41
%	8.0 ± 1.9	8.1 ± 2.1	8.1 ± 1.6	7.9 ± 2.1	0.41
Haematuria‡, %	7.1	7.9	2.6	10.8	0.35
Triglycerides, mg/dl	171.0 ± 92.2	168.1 ± 95.5	179.6 ± 106.6	165.3 ± 72.5	0.87
Total cholesterol, mg/dl	217.7 ± 58.7	204.6 ± 52.7	230.5 ± 56.0	217.9 ± 65.5	0.10
LDL cholesterol, mg/dl	141.0 ± 52.4	128.3 ± 44.1	150.1 ± 50.7	144.5 ± 60.4	0.15
ACE-I or ARB treatment, %	64.6	50.0	68.4	75.7	0.056
Number of anti-hypertensive agents	1.8 ± 1.3	1.3 ± 1.4	1.9 ± 1.0	2.3 ± 1.3	<0.01
Oral hypoglycaemic agent therapy, %	34.5	28.9	36.8	37.8	0.67
Insulin therapy§, %	41.6	36.8	42.1	45.9	0.72
Iron deficiency anaemia, %	4.4	5.3	5.3	2.7	1.0
Oral iron supplement treatment, %	1.8	0	2.6	2.7	1.0

Histopathological findings	All patients (n = 113)	Haemoglobin decrease tertile: Non-decliner <0.14 g/dl per year (n = 38)	Moderate decliner 0.14 to 0.51 g/dl per year (n = 38)	Rapid decliner > 0.51 g/dl per year (n = 37)	P
Glomerular class, %					
I	10.6	21.1	10.5	0	<0.01
IIA	31.9	39.5	14.2	13.5	
IIB	31.0	23.7	23.7	45.9	
III	18.6	13.2	21.1	21.6	
IV	8.0	2.6	2.6	18.9	

Table 1 (*Continued*)

Histopathological findings	All patients (n = 113)	Haemoglobin decrease tertile: Non-decliner <0.14 g/dl per year (n = 38)	Moderate decliner 0.14 to 0.51 g/dl per year (n = 38)	Rapid decliner > 0.51 g/dl per year (n = 37)	P
IFTA score, %					
0	13.3	34.2	5.3	0	<0.01
1	39.8	39.5	57.9	21.6	
2	31.9	21.1	28.9	45.9	
3	15.0	5.3	7.9	32.4	
Interstitial inflammation score	0.9 ± 0.5	0.6 ± 0.6	1.0 ± 0.4	1.0 ± 0.3	<0.01
Arteriolar hyalinosis score	1.7 ± 0.7	1.4 ± 0.8	1.7 ± 0.7	1.9 ± 0.3	<0.01
Arteriosclerosis score	1.3 ± 0.6 (n = 105)	1.1 ± 0.7 (n = 35)	1.2 ± 0.6 (n = 37)	1.5 ± 0.5 (n = 33)	0.11
Exudative lesions, %	43.4	23.7	42.1	64.9	<0.01
Number of glomeruli obtained by renal biopsy	15.1 ± 9.5	14.7 ± 10.3	14.5 ± 8.5	16.0 ± 9.9	0.67

Data are mean ± SD or number (%).
eGFR, estimated glomerular filtration rate; IFTA, interstitial fibrosis and tubular atrophy; ACE-I, angiotensin-converting enzyme inhibitor; ARB, angiotensin II receptor blocker.
*Average of the initial two haemoglobin levels. †Annual haemoglobin decrease rate. ‡>5 red blood cells/HPF in urine sediment. §Treatment with insulin (including basal-supported oral therapy).
For all histopathological findings, P values were obtained by comparison among the haemoglobin decrease tertiles.

HbA_{1c}, and eGFR. In model 2, higher IFTA scores showed higher standardized coefficients, and an IFTA score of 3 had a significantly stronger association with the decrease in haemoglobin than did an IFTA score of 0 [standardized coefficient for IFTA scores 1–3: 0.20 (P = 0.32), 0.34 (P = 0.16), and 0.47 (P = 0.036), respectively]. By contrast, a higher glomerular class did not always show a higher standard coefficient than a lower glomerular class. In the same model, higher scores for interstitial inflammation, arteriolar hyalinosis and arteriosclerosis, as well as the presence of exudative lesions, did not show a significant relationship with the haemoglobin decrease. With respect to clinical variables, female gender and lower BMI were significantly associated with haemoglobin decrease in model 2, whereas urinary protein excretion was not significantly associated with the haemoglobin decrease (standardized coefficient 0.19; P = 0.093). Although IFTA score, BMI and sex were strongly correlated with the decrease in haemoglobin, another multivariate model that included IFTA score, BMI, sex and rate of eGFR decrease as covariates showed that the eGFR decline was most strongly correlated with the decrease in haemoglobin level [standardized coefficient 0.70; $P <$ 0.001 (Table S1)].

To investigate the influence of the very short follow-up period for some patients, we excluded 18 patients with a follow-up period < 2 years and compared the findings obtained in this adjusted model with our original results as a sensitivity analysis. The outcome of the multivariate linear regression analysis in the 95 patients with a follow-up period ≥ 2 years was similar to our original results, and showed that IFTA score, BMI, urinary protein excretion rate and sex were strong predictors of haemoglobin decline (Table S2). There were also significant interactions of baseline haemoglobin with urinary protein excretion and IFTA score in this multivariate model; however, we found that the results were similar when an interaction term was added to the model or when baseline haemoglobin was removed from it (Table S3).

Additional analysis

Another multivariate linear regression analysis was performed to investigate the relationship between baseline haemoglobin and renal pathological findings in 108 patients without definite iron deficiency anaemia. In the adjusted regression model, the dependent variable was baseline haemoglobin, while age, sex, BMI and renal pathological findings (glomerular class, IFTA score, interstitial inflammation score, arteriolar hyalinosis score, arteriosclerosis score and exudative lesions) were included as categorical covariates. As a result, IFTA score was found to be strongly correlated with baseline haemoglobin independently of other pathological findings, as well as with decrease in haemoglobin [standardized coefficients for IFTA scores of 1–3: 0.18 (P = 0.31), 0.31 (P = 0.16), and 0.46 (P = 0.022), respectively (Table 4)].

Table 2 Clinical variables during the follow-up period and at final follow-up in all patients and in groups stratified according to haemoglobin decrease tertile

	Haemoglobin decrease tertile				
	All patients (N = 113)	Non-decliner < 0.14 g/dl per year (n = 38)	Moderate decliner 0.14 to 0.51 g/dl per year (n = 38)	Rapid decliner > 0.51 g/dl per year (n = 37)	P
eGFR decrease ml/min/1.73 m^2per year	6.3 ± 6.8	2.1 ± 3.2	5.0 ± 4.1	11.9 ± 7.9	< 0.01
eGFR decrease, % per year	12.7 ± 13.8	3.6 ± 6.0	8.6 ± 7.9	26.3 ± 14.1	< 0.01
Urinary protein excretion rate, g/day or g/g creatinine	2.9 ± 2.7	1.2 ± 1.2	2.6 ± 2.2	5.1 ± 2.8	< 0.01
Systolic blood pressure, mmHg	138.4 ± 13.3	132.0 ± 13.5	139.7 ± 11.7	143.6 ± 12.2	< 0.01
Variation in systolic blood pressure*, mmHg	−4.8 ± 12.7	−5.5 ± 14.5	−3.9 ± 12.5	−5.2 ± 11.2	0.84
Diastolic blood pressure, mmHg	77.3 ± 8.1	75.5 ± 8.0	77.6 ± 6.2	78.9 ± 9.6	0.19
Variation in diastolic blood pressure†, mmHg	−4.3 ± 9.2	−4.8 ± 10.4	−4.1 ± 10.3	−3.9 ± 6.6	0.91
HbA_{1c}					
mmol/mol	59 ± 15	62 ± 14	60 ± 12	55 ± 17	0.028
%	7.6 ± 1.3	7.9 ± 1.2	7.7 ± 1.1	7.2 ± 1.6	0.029
Variation in HbA_{1c}‡					
mmol/mol	−5 ± 15	−3 ± 20	−5 ± 11	−7 ± 12	0.012
%	−0.5 ± 1.4	−0.3 ± 1.9	−0.5 ± 1.0	−0.7 ± 1.1	0.022
Haemoglobin, g/dl	12.7 ± 1.9	13.9 ± 1.4	12.5 ± 1.5	11.8 ± 2.2	< 0.01
ACE-I or ARB treatment§, %	81.4	73.7	84.2	86.4	0.40
Final number of anti-hypertensive agents	2.9 ± 1.6	2.1 ± 1.6	3.3 ± 1.2	3.4 ± 1.6	< 0.01
Final oral hypoglycaemic agent therapy, %	30.1	31.6	31.6	27.0	0.89
Final insulin therapy, %	56.6	50.0	63.2	56.8	0.51
Erythropoiesis-stimulating agents¶, %	37.2	2.6	44.7	64.9	< 0.01
Renal death**	6	0	2	4	0.083
Death††	11	3	4	4	0.93

Data are mean ± SD, number (%), or number.
eGFR, estimated glomerular filtration rate; ACE-I, angiotensin-converting enzyme inhibitor; ARB, angiotensin II receptor blocker.
*Mean annual systolic blood pressure–baseline systolic blood pressure. †Mean annual diastolic blood pressure–baseline diastolic blood pressure, ‡Mean annual HbA_{1c}–baseline HbA_{1c}. §Treatment for more than half of the follow-up period. ¶Endpoint was commencement of erythropoietin-stimulating agents. **Endpoint was commencement of dialysis due to end-stage renal disease. ††Endpoint was the time of death.

Discussion

Some recent studies have investigated the associations between haemoglobin or haemoglobin decrease and clinical variables in patients with diabetes [10,15]; however, for individual patients with diabetes (especially those with diabetic nephropathy), it remains unclear which clinical and histopathological variables can predict a decrease in haemoglobin and which clinical variables are closely associated with haemoglobin decline.

According to the present multiple linear regression model adjusted for clinical and histopathological variables, IFTA was the strongest pathological predictor of the decrease in haemoglobin level, and higher IFTA scores were found to have higher standardized coefficients for the haemoglobin decrease. There are some possible mechanisms by which tubulointerstitial lesions could influence anaemia. One is reduced production of EPO by myofibroblasts as a result of interstitial fibrosis. Asada *et al.* [7] showed that EPO-producing fibroblasts undergo transformation into myofibroblasts, which impairs EPO production in fibrotic kidneys *in vivo*. In general, up to 90% of EPO is produced by peritubular fibroblasts in the kidneys [16]. Human studies have shown that interstitial damage and relative EPO deficiency leading to anaemia occur in the early stage of diabetic nephropathy [6,17,18]; therefore, progression of interstitial fibrosis from the early stage of diabetic nephropathy could lead to the development of anaemia via reduced EPO production. Our results were consistent with this mechanism of renal anaemia, although the EPO level at the time of renal biopsy was not available in all of our cohorts. Alternatively, anaemia could aggravate renal fibrosis by causing renal tissue hypoxia [2,19]. Renal hypoxia is known

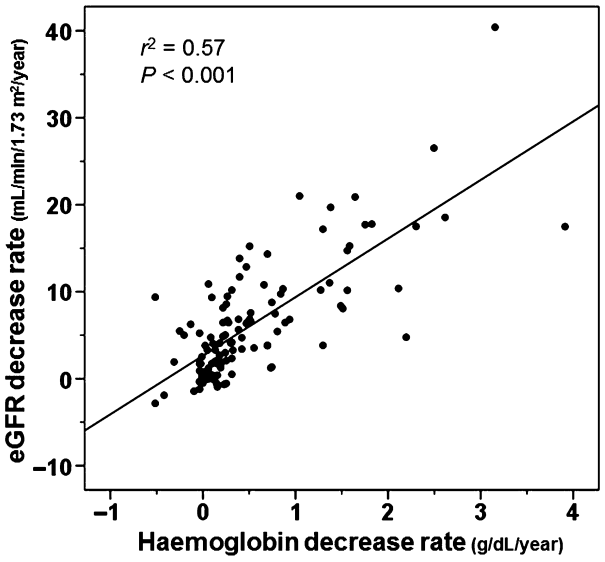

FIGURE 1 Correlation between the rate of decrease in estimated glomerular filtration rate (eGFR) and the rate of decrease in haemoglobin level. [y (eGFR decrease rate) = 2.64 + 6.73 × (haemoglobin decrease rate), r^2 = 0.57, P < 0.001].

to stimulate the production of cytokines such as hypoxia-inducing factor-1, which contributes to renal scarring [20]. In addition, hypoxia stimulates renal sympathetic activity, resulting in reduction of renal blood flow and eGFR over time [21]; therefore, anaemia and renal interstitial fibrosis could have a cause-and-effect relationship with each other. Our multivariate analysis showed that IFTA score was strongly associated with both the decrease in haemoglobin and the baseline haemoglobin level, which may support such a cause-and-effect relationship. In addition to EPO deficiency, other causes of anaemia, especially iron deficiency, could accelerate this vicious circle that promotes anaemia. Moreover, as recent studies have shown, progression of tubulointerstitial lesions is strongly associated with a worse renal prognosis [8,22]. Accordingly, the vicious circle interaction between renal anaemia and interstitial fibrosis may occur in parallel with a decline in renal function, which is consistent with our finding that the haemoglobin decrease was strongly correlated with the eGFR decrease (Figure 1; Table S1).

With respect to the role of clinical variables, our multiple linear regression model adjusted for baseline clinical variables showed that the significant predictors of haemoglobin decrease were more severe proteinuria and a lower BMI. Lower BMI was also a strong predictor of the haemoglobin decrease, even after adjustment for clinical and pathological variables, although proteinuria was not significantly associated with it. In the 5-year prospective cohort study of anaemia in patients with Type 2 diabetes performed by Thomas *et al.* [10], albumin excretion rate, eGFR and macrovascular disease were independent predictors of the haemoglobin decrease in patients without baseline anaemia. The authors stated that markers of renal injury were independently associated with a decrease in haemoglobin level, which is consistent with the results of the present study. After adjusting for pathological factors in our multiple linear regression model, we found that urinary protein excretion rate and eGFR had a smaller impact on the haemoglobin decrease, and eGFR at baseline was one of the least significant predictors of haemoglobin decrease (P = 0.74); therefore, it is possible that IFTA score, which indicates the degree of tubulointerstitial injury, is the best prognostic factor for decrease in haemoglobin levels in patients with diabetic nephropathy.

We cannot clearly explain why a low BMI was independently associated with the decrease in haemoglobin. In the above-mentioned study by Thomas *et al.* [10], patients with anaemia at baseline and a further decrease of haemoglobin were less likely to be obese at baseline. Moreover, the effect of obesity in these patients was independent of serum albumin, transferrin saturation, other nutritional indices and glycaemic therapy. Furthermore, in the present study, a lower BMI showed a significant association with decrease in haemoglobin, independently of serum albumin level and glycaemic therapy (data not shown). Further investigation of the reason why a lower BMI predicts the haemoglobin decrease is needed in the future.

In our model adjusted for clinical and pathological variables, female gender was significantly correlated with the haemoglobin decrease, which was not consistent with the findings of Thomas *et al.* [10]. One reason for this discrepancy may be the differing numbers in each study of women aged < 55 years who are more likely to be menstruating. In the study by Thomas *et al.*, only 16% of the women were aged < 55 years compared with 42% in the present study.

The present study has several limitations. First, it was a retrospective investigation of a relatively small cohort performed at a single centre and the indications for renal biopsy were not standardized, suggesting that there might have been selection bias; however, this was the first investigation of the relationship between the progression of anaemia and renal pathological findings in patients with biopsy-proven diabetic nephropathy. Second, potential causes of anaemia and complications, such as malignancy, gastrointestinal tract lesions and sleep apnoea syndrome, were not investigated sufficiently. The major causes of anaemia in patients with diabetes and chronic kidney disease, however, are deficiency of EPO and iron, as well as hyporesponsiveness to the actions of EPO [1]. In the present study, the presence of iron deficiency anaemia at the start of follow-up was examined, but baseline EPO level and iron therapy during follow-up were not investigated, therefore, we could not determine whether IFTA score was associated with progression of anaemia as a result of EPO deficiency or not. Third, the decrease in haemoglobin level was not linear in some patients. When we compared r^2 values and statistical significance for the decrease in haemoglobin over time in each patient among various regression equations, we obtained a linear relation in 81 of the 113 patients. Although this confirmed that the haemoglobin decline was not linear in

Table 3 Independent predictors of annual haemoglobin decrease by multivariate analysis

Model 1 Clinical variables		Standardized coefficient	95% CI	*P*
Age (year)		−0.08	−0.02–0.008	0.49
Sex (female vs male)		0.18	−0.05–0.72	0.090
Type of diabetes (Type 1 vs Type 2)		−0.16	−1.05–0.13	0.12
BMI (kg/m^2)		−0.27	−0.09–−0.01	0.008
Systolic blood pressure (mmHg)		0.05	−0.006–0.01	0.58
Retinopathy (yes/no)		0.08	−0.17–0.40	0.43
eGFR (ml/min/1.73 m^2)		−0.16	−0.01–0.002	0.16
Urinary protein excretion rate (g/day)		0.28	0.02–−0.14	0.007
HbA_{1c} (%)		0.005	−0.07–0.07	0.96
Baseline haemoglobin (g/dl)		0.12	−0.04–0.15	0.27
ACE-I or ARB treatment (yes/no)		0.16	−0.04–0.55	0.093
Current or previous smoking habit (yes/no)		0.07	−0.21–0.41	0.51
Model 2 Clinical or histopathological variables		Standardized coefficient	95% CI	*P* Value
Age (year)		−0.07	−0.02–0.01	0.61
Sex (female vs male)		0.26	0.05–0.96	0.029
Type of diabetes (Type 1 vs Type 2)		−0.14	−1.2–0.33	0.26
BMI (kg/m^2)		−0.25	−0.09–−0.007	0.022
Systolic blood pressure (mmHg)		0.05	−0.007–0.01	0.61
Urinary protein excretion rate (g/day)		0.19	−0.01–0.13	0.093
Baseline haemoglobin (g/dl)		0.17	−0.03–0.18	0.16
ACE-I or ARB (yes/no)		0.10	−0.18–0.51	0.34
Current or previous smoking habit (yes/no)		0.13	−0.15–0.57	0.25
Glomerular class I (reference)	(n = 11)			
Glomerular class IIA	(n = 35)	0.10	−0.45–0.80	0.59
Glomerular class IIB	(n = 30)	0.14	−0.49–0.96	0.53
Glomerular class III	(n = 21)	0.08	−0.58–0.89	0.68
Glomerular class IV	(n = 8)	0.02	−0.88–0.97	0.92
IFTA score 0 (reference)	(n = 13)			
IFTA score 1	(n = 41)	0.20	−0.31–0.93	0.32
IFTA score 2	(n = 35)	0.34	−0.22–1.34	0.16
IFTA score 3	(n = 16)	0.47	−0.07–1.96	0.036
Interstitial inflammation score 0 (reference)	(n = 20)			
Interstitial inflammation score 1	(n = 80)	−0.02	−0.63–0.56	0.92
Interstitial inflammation score 2	(n = 5)	0.08	−0.62–1.22	0.52
Arteriolar hyalinosis score 0 (reference)	(n = 12)			
Arteriolar hyalinosis score 1	(n = 12)	−0.02	−0.71–0.63	0.90
Arteriolar hyalinosis score 2	(n = 81)	−0.17	−0.96–0.34	0.35
Arteriosclerosis score 0 (reference)	(n = 10)			
Arteriosclerosis score 1	(n = 58)	0.04	−0.51–0.65	0.82
Arteriosclerosis score 2	(n = 37)	0.10	−0.47–0.80	0.60
Exudative lesions (yes[n = 47]/no[n = 58])		0.12	−0.18–0.54	0.32

eGFR, estimated glomerular filtration rate; IFTA, interstitial fibrosis and tubular atrophy; ACE-I, angiotensin-converting enzyme inhibitor; ARB, angiotensin II receptor blocker.
In this multivariate linear regression model, the dependent variable was the annual haemoglobin decrease, which was included as a continuous variable. Standardized (β) coefficients were estimated by analysis of variables that were standardized to have a variance of 1. Accordingly, standardized coefficients show how many standard deviations a dependent variable changes per standard deviation increase (or decrease) in the predictor variable.

all patients, the results of multivariate regression analysis in these 81 patients were similar to our original results (data not shown). Moreover, in a similar previous study, it was not formally checked whether the change in haemoglobin level over time was linear [10]. Finally, the management of diabetic nephropathy during follow-up, including the use of renin-angiotensin inhibitors, glycaemic control and blood pressure control, was not sufficiently examined in this study or adjusted in analyses. Management of diabetic nephropathy has an important influence on anaemia because it can affect the progression of tubulointerstitial lesions that promote anaemia via EPO deficiency. In the present study, there were no significant differences in angiotensin-converting enzyme inhibitor or angiotensin II receptor blocker treatment during follow-up among the haemoglobin decrease tertiles. With respect to glycaemic control, the mean HbA_{1c} level was significantly lower in the more rapid haemoglobin decrease tertiles than in the slowest decrease tertile; however, the mean haemoglobin level was also significantly lower in the more rapid decrease tertiles, therefore, there might not be any major

Table 4 Independent pathological predictors of baseline haemoglobin according to multivariate analysis

Model Histopathological variables		Standardized coefficient	95% CI	*P*
Glomerular class I (reference)	(n = 11)			
Glomerular class IIA	(n = 34)	−0.06	−1.50–1.02	0.71
Glomerular class IIB	(n = 28)	−0.10	−1.90–1.09	0.59
Glomerular class III	(n = 20)	−0.34	−3.10– −0.06	0.042
Glomerular class IV	(n = 8)	−0.14	−2.85–1.01	0.35
IFTA score 0 (reference)	(n = 13)			
IFTA score 1	(n = 38)	−0.18	−1.95–0.62	0.31
IFTA score 2	(n = 35)	−0.31	−2.80–0.46	0.16
IFTA score 3	(n = 15)	−0.46	−4.28– −0.35	0.022
Interstitial inflammation score 0 (reference)	(n = 20)			
Interstitial inflammation score 1	(n = 76)	0.02	−1.10–1.28	0.88
Interstitial inflammation score 2	(n = 5)	−0.11	−2.82–0.93	0.32
Arteriolar hyalinosis score 0 (reference)	(n = 12)			
Arteriolar hyalinosis score 1	(n = 12)	−0.16	−2.13–0.39	0.17
Arteriolar hyalinosis score 2	(n = 77)	−0.20	−2.20–0.50	0.21
Arteriosclerosis score 0 (reference)	(n = 10)			
Arteriosclerosis score 1	(n = 54)	−0.02	−1.23–1.06	0.88
Arteriosclerosis score 2	(n = 37)	0.07	−1.02–1.52	0.70
Exudative lesions [yes (n = 45)/no (n = 56)]		0.13	−0.25–1.20	0.20

IFTA, interstitial fibrosis and tubular atrophy.
In this multivariate linear regression model, the dependent variable was baseline haemoglobin, which was included as a continuous variable. Standardized coefficients for glomerular classes IIA, IIB, III, and IV were calculated compared with that for glomerular class I. To calculate standardized coefficients for the IFTA score, interstitial inflammation score, arteriolar hyalinosis score, and arteriosclerosis score, the reference values were an IFTA score of 0, interstitial inflammation score of 0, arteriolar hyalinosis score of 0, and arteriosclerosis score of 0, respectively.

differences in glycaemic control among the haemoglobin tertiles. Investigation of blood pressure control showed that the mean systolic blood pressure and diastolic blood pressure during follow-up were lower than at baseline for each haemoglobin decrease tertile, and the number of anti-hypertensive agents at final follow-up was increased in each tertile.

In conclusion, a higher IFTA score was strongly associated with the decrease in haemoglobin levels in patients with biopsy-proven diabetic nephropathy after adjusting for numerous clinical and pathological covariates; therefore, more severe tubulointerstitial lesions can predict more rapid progression of anaemia in patients with diabetic nephropathy. As the decrease in haemoglobin closely paralleled the decrease in eGFR, tubulointerstitial lesions may be associated with the renal prognosis of diabetic nephropathy, both directly and indirectly via anaemia.

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Competing interests

None declared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1 Multivariate analysis of strong predictors of the annual decrease in haemoglobin levels.
Table S2 Independent predictors of the annual decrease in haemoglobin level by multivariate analysis in patients with a follow-up period ≥ 2 years.
Table S3 Comparison between the original model (follow-up periods ≥ 1 year) and the new model (follow-up periods ≥ 2 years) when baseline haemoglobin was removed from the models.