

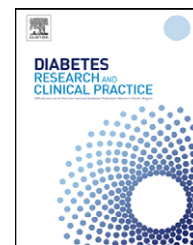


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Are low erythropoietin and 1,25-dihydroxyvitamin D levels indicative of tubulo-interstitial dysfunction in diabetes without persistent microalbuminuria?

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ABSTRACT

Aims: To investigate the relationship between Erythropoietin (EPO) and 1,25-dihydroxyvitamin D levels, and tubular damage in patients with diabetes mellitus (DM) without persistent microalbuminuria.

Methods: We measured serum EPO and 1,25-dihydroxyvitamin D levels and tubular injury markers such as urinary N-acetyl- β -D-glucosaminidase (NAG) and retinol binding protein (RBP) levels in 41 non-diabetic controls, 40 patients with Type 1 and 40 with Type 2 DM.

Results: Median serum EPO levels were lower in Type 1 (2.57 mIU/ml; $p < 0.001$) and Type 2 DM (5.69 mIU/ml; $p = 0.044$) than in controls (8.76 mIU/ml), though haemoglobin levels did not differ. Median 1,25-dihydroxyvitamin D levels were lower in Type 1 (41.0 pmol/l; $p = 0.001$) and Type 2 DM (41.8 pmol/l; $p = 0.035$) than in controls (56.1 pmol/l), though serum creatinine, calcium, phosphate and PTH levels did not differ. Median RBP excretion was higher in Type 2 DM (0.35 mg/l vs. 0.23 mg/l; $p = 0.013$) than in controls. Median NAG excretion was higher in Type 1 DM (1079 μ mol/h vs. 1030 μ mol/h; $p = 0.048$) compared to controls.

Conclusions: Tubulo-interstitial damage with low levels of EPO and 1,25-dihydroxyvitamin D occurs early in Type 1 and Type 2 DM before persistent microalbuminuria.

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1. Introduction

Diabetic nephropathy (DN) is a major microvascular complication of diabetes, and is now the leading cause of end stage renal disease (ESRD) worldwide [1]. Classically, DN has been described as a glomerular disease, with five different stages, starting with hyperfiltration, and progressing through a silent or incipient phase, through microalbuminuric and macroalbuminuric stages to a final stage of ESRD [2]. In contrast to

this traditional understanding, histological studies have suggested a major pathogenetic role for the tubulo-interstitium [3,4]. Altered tubular function, suggesting early tubulo-interstitial injury has been reported in normoalbuminuric patients with Type 2 DM [5].

N-acetyl- β -D-glucosaminidase (NAG) and retinol-binding protein (RBP) are markers of renal tubular injury [6]. Early tubular dysfunction may be seen in diabetes subjects, before the onset of microalbuminuria. Increased urinary excretion of

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NAG has been reported in patients with Type 1 DM without microalbuminuria [7]. Normoalbuminuric patients with Type 2 DM have increased urinary excretion of RBP [8]. These suggestions of tubular dysfunction in the absence of microalbuminuria suggest early involvement of renal tubulo-interstitium in the genesis of DN.

The renal tubulo-interstitium is the primary site of hydroxylation of 25-hydroxyvitamin D to 1,25-dihydroxyvitamin D [9] and EPO synthesis is primarily carried out by the peritubular fibroblasts [10]. Anaemia, due to low levels of EPO, develops early in patients with DN [11]. Low EPO levels in these patients may result from damage to the EPO-producing fibroblasts [10]. Abnormalities in mineral metabolism have also been described in DM patients, early in the course of chronic kidney disease (CKD) and have been associated with reduced serum levels of 1,25-dihydroxyvitamin D [12].

We investigated the relationship between markers of tubular injury (NAG and RBP) and tubulo-interstitial hormone (EPO and 1,25-dihydroxyvitamin D) synthesis in non-diabetic healthy controls and patients with Type 1 and Type 2 diabetes without persistent microalbuminuria.

2. Research design and methods

2.1. Subjects

Patients with Type 1 and Type 2 DM were recruited from local General Practices (GP) in East and North Hertfordshire according to predefined inclusion and exclusion criteria. Inclusion criteria were age >18 years, normal estimated creatinine clearance (>90 ml/min) by Cockcroft–Gault formula [13], no history of microalbuminuria within previous 3 months on screening, no evidence of documented peripheral neuropathy or retinopathy at screening. Exclusion criteria were history of renal disease, malignancy, current pregnancy, current immunosuppressive therapy and use of non-steroidal anti-inflammatory drugs within the previous month.

Non-diabetic controls were mainly recruited from hospital staff at Lister Hospital. Controls were age and sex-matched with Type 1 DM and sex-matched with Type 2 DM subjects. It was not possible to age match controls and Type 1 DM with Type 2 DM subjects. Neither the presence of hypertension nor the use of antihypertensive medications was a bar to recruitment either in the control or diabetic groups. The study was approved by the Hertfordshire research ethics committee. After screening the medical records, suitable patients were approached with the patient information sheet and explained about the study. The prospective study subjects, who agreed to participate, gave written informed consent and there were no drop-outs post-consent. Forty-one non-diabetics, 40 Type 1 DM and 40 Type 2 DM, met criteria (Total 121 subjects). The study was carried out in the summer months (July and August).

2.2. Data collection

A detailed medical history was recorded including, age, gender, race, type of diabetes, duration of diabetes, treatment for diabetes with insulin or oral hypoglycemics, history of

hypertension, duration of hypertension, type of antihypertensive therapy including angiotensin converting enzyme inhibitors (ACE-I) or angiotensin receptor blockers (ARB) and finally all other concomitant medication.

2.3. Laboratory investigations

A single overnight fasting blood sample was collected to measure baseline blood chemistry including fasting blood glucose (FBG), HbA_{1c}, full blood count, blood urea, serum ferritin, B₁₂ and folate levels, serum creatinine, serum albumin, C-reactive protein, serum bilirubin serum alanine transferase (ALT), serum alkaline phosphatase, serum parathyroid hormone (PTH), serum calcium, serum phosphate, and serum magnesium. Samples were also taken for serum EPO, 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D estimation. A spot urine sample was collected to assess levels of urinary albumin excretion, urine phosphate, creatinine, magnesium, NAG and RBP excretion.

2.4. Laboratory methodology

Full blood count measurement was performed on the ABX Pentra[®] (Horiba Diagnostics, Northampton, UK) according to manufacturer specifications. Routine biochemistry analyses (sodium, potassium, urea, creatinine, alanine transferase (ALT), gamma glutamyl transferase (GGT), full lipid profile, C-reactive protein (CRP), glucose and urine microalbumin) were performed on the Olympus AU 2700[®] multi-analyser (Olympus Diagnostics, Watford, UK) according to manufacturer specifications. PTH concentrations were measured on the Beckman Access[®] 2 immunoassay system (Beckman Coulter, High Wycombe, UK). Urine retinol binding protein (RBP), serum EPO, 25 hydroxyvitamin D and 1,25 dihydroxyvitamin D were performed in duplicate on the automated ELISA (enzyme linked immunosorbent assay) analyser, Triturus[®] (Grifols, Cambridge, UK).

The reagents were obtained from the following manufacturers: RBP (Immunodiagnostik AG, Bensheim, Germany), EPO (IBL, Hamburg, Germany), 25 hydroxyvitamin D and 1,25-dihydroxyvitamin D (Immunodiagnostic Systems Ltd, Boldon, UK). Urine NAG was also measured on the Triturus[®] (Grifols, Cambridge, UK) employing colorimetric detection at 505 nm, using reagents supplied by PPR Diagnostics Ltd (London, UK).

All analyses were performed as a single batch. The following within-run analytical coefficients of variation ranges were obtained for the respective concentration ranges. These analytes were also measured in duplicate to exclude the occurrence of random analytical; EPO (range 0.07–47.8 mIU/ml, CV 1.9–6.7%), 25-dihydroxyvitamin D (range 29.7–99.0 nmol/l, CV 3.5–11.2%), 1,25-dihydroxyvitamin D (range 15.4–89.3 pmol/l, CV 0.4–10.2%), RBP (range 0.01–0.94 mg/l, CV 0.6–8.3%), and NAG (range 609–1459 μ mol/h, CV 0.2–6.8%).

2.5. Statistical analysis

The statistical analysis was performed by SPSS (Statistical Package for Social Sciences) Version 16 (SPSS Inc., Chicago, USA). The results of the analysis have been presented as mean (95% CI) when data were normally distributed, and as median

Table 1 – Patient characteristics and demographics.

Variables	Groups			Comparison of groups		
	Controls (C)	Type 1 DM (T1)	Type 2 DM (T2)	C vs. T1 (p-value)	C vs. T2 (p-value)	T1 vs. T2 (p-value)
Number	41	40	40	NS	NS	NS
Age (years)	43.1 ± 9.5	42.4 ± 11.6	56.3 ± 9.0	NS	<0.001	<0.001
Gender (M:F)	20/21	20/20	21/19	NS	NS	NS
White/non-white	29/12	40/1	33/7	0.002	NS	0.057
DM Duration (years)	NA	19.9 ± 10.7	6.6 ± 3.7	<0.001	<0.001	<0.001
Systolic BP (mmHg)	124 ± 15	132 ± 18	138 ± 20	NS	0.004	NS
Diastolic BP (mmHg)	85 ± 10	84 ± 10	90 ± 9	NS	NS	NS
BMI	26.4 ± 4.3	27.0 ± 4.3	33.4 ± 6.2	NS	<0.001	<0.001
Ever-smoked (%)	39	32.5	58.5	NS	NS	0.042
ACEI/ARB (%)	7.3	30	56.1	0.011	<0.001	0.041

Demographic and clinical characteristics in control subjects and patients with Type 1 and Type 2 Diabetes. BMI = body mass index; ACE_I = angiotensin converting enzyme inhibitor; ARB = angiotensin receptor blocker; DM = diabetes mellitus; NS = non-significant.

values, when the distribution was not normal. Thus routine biochemical measurements have been reported as means, whereas, median values has been reported for EPO, 1,25 dihydroxyvitamin D, NAG and RBP. Comparisons between groups were carried out using the Kruskal–Wallis and Mann–Whitney *U*-tests for non-normally distributed data and One-Way Analysis of Variance (ANOVA) followed by Bonferroni post hoc testing when data was normally distributed. Differences between the groups with respect to the distribution of categorical variables were examined using the Chi-squared test. *p*-Values ≤0.05 were considered to be statistically significant.

3. Results

3.1. Demographics and clinical factors (Table 1)

There was no difference in the age of Type 1 DM patients and controls. However, patients with Type 2 DM were significantly older than other groups ($p < 0.001$ in both cases). There were also racial differences between the groups, the proportion of non-whites being lower in patients with Type 1 DM than in those with Type 2 disease ($p = 0.057$) and in controls ($p = 0.002$). There was no difference in the gender ratio across the groups. More patients with Type 2 than with Type 1 DM gave a history of ever smoking ($p = 0.042$), the difference with controls was not significant. The proportion of current smokers did not differ significantly across the groups. Patients with Type 2 DM were significantly heavier than those with Type 1 disease ($p = 0.001$) and controls ($p < 0.001$). Systolic blood pressure was significantly higher in patients with Type 2 DM (137 mm of Hg: $p = 0.002$) than in controls (124 mm of Hg). A greater proportion of patients with Type 2 DM took ACEI or ARB than Type 1 ($p = 0.041$) or controls ($p = 0.011$).

3.2. Baseline biochemistry and haematology (Table 2)

Patients with Type 2 DM had significantly lower HbA_{1c} ($7.6 \pm 1.5\%$ vs. $8.3 \pm 1.4\%$: $p = 0.022$) compared to those with Type 1 DM. There was no difference in renal function between the groups. Serum creatinine and eGFR by the MDRD-4 [14]

formula did not differ significantly across the groups, though creatinine clearance calculated by the Cockcroft–Gault method [13] was slightly higher in Type 2 DM when compared with controls ($p = 0.018$). Haemoglobin, serum calcium, phosphate and parathyroid hormone levels did not differ significantly between the three groups. Serum magnesium levels were lower in Type 1 DM (0.84 mmol/l: $p = 0.009$) and Type 2 DM (0.82 mmol/l: $p < 0.001$) compared to controls (0.88 mmol/l).

Serum ferritin and vitamin B₁₂ levels did not differ across the groups, but folate levels in patients with Type 1 DM were significantly higher than in controls (474 ng/ml vs. 349 ng/ml: $p = 0.004$). Serum alkaline phosphatase levels were higher in patients with Type 1 DM (94.2 IU/l) than in those with Type 2 DM (77.9 IU/l: $p = 0.007$) and controls (74.6 IU/l: $p = 0.001$), though ALT levels did not differ. In addition, serum albumin was lower in Type 2 DM (43.9 g/l vs. 45.4 g/l: $p = 0.033$) as compared to controls.

3.3. Erythropoietin levels

Median serum EPO levels were lower in Type 1 (2.57 mIU/ml: $p < 0.001$) and Type 2 DM (5.69 mIU/ml: $p = 0.044$) compared to controls (8.76 mIU/ml) (Fig. 1), though there were no significant differences in haemoglobin levels between the groups (Table 2). EPO levels did not correlate with age, sex, HbA_{1c} and fasting blood glucose in any of the groups. There were no significant differences in EPO levels in patients on ACE/ARB therapy and those not on this treatment.

3.4. Serum vitamin D levels

Median serum 25-hydroxyvitamin D levels in patients with Type 2 DM (63.8 nmol/l) were lower than those in controls (69.1 nmol/l: $p = 0.034$). Median levels in patients with Type 1 DM (65.5 nmol/l) did not differ from control values. Median 1,25-dihydroxyvitamin D levels were lower in Type 1 (41.0 pmol/l: $p = 0.001$) and Type 2 DM (41.8 pmol/l: $p = 0.035$) compared to controls (56.1 pmol/l) (Fig. 1), though the groups did not differ with respect to calcium, phosphate and PTH levels (Table 2). There was no effect of age, sex, race, smoking, BMI, ACEI and ARB therapy on 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D levels.

Table 2 – Baseline biochemical and haematological parameters.

Variables	Groups			Comparison of groups		
	Controls (C)	Type 1 DM (T1)	Type 2 DM (T2)	C vs. T1 (p-value)	C vs. T2 (p-value)	T1 vs. T2 (p-value)
Serum parameters						
Erythropoietin (mIU/ml)	8.76 (24.5)	2.57 (24.7)	5.69 (47.6)	<0.001	0.044	<0.001
25 Vit D (nmol/l)	69.1 (54.1)	65.5 (60)	63.8(54.3)	NS	0.034	NS
1,25 Vit D (pmol/l)	56.1(59.2)	41(58)	41.8 (68)	0.001	0.035	NS
Glucose (mmol/l)	4.9 ± 0.6	8.6 ± 3.9	7.8 ± 2.7	<0.001	<0.001	NS
HbA _{1c} (%)	5.5 ± 0.4	8.3 ± 1.4	7.6 ± 1.5	<0.001	<0.001	0.022
Creatinine (μmol/l)	84 ± 10	82 ± 11	79 ± 14	NS	NS	NS
Creat. clearance (C-G)	100 ± 24	106 ± 18	115 ± 31	NS	0.018	NS
Urea (mmol/l)	4.9 ± 1.3	5.2 ± 1.3	5.3 ± 1.3	NS	NS	NS
Haemoglobin (g/dl)	14.1 ± 1.3	14.2 ± 1.2	14.0 ± 1.3	NS	NS	NS
Ferritin (ng/ml)	66.7 ± 59.4	63.7 ± 57	89.6 ± 82.6	NS	NS	NS
Vitamin B ₁₂ (pg/ml)	377 ± 163	386 ± 152	364 ± 249	NS	NS	NS
Folate (ng/ml)	349 ± 121	474 ± 206	407 ± 167	0.004	NS	NS
Corrected calcium (mmol/l)	2.29 ± 0.08	2.29 ± 0.07	2.31 ± 0.07	NS	NS	NS
Phosphate (mmol/l)	1.05 ± 0.16	1.1 ± 0.18	1.08 ± 0.15	NS	NS	NS
PTH (pmol/l)	4.4 ± 2.5	4.1 ± 1.8	4.0 ± 1.8	NS	NS	NS
Magnesium (mmol/l)	0.88 ± 0.05	0.84 ± 0.06	0.82 ± 0.08	0.009	<0.001	NS
Alkaline phosphatase (IU/l)	74.6 ± 23.9	94.2 ± 25.7	77.9 ± 20.7	0.001	NS	0.007
ALT (IU/L)	26 ± 12.1	33.6 ± 23.8	34.5 ± 15.6	NS	NS	NS
Albumin (g/l)	45.4 ± 2.5	44.6 ± 2.6	43.9 ± 2.7	NS	0.033	NS
Urinary parameters						
Median NAG (μmol/h)	1030 (670)	1079 (756)	989 (780)	0.048	NS	NS
Median NAG:creat. ratio	67.9 (848)	83.1 (373)	93.5 (411)	NS	NS	NS
Median RBP (mg/l)	0.23 (0.73)	0.27 (0.85)	0.35 (0.93)	NS	0.013	NS
Median RBP:creat. ratio	0.02 (0.41)	0.02 (0.15)	0.03 (0.25)	NS	0.023	NS

Baseline biochemical and haematological parameters in control subjects and patients with Type 1 and Type 2 Diabetes. Normally distributed parameters represented as mean ± standard deviation. Non-normally distributed parameters represented as median (range). 25 Vit D = 25 hydroxyvitamin D; 1,25 Vit D = 1,25 dihydroxyvitamin D; Creat. clearance (ml/min) = creatinine clearance estimated by Cockcroft–Gault method (C–G), PTH = parathyroid hormone; ALT = alanine transaminase, NAG = N-acetyl-β-D-glucosaminidase; RBP = retinol binding protein; NAG:creat. ratio = N-acetyl-β-D-glucosaminidase: creatinine ratio; RBP:creat ratio = retinol binding protein: creatinine ratio.

3.5. Tubular dysfunction markers (Table 2)

Median RBP excretion was significantly higher in patients with Type 2 DM (0.35 mg/l; $p = 0.013$) as compared to controls (0.23 mg/l). In Type 1 DM (0.27 mg/l) patients, the median RBP excretion, although higher than in controls was not significantly different. Median RBP:creatinine ratio was significantly higher in patients with Type 2 DM than in controls (0.027 mg/l vs. 0.018 mg/(l mmol); $p = 0.023$). Median NAG excretion was higher in Type 1 (1079 μmol/h; $p = 0.048$) as compared to controls (1030 μmol/h). There was no difference between the groups with respect to urinary NAG:creatinine ratio.

In all subjects, NAG:creatinine ratio correlated significantly with RBP:creatinine ratio ($r = 0.676$, $p < 0.001$), and with fractional excretion of magnesium ($r = 0.179$, $p = 0.05$). In the diabetes group, NAG:creatinine ratio correlated significantly only with RBP:creatinine ratio ($r = 0.676$, $p < 0.001$). Correlations were similar in the DM groups singly and as a whole. There were no significant correlations between urinary NAG, NAG:creatinine ratio, RBP or RBP:creatinine ratio and EPO levels or 1,25-dihydroxyvitamin D in the group as a whole or within individual subgroups.

In the whole group, urinary RBP:creatinine ratio correlated significantly with age ($r = 0.195$, $p = 0.032$) and creatinine clearance (Cockcroft–Gault) ($r = -0.195$, $p = 0.032$). Urinary

RBP:creatinine ratio was higher in smokers (0.027 vs. 0.018; $p = 0.021$), but sex, race, BMI and the use of ACEI or ARB therapy had no effect on this ratio. Neither age nor smoking was significant determinants of urinary RBP:creatinine ratios in multivariate analysis. Sex, race, smoking, BMI and being on ACEI or ARB therapy had no effect on urinary RBP and NAG levels or on urinary NAG:creatinine ratios.

3.6. Urinary albumin levels

Although patients with diabetes were selected on the basis of having no microalbuminuria on screening (defined as albumin creatinine ratio (ACR) <2.5 in males and <3.5 mg/mmol in females on two or more consecutive tests, within a period of 1–3 months) [15], when urinary ACR estimates were repeated as part of the study protocol, 13 subjects were found to have high ACR levels—4 controls, 5 patients with Type 1 DM, and 4 patients with Type 2 DM. Mean ACR levels were minimal and did not differ between the groups (non-diabetics 0.10 ± 0.30 , Type 1 DM, 0.11 ± 0.33 , Type 2 DM 0.10 ± 0.30 mg/mmol; $p = \text{NS}$). There was no correlation between urinary RBP, NAG, RBP:creatinine ratio, NAG:creatinine ratio and ACR levels. The exclusion of those patients with high ACR from the analysis, did not significantly impact upon the overall findings.

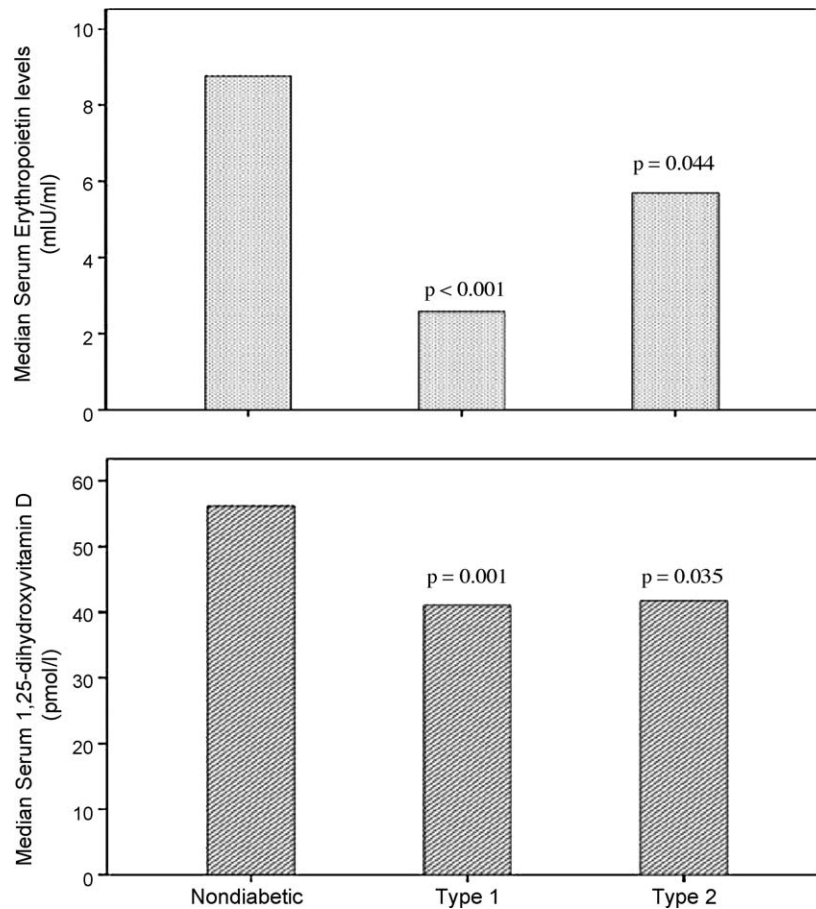


Fig. 1 – Serum erythropoietin and 1,25-dihydroxyvitamin D levels. Median serum erythropoietin and 1,25-dihydroxyvitamin D levels in patients with Type 1 and Type 2 diabetes and in non-diabetic controls. p-Values quoted reflect significance of differences from controls.

4. Discussion

The major findings in this study are that both Type 1 and Type 2 DM patients have low EPO and 1,25-dihydroxyvitamin D levels with normal estimated creatinine clearance and prior to the onset of persistent microalbuminuria. These subjects with diabetes also had evidence of early tubulo-interstitial injury as reflected by higher excretion of NAG in Type 1 DM and RBP in Type 2 DM patients.

Though serum EPO levels were lower in Type 1 and Type 2 DM patients compared to controls, there were no significant differences in haemoglobin levels between the groups. Low EPO levels with normal haemoglobin have been reported in normoalbuminuric [11,16,17] and microalbuminuric [18] Type 2 DM patients. However, the previous studies in normoalbuminuric Type 2 DM were limited by non-characterization of patient groups in terms of GFR and albuminuria [17] and/or absence of a control group [16], and lack of data on haematitic levels. Also, none of the previous studies included patients with Type 1 DM or examined the status of the tubulo-interstitium [11,16–18].

EPO stimulates erythropoiesis and has other pleiotropic properties. We found that low serum EPO levels were not associated with low haemoglobin levels perhaps because the

levels in the diabetes groups remained above the threshold required to maintain normal haemoglobin. It may be, though, that these low levels of EPO could potentially influence its pleiotropic properties in some individuals. In agreement with previous observations, EPO levels did not differ significantly in patients who were on ACE-I/ARB therapy compared to those who were not [16].

Low 25-hydroxyvitamin D levels have been reported and implicated in the pathogenesis of Type 1 [19] and Type 2 DM [20]. This is the first study to report low levels of 1,25-dihydroxyvitamin D in Type 1 and Type 2 diabetes before the onset of persistent microalbuminuria. It is noteworthy that these changes occurred in the absence of changes in calcium, phosphate, or parathyroid hormone levels, though magnesium levels were lower in both DM groups. Magnesium deficiency has been associated with low serum levels of 1, 25-dihydroxyvitamin D and impaired secretion of parathyroid hormone [21].

The observations of higher excretion of tubular injury markers are in agreement with previous studies which have shown increased excretion of RBP in Type 2 DM [8] and NAG in Type 1 DM [7]. The differences in RBP and NAG excretion in Type 1 and Type 2 DM may reflect different pathogenetic factors operating in these conditions, and merit further study.

As discussed above, the reductions in EPO and 1, 25-dihydroxyvitamin D levels were more marked in patients with Type 1 DM, whilst the evidence for tubular dysfunction was stronger in Type 2 patients. This dissociation also merits further attention.

Microalbuminuria has been considered to be the first significant marker of DN. Up to 30% of patients with Type 2 DM may have microalbuminuria or proteinuria at diagnosis [22]. Early microalbuminuria may result from both glomerular and proximal tubular dysfunction [23]. In DM patients with normal renal function, increased urinary excretion of NAG and RBP may indicate proximal tubular injury and potentially help in identifying patients at high risk of developing DN [7].

Recently, we suggested that the renal tubule may be damaged prior to the glomerulus in early DN, due to the chronic hypoxic milieu, the consequence of sustained hyperglycaemia [24]. Early loss of tubular and peritubular cells could impair the synthesis of 1,25-dihydroxyvitamin D and EPO which, together with dysfunction of their receptors caused by the diabetic state, and other mechanisms, such as early removal of EPO from circulation as a result of abnormal glycosylation [17], might diminish the local trophic effects of these hormones. This could potentially result in further compromise to the functional and structural integrity of the renal parenchyma, and contribute to the gradual decline of renal function. The findings of the current study are compatible with this hypothesis.

Although the excretion of NAG and RBP was elevated in patients with diabetes and the serum levels of EPO and 1,25-dihydroxyvitamin D were low, there were no significant correlations between the markers of tubulo-interstitial injury and those reflecting diminished functional capacity, that is, low serum levels of EPO and 1,25-dihydroxyvitamin D. This lack of correlation may be accounted for by: (1) the different cellular locations of the hormone function (EPO synthesis and 1 α hydroxylation of 25-hydroxyvitamin D) and the markers of tubular injury; (2) a dissociation between the mechanisms of tubular and peritubular injury; (3) lack of sensitivity of urinary NAG and RBP excretion as markers of tubular damage. If this is the case our study would suggest that low serum levels of EPO and 1,25-dihydroxyvitamin D may be better indicators of early tubulo-interstitial dysfunction in this setting than more traditional markers, such as urinary NAG and RBP.

Our study has a number of limitations. These include small patient numbers and incomplete matching of the groups particularly with respect to age—the group with Type 2 DM being older than the other two groups. There were also differences between duration of diabetes in the groups with Type 1 and Type 2 disease. In addition, although the diabetes groups were selected on the basis of having been screened negative for microalbuminuria, when, during the course of the study, microalbuminuria was retested on the basis of ACR in the spot urine sample, similar numbers in all groups (4–5 patients, 10–12.5%) were found to be in microalbuminuric range. The diagnosis of microalbuminuria requires ACR levels exceeding threshold values on two or more consecutive occasions, ideally within 1–3 months [15]. Our finding that 10–12.5% of patients with diabetes had a single ACR value in the microalbuminuric range may reflect a natural variability in urinary albumin excretion these patients or may indicate

evolving microalbuminuria. Interestingly, the prevalence of microalbuminuria, based on a single ACR sample, in our control group was also 10%. This was lower compared with a previous study, which reported the prevalence of microalbuminuria in non-diabetic general population of up to 13% [25].

We also recognize that NAG and RBP may not be the gold standard markers of tubular injury. However, NAG and RBP are most widely assessed tubular injury markers. It may be though that further studies in this area will need to employ a range of markers of tubular function, and include timed collections of urine to improve sensitivity.

In conclusion, we have shown diminished functional ability of tubulo-interstitium in DM patients with normal levels of serum creatinine and estimated GFR, and in the absence of persistent microalbuminuria. This is reflected in reduced levels of EPO and 1,25-dihydroxyvitamin D, in the presence of significantly higher excretion of NAG and RBP. EPO and 1,25-dihydroxyvitamin D levels in this setting may be useful markers for the diagnosis and monitoring of early DN. Our findings are also compatible with the hypothesis that in the evolution of DN, significant tubulo-interstitial damage may precede clinically evident glomerular injury. Further studies with larger patient groups are required to confirm and extend our findings.

Conflict of interest

There are no conflicts of interest.

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