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The impact of diabetes mellitus on vitamin D metabolism in predialysis patients

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ABSTRACT

Although diabetes mellitus (DM) disturbs bone metabolism, little is known concerning its effects on laboratory abnormalities in chronic kidney disease-mineral and bone disorders (CKD-MBD). We extracted data for 602 patients from the Osaka Vitamin D Study in patients with CKD (OVIDS-CKD), an observational study enrolling predialysis outpatients. No enrolled patients received vitamin D, bisphosphonate, estrogen or raloxifene. We measured 1- 84 PTH, 25-hydroxyvitamin D (25D), calcitriol, fibroblast growth factor-23 (FGF-23), calcium (Ca), and phosphate (P). Since there were 112 DM patients (group D), we extracted 112 age-, sex-, and eGFR-matched non-DM counterparts (group N). We compared biochemical markers between groups, and then performed multiple regression analyses for all 602 subjects to confirm the results obtained. Group D had significantly higher corrected Ca and P than group N throughout all stages of CKD. In group D, 25D decreased as renal function declined, while in group N it remained constant (interaction $P<0.05$). Despite higher P and poorer vitamin D status in DM, there were no differences in 1- 84 PTH level between group D and group N stratified by stage of CKD, resulting in significantly lower calcitriol levels in group D in late CKD. Multiple regression analyses revealed that DM was significantly associated with low vitamin D status even with adjustment for urinary protein, and that this poorer vitamin D status in DM was responsible for lower calcitriol level associated with DM. Despite higher P, lower FGF-23 in early CKD (stages 1+2) and comparable level of FGF-23 in late stages of CKD (stages 3, 4, and 5) were observed in group D. We interpreted these results to indicate that inappropriate production of FGF-23 in DM might explain higher serum phosphate in DM. Multiple regression analysis with adjustment for covariates confirmed an independent relationship between DM and low FGF-23, implying the existence of dysfunction or decreased density of osteocytes in DM. Given the origin of these phosphaturic hormones, DM may thus have markedly deleterious effects on parathyroid and bone. Poorer vitamin D status and higher CaP product might be partly responsible for functional and structural changes of vasculature, respectively, in DM.

Introduction

Use of the new umbrella concept chronic kidney disease-mineral and bone disorders (CKD-MBD) was advocated by Kidney Disease Improving Global Outcomes (KDIGO) [1]. Three factors, ectopic calcification, renal osteodystrophy, and laboratory abnormalities, are included in this novel concept. It is known that in predialysis patients diabetes mellitus (DM) significantly worsens vascular calcification, one of the types of ectopic ossification reported to be associated with morbidity and mortality in hemodialysis patients [2–5]. Moreover, DM has a marked impact on renal osteodystrophy, including suppression of serum parathyroid hormone (PTH) in hemodialysis patients [6]. However, no studies have reported the differences in laboratory abnormalities between diabetic and non-diabetic predialysis patients.

It is known that DM disturbs bone metabolism in subjects without CKD [7,8]. One of the characteristics of bone metabolism in DM patients is low bone turnover. Although Type 2 DM has no deleterious effect on bone mineral density because of this low rate of bone turnover, risk of bone fracture is reported to be higher in diabetic patients [9–11]. Diabetic hemodialysis patients have also been reported to have an increased incidence of hip fracture [12]. Osteoblast dysfunction induced by oxidative stress might explain the disturbance of bone metabolism in DM, since it would lead to impairment of bone formation [13].

In this study, we focused on the effects of DM on serum calcium, phosphate, and vitamin D metabolism with adjustment for estimated glomerular filtration rate (eGFR). In investigating vitamin D regula-

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tion, we measured two phosphaturic hormones, PTH and fibroblast growth factor-23 (FGF-23). In this cross-sectional, observational study enrolling DM patients and sex-, age-, and eGFR-matched non-DM patients, we found that DM patients had higher serum corrected calcium and phosphate levels and poorer vitamin D status with comparable levels of 1–84 PTH, resulting in lower serum calcitriol levels in advanced CKD. We also found that DM was a significant negative determinant for serum FGF-23.

Methods

Study population

We extracted data for 602 patients from the Osaka Vitamin D Study in patients with CKD (OVIDS-CKD), a multicenter cross-sectional observational study enrolling 751 CKD outpatients in Japan. All patients gave written informed consent to participate in the study. The Ethics Committee at Osaka University Graduate School of Medicine approved this study (approval number 07142). Subjects were eligible for enrollment if they had never received steroids, bisphosphonates, native or active vitamin D, or raloxifene as determined by medical history. Since there is a report that statins do not affect mineral metabolism in CKD including calcium, phosphorus and iPTH [14], we included them into the analysis. Of the total of 602 subjects enrolled, 112 patients had DM (group D) and met the criteria of the American Diabetes Association. We selected one sex-, age-, and eGFR-matched control patient without diabetes for each enrolled diabetic patient (group N) to obtain a new cohort of 224 patients. We selected them by computerization, leading to least possibility of bias. In brief, each diabetic patient was assigned a 4-digit index number which includes the information of sex, eGFR category in 10 mL/min/1.73 m^2 band, and age category in 10 year band. And for each diabetic patient, one non-diabetic patient with the same 4-digit index number was selected from the entire non-diabetic population by using computer (a 1:1 match). If this could not be done, the algorithm then proceeded sequentially to the next highest digit match (a 3-, 2-digit) on index number to make "next-best" matches.

In order to exclude the selection bias, we further performed sensitivity analysis, in which we created another cohort of non-diabetic patients by another equation formula.

Measured variables

Blood and urine samples were obtained at enrollment. Blood samples were drawn from ambulatory patients after an overnight fast. After 30 minute incubation, blood samples were centrifuged for serum separation and the serum was stored frozen at −80 °C until analysis. Blood chemistry parameters (blood urea nitrogen, creatinine, albumin, calcium, and inorganic phosphate) were measured by standard automated techniques. Estimated GFR was calculated according to revised MDRD formula [15]. Full-length 1–84 PTH was measured by a third-generation assay (Whole PTH, Scantibodies, Santee, CA, USA). The biologically active form of FGF-23 (intact FGF-23) was measured by a sandwich enzyme-linked immunosorbent assay system (Kainos Laboratories, Inc., Tokyo, Japan). Levels of serum calcitriol and 25-hydroxyvitamin D were measured using a TFB 1,25-hydroxyvitamin D RIA kit (Immunodiagnostic Systems Ltd., Boldon, UK) and a ^{125}I RIA kit (DiaSorin Inc., Stillwater, MN, USA), respectively. Bone-specific alkaline phosphatase (bone ALP) and serum cross-linked N-telopeptide of type I collagen (NTX) were assayed by using the Osteolinks-Bone ALP high-sensitivity diagnostic enzyme immunoassay (EIA) kit (Sumitomo Pharmaceuticals, Co., Osaka, Japan) and an OSTEOMARK ELIZA kit (Mochida pharmaceutical Co., Tokyo, Japan), respectively. We measured the bone mineral density (BMD) of the second to fourth lumbar vertebrae (L2–4) in addition to the femoral neck with a dual-energy X-ray absorptiometer (Discovery A, Hologic Inc., Bedford, MA, USA) in the posterior–anterior projection. Serum calcium level was corrected for Alb using the formula corrected calcium = total calcium + (4.0 − albumin) * 0.8 if Alb < 4.0 mg/dL. Urinary protein was measured semiquantitatively with a dipstick test.

Statistical analyses

Demographic factors and laboratory data for groups D and N were compared using *t*-tests or Wilcoxon rank-sum tests for continuous variables as appropriate, and the Pearson chi-square test for categorical variables. When dependent or explanatory variables had no linearity, they were log transformed (e.g. 1–84 PTH, FGF-23).

First, between-group analyses of these laboratory parameters were performed using a multiple linear regression model with eGFR and group as explanatory variables. This model involves analysis of covariance (ANCOVA) only when there is no interaction between eGFR and group. We performed post-hoc analysis regarding the difference within each CKD stage only if significant interaction was observed.

Second, we confirmed between-group differences in laboratory parameters by stepwise forward multivariate linear regression analyses for the total of 602 patients enrolled. Factors adjusted in the analysis of FGF-23 were age, sex, body mass index, corrected Ca, P, eGFR, 1–84 PTH, 25D, and 1,25D. In the analysis of 25D, two models were used, with and without urinary protein, with adjustment for the season of collection of blood samples, age, sex, BMI, corrected Ca, P, eGFR, 1–84 PTH, and FGF-23. The prevalences of semiquantitative urinary protein (uPro) dipstick testing across CKD stages were compared by chi-square test separately in each group. If a difference was significant, we proceeded to Cochrane–Armitage trend analysis to examine the association between CKD stage and the prevalence of uPro ≥3+. To take into account the contributions of urinary protein [16] and season [17,18] to 25D level, we converted them to dummy variables in multivariable analyses for vitamin D status. Dummy variables were constructed with no urinary protein and winter as references, respectively. Herein, spring denotes March to May, summer June to August, autumn September to November, and winter December to February. In another analysis of calcitriol, we also prepared two models with or without 25D, to determine whether poorer calcitriol levels in diabetic patients were actually due to poorer

Table 1
Demographic factors and laboratory findings for all subjects and matched groups.

Variable	All subjects	Group D	Group N	*P* value between groups
n [female%]	602 [34.7%]	112 [25%]	112 [25%]	1
Age (years)	64 (54–71)	66 (57–73)	66 (57–73)	0.9
BMI (kg/m^2)	23.2 (21.2–25.5)	23.9 (22.0–26.7)	22.6 (20.9–25.1)	0.02
Cr (mg/dL)	1.4 (1.1–2.1)	1.9 (1.3–3.2)	1.8 (1.2–3.0)	0.6
Alb (g/dL)	4.0 (3.7–4.2)	3.9 (3.5–4.2)	4.0 (3.7–4.2)	0.08
eGFR (mL/min/1.73 m^3)	48.1 (30.3–65.9)	34.6 (19.0–55.1)	35.6 (20.5–58.7)	0.6
$uPro^N$ (−/±/+/2+/>3+)	126/83/116/93/61	13/17/26/22/32	19/15/24/11/8	0.007

n, numbers of patients; DM, diabetes mellitus; values are the mean ± SD or median (interquartile range) as appropriate.
Note. serum creatinine in mg/dL may be converted to μmol/L by multiplying by 88.4, albumin in g/dL to g/L by multiplying by 10, eGFR in mL/min/1.73 m^2 to mL/s/1.73 m^2 by multiplying by 0.01667.

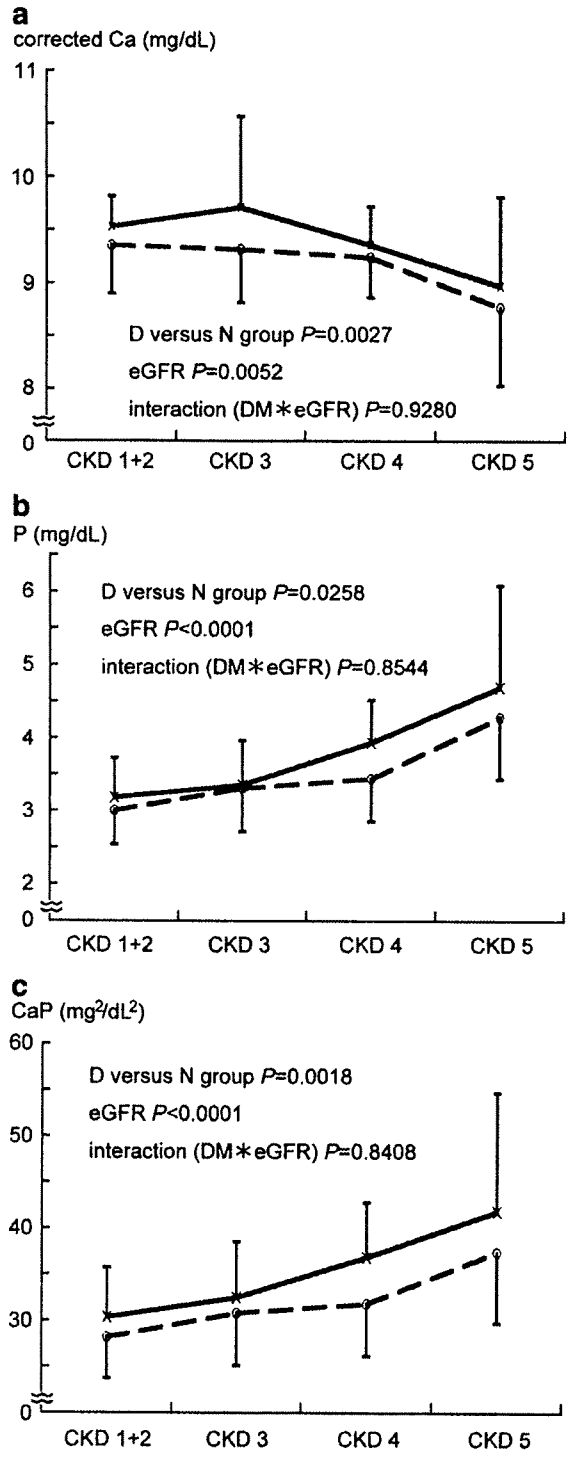

Fig. 1. Differences in laboratory findings between the two groups stratified by CKD stage. Differences between the two groups in corrected Ca (a), P (b), and CaP product (c) levels by CKD stage. The broken line represents group N and the solid line group D. Each bar represents the mean + SD for group D or mean-SD for group N. Serum corrected Ca in mg/dL may be converted to mmol/L by multiplying by 0.2495, P in mg/dL to mmol/L by multiplying by 0.3229.

vitamin D status. In all analyses, 1–84 PTH and FGF-23 levels were log-transformed because their distributions were skewed. Statistical tests were two-sided, and P values of less than 0.05 were considered significant. All statistical analyses were performed using JMP software, version 7.0.2 (SAS Institute, NC, USA).

Results

Patient characteristics

A total of 602 subjects were analyzed, 34.7% ($n=209$) of whom were female. Since there were 112 DM patients (group D), we extracted age-, sex-, and eGFR-matched non-DM counterparts (group N) to obtain a new cohort of 224 patients. The demographic factors and laboratory data in each group are shown in Table 1. Compared with group N, group D had higher body mass index (BMI) and more severe proteinuria, though the difference between the groups in serum albumin (Alb) was not statistically significant. Thirteen percent of non-diabetic patients (14 out of 112) received statins, whereas 38% of the diabetic patients (42 out of 112) received them. Only small portion of diabetic patients (8 out of 112) had thiazolidinedione prescribed, and 40% of the diabetic patients (46 out of 112) received insulin therapy. Regarding diuretics, thiazide and loop diuretics were

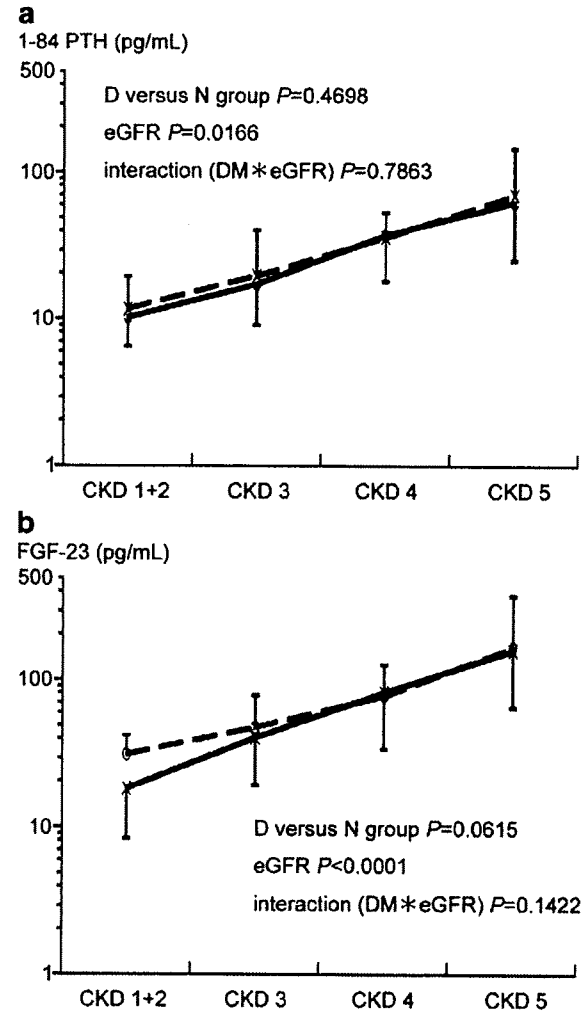

Fig. 2. Differences in phosphaturic hormones between the two groups by CKD stage. Differences between the two groups in 1–84 PTH (a) and FGF-23 (b) level by CKD stage. The broken line represents group N and the solid line group D. Each bar represents the mean + SD for group N or mean-SD for group D. Serum 1–84 PTH levels in pg/mL and ng/L are equivalent.

Table 2
Multiple linear regression analyses for FGF-23.

Independent variable	β	SE	T	P
DM	−0.1242	0.0312	−3.98	<0.001
Sex (female)	−0.0637	0.0264	−2.42	0.02
eGFR	−0.0009	0.0015	−6.06	<0.001
Log (1–84 PTH)	0.1357	0.0363	3.74	<0.001
P	0.3071	0.0394	7.80	<0.001
25D	0.0127	0.0044	2.89	0.004
1,25D	−0.0092	0.0016	−5.60	<0.001
	Intercept = 2.82		$R^2 = 0.53$	

25D, 25-hydroxyvitamin D; 1,25D, 1,25-dihydroxyvitamin D; PTH, parathyroid hormone.

significantly more often used in group D (thiazide; 12% in group D, 4% in group N, $P<0.05$, loop; 47% in group D, 13% in group N, $P<0.0001$).

Differences in serum Ca and P between groups stratified by renal function

We compared biochemical laboratory findings in the two groups. Corrected calcium (Ca) was higher in group D ($P=0.0027$) than group N through all CKD stages, and decreased in both groups as renal function declined ($P=0.0052$). No significant interaction was detected (Fig. 1a). Serum phosphate (P) was also significantly higher in group D ($P=0.0258$), but increased in both groups as renal function declined (Fig. 1b); once again, interaction was not significant. Consequently, Ca P product, which was reported to be associated with the extent of vascular calcification in hemodialysis patients [19–21], was significantly higher in group D ($P=0.0018$), and increased in both groups as renal function declined. Again, no significant interaction was observed (Fig. 1c).

Two phosphaturic hormones

In the same fashion, we examined the difference between groups in two phosphaturic hormones, 1–84 PTH and full length FGF-23. Levels of both hormones increased as renal function declined, with no interaction observed. No significant differences were found in levels of either phosphaturic hormone between the groups (Figs. 2a, b), despite higher serum phosphate level in group D. Moreover, there was a trend toward lower FGF-23 in group D in early CKD (stages 1 + 2), though this difference decreased in late CKD. Thus, since group D had significantly higher phosphate than group N, our finding that group D had a borderline significantly lower FGF-23 overall ($P=0.06$) was paradoxical. In this context, we examined the relationship between DM and low FGF-23 by multiple regression analysis of FGF-23 in the group of all enrolled patients, with adjustment for serum phosphate (Table 2), and found that DM was significantly and

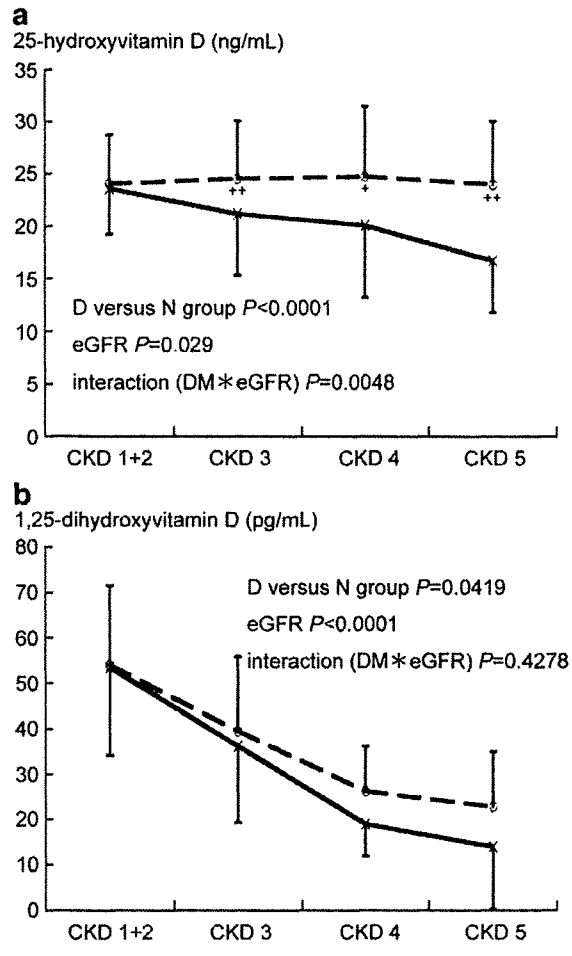

Fig. 3. Differences in vitamin D status and serum calcitriol level between the two groups by CKD stage. Differences between the two groups in 25-hydroxyvitamin D (a) and 1,25-dihydroxyvitamin D (b) level by CKD stage. The broken line represents group N and the solid line group D. Each bar represents the mean + SD for group N or mean-SD for group D. +, $P<0.05$; ++, $P<0.01$: results of post-hoc analysis for comparison between groups within each CKD stage. Serum 25-hydroxyvitamin D in ng/mL may be converted to nmol/L by multiplying by 2.496; serum 1,25-dihydroxyvitamin D in pg/mL to pmol/L by multiplying by 2.6.

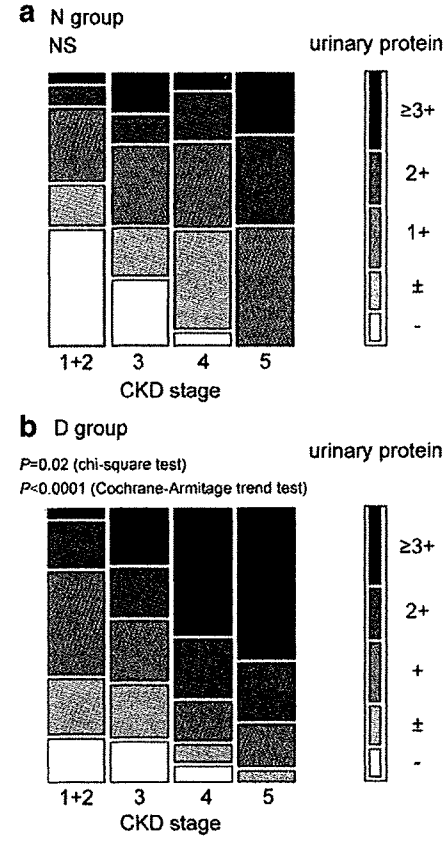

Fig. 4. The prevalence of proteinuria category as determined by dipstick test in each CKD stage by DM status. Urinary protein was measured semiquantitatively by dipstick test. (b) The differences in amount of proteinuria across CKD stages were significant in group D ($P=0.02$ by chi-square test) but not in group N (a). There was also a significant trend toward increase in percentage of patients with urinary protein ≥ 3+ with progression of CKD stage ($P<0.0001$ by Cochrane-Armitage trend test). NS means not significant.

Table 3
Multiple linear regression analyses for 25-hydroxyvitam D.

Independent variable	Model 1		Model 2	
	β	P	β	P
DM	−1.6547	<0.001	−1.1502	<0.001
Sex (female)	−0.8790	<0.001	−1.0879	<0.001
Log (1–84 PTH)	−1.1829	<0.001	−0.9413	0.001
Season (spring and autumn)	0.0843	0.8	0.1139	0.7
Season (summer)	1.2824	<0.001	1.3004	<0.001
uPro (±)	-	-	−0.3564	0.3
uPro (1+)	-	-	−0.4953	0.1
uPro (2+)	-	-	−0.8654	0.01
uPro (≥3+)	-	-	−2.3371	<0.001
	$R^{2\#} = 0.11$		$R^{2\#} = 0.15$	

#, corrected R^2 for degree of freedom.
uPro, urinary protein; PTH, parathyroid hormone.
Reference of the seasons was set at winter, reference of uPro was no urinary protein, i.e. (−) by dipstick tests.
Model 1 is a model without uPro, Model 2 with uPro.

independently associated with low FGF-23, with adjustment for numerous confounders such as eGFR and serum phosphate.

Impact of DM and renal function on vitamin D status and calcitriol

Group D had poorer vitamin D status (Fig. 3a) with comparable levels of 1–84 PTH (Fig. 2b), leading to lower calcitriol levels in comparison with group N (Fig. 3b). Marked difference between the groups was observed in the association between CKD stage and serum 25-hydroxyvitamin D (25D) level. In group D, 25D decreased as renal function declined, while in group N it was constant through all CKD stages (Fig. 3a, interaction $P = 0.048$). The percentage of patients with proteinuria $\geq +3$ increased as renal function declined in group D ($P < 0.0001$ by Cochrane–Armitage trend analysis), but was constant in group N (Fig. 4). It is known that in nephrotic syndrome 25D is lost in urine with vitamin D binding protein [22]. Therefore, attributing the difference in vitamin D status between the groups to their difference in degree of proteinuria, we performed multivariate linear regression analyses for 25D with adjustment for proteinuria. Model 2 including proteinuria as an explanatory variable had a higher corrected R^2 for degrees of freedom than model 1, which did not include proteinuria. In both models, however, DM remained an independent factor for poor vitamin D status (Table 3).

In order to confirm that lower serum calcitriol level in DM was due to poorer vitamin D status, we constructed two multivariate models for serum calcitriol with or without 25D, the substrate of 1,25-hydroxyvitamin D (1,25D). Inclusion of 25D (Model 2) eliminated the significant effect of DM on 1,25D found in Model 1 (Table 4).

Impact of DM on bone markers and bone mineral density

We could not observe any difference in serum BSAP or NTX level between the two groups (data not shown). Neither did we observe any difference in bone mineral density Z-score (at femoral neck or lumbar spine) (data not shown), although body mass index was significantly higher in diabetic patients.

The percentage of prior cardiovascular disease in both groups

Twenty-three percent of diabetic patients (26 out of 112) had prior cardiovascular disease (CVD) including myocardial infarction, angina pectoris, myocardial ischemia, whereas only 5% of non-diabetic patients (6 out of 112) had prior CVD.

Sensitivity analysis

As a sensitivity analysis, we made another matched non-DM group consisted of 112 patients. The same results were obtained regarding the differences in laboratory data between groups (data not shown).

Discussion

We investigated the effects of DM on laboratory parameters related to CKD-MBD in this cross-sectional study with enrollment of a sex-, age-, and eGFR-matched CKD cohort. The subjects of our study, who were mainly ambulatory patients who had never been treated for CKD-MBD, had never received vitamin D, estrogen, raloxifene, or bisphosphonate.. The following findings were obtained: (1) Compared with non-diabetic subjects, diabetic patients had higher serum corrected Ca and P levels and comparable levels of 1–84 PTH despite poor vitamin D status and higher serum P. (2) Multiple regression analysis adjusted for serum P and eGFR revealed a relationship between DM and low FGF-23. (3) Whereas serum 25D level was constant across CKD stages in non-DM patients, it deceased in patients with DM as CKD stage progressed. The relationship between poor vitamin D status and DM remained robust even after adjustment for degree of proteinuria. (4) Serum calcitriol was also significantly decreased in DM patients, especially in advanced CKD. Multiple

Table 4
Multiple linear regression analyses for 1,25-dihydroxyvitamin D.

Independent variable	Model 1		Model 2	
	β	P	β	P
DM	−2.4657	0.007	−1.0258	0.2
eGFR	0.3864	<0.001	0.3941	<0.001
Log (FGF-23)	−6.7567	<0.001	−7.1915	<0.001
Log (1–84 PTH)	2.3939	0.02	3.7110	<0.001
25D	-	-	0.8793	<0.001
	$R^{2\#} = 0.40$		$R^{2\#} = 0.47$	

#, corrected R^2 for degree of freedom.
25D, 25-hydroxyvitamin D; FGF-23, fibroblast growth factor-23; PTH, parathyroid hormone.
Model 1 is a model without 25D, Model 2 with 25D.

regression analysis implied that poor vitamin D status was a reason for the low serum calcitriol levels in DM.

Higher corrected serum Ca in DM was also reported in elderly patients above age 70 in nursing homes [23]. Higher CaP product in DM might be one of the reasons for the higher coronary artery calcification score reported in predialysis DM patients [5]. Although it is unclear how DM contributes to higher serum Ca despite lower vitamin D status. More frequent prescription of thiazide diuretics in group D might play a role in it. However, loop diuretics associated with negative calcium balance was also used more often in group D. Another possibility might be low bone turnover in DM. Inaba et al. reported in an *in vitro* study that exposure to high glucose impaired the response of human osteoblast-like cells to PTH [24] and 1,25D [25]. They also found that poor glycemic control impaired the response of bone formation markers to exogenous calcitriol in patients with type 2 DM [26]. Although BSAP and NTX level was comparable between the groups, we cannot say safely that low bone turnover was not present in group D, since we lack data on serum osteocalcin, which is reported to be negatively correlated with by $HbA1_C$ in diabetic patients [23]. Therefore potential low bone turnover in DM may lead to low buffering capacity of bone, resulting in higher serum Ca and P. Since FGF-23, one of the phosphaturic hormones, is produced mainly in osteocytes [27] under conditions of high serum P level [28,29]. Considering a report that body weight is positively associated with serum FGF-23 [30], group D patients with higher body weight should have had higher serum FGF-23, however, we found a independent link between DM and low FGF-23. Given that the plasma half-life of this hormone is in the range of 46–58 min [31], low serum FGF-23 can be attributed to its reduced production. Therefore, the finding of lower FGF-23 level in DM in early stages of CKD despite higher serum P implies that the function of osteocytes is disturbed or osteocyte density is reduced in DM. This result can be otherwise interpreted as indicating that impaired osteocyte function in DM resulted in relatively low FGF-23 levels, leading to higher serum P. In fact, osteocyte density was reported to be reduced in a rat model of acute DM [32], the mechanism of which may involve oxidative stress, as reported in osteoblasts [33]. It seems likely that uremia overcomes the effects of DM on FGF-23, resulting in comparable levels of FGF-23 in subjects with and without DM in late stages of CKD.

In our analysis, we revealed the link between lower FGF-23 and DM, which predisposes patients to high morbidity and mortality. Our results are not inconsistent with previous literature arguing that high serum cFGF-23 level at the initiation of hemodialysis therapy was reported to be associated with higher mortality risk [34]. They adjusted for DM in this brilliant manuscript, meaning that higher serum cFGF-23 was associated with higher mortality risk in DM and also in non-DM (regardless of diabetic state). They did not insist that higher mortality in DM was due to higher FGF-23 level in them. In fact, they did not show a result such that inclusion of FGF-23 into the model attenuated or disappeared the deleterious effect of DM on mortality.

A large difference in the dietary recipe between the group D and group N might explain the difference in serum P. In early CKD stages, no protein restriction was performed in diabetic patients, leading to higher protein intake than in non-diabetic CKD patients. Because of lower serum level of FGF-23 in CKD stage 1 + 2, urinary excretion of phosphate might not compensate for this higher phosphate intake, resulting in higher serum phosphate in this population.

Even though diabetic patients had higher serum P and lower 25D and 1,25D levels, they had levels of 1–84 PTH comparable to those in non-diabetic patients, suggesting that parathyroid function is also impaired in predialysis DM patients. These results accord with the finding of *in vitro* analysis that increasing medium concentration of glucose caused suppression of PTH secretion by cultured bovine parathyroid cells [35]. These findings together suggest that the function of parathyroid cells and bone cells may be impaired in DM.

The progression of proteinuria with decrease in eGFR in DM suggested that massive proteinuria is one of the reasons for poor vitamin D status in advanced CKD in patients with DM. In fact, inclusion of amount of proteinuria in the model increased the corrected R^2 for degrees of freedom. However, the relationship between DM and poor vitamin D status remained robust even after adjustment for proteinuria. The relationship between low vitamin D status and low insulin sensitivity reported for both the general population [36] and CKD patients [37] is consistent with our findings. As confirmed by multiple regression analysis, the low calcitriol level in advanced CKD in patients with DM was due to low 25D level. A clinical study reported that low 25D level at the initiation of hemodialysis is associated with poor prognosis [38], and another observational study by London et al. reported that nutritional vitamin D deficiency and low 1,25D could be associated with arteriosclerosis and endothelial dysfunction [39]. Therefore, the poorer vitamin D status and lower calcitriol level found in DM might contribute to the increased likelihood of cardiovascular disease in diabetic patients. In fact, the prevalence of the patients with prior CVD was as high as 23% in group D, whereas only 5% in group N.

One of the limitations of our study is that it was cross-sectional. We lack findings regarding the effects of control of blood glucose on the parameters we measured, and are thus unable to determine whether the DM-specific findings for laboratory parameters we detected are due to high insulin resistance, low serum level of insulin like growth factor-1, oxidative stress in DM, or high glucose level itself. Since an experimental study revealed that intensive control of blood glucose by insulin therapy resulted in improvement of bone turnover in a mouse model of type 1 DM [13], a clinical study in CKD is clearly needed to determine whether lowering of blood glucose by any agent will affect bone turnover or biochemical parameters associated with it.

Our examination of predialysis patients revealed some impacts of DM on laboratory abnormalities, one of the characteristics of CKD-MBD, including higher CaP product through all stages of CKD, poorer vitamin D status, lower serum calcitriol level in advanced CKD, and lower FGF-23 level. Poor vitamin D status and low calcitriol level in DM could bring about functional changes in the vasculature mediated by endothelial dysfunction, and high CaP product in DM could bring about structural changes in the vasculature, i.e. vascular calcification, another aspect of CKD-MBD. The finding of comparable levels of two phosphaturic hormones in subjects with and without DM despite higher phosphate and poor vitamin D status in those with DM implies impairment of bone and parathyroid function in DM.

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ELSEVIER

Clinicopathological Study of Expression of Lymphatic Vessels in Renal Allograft Biopsy After Treatment for Acute Rejection

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ABSTRACT

Background. Lymph vessel expression is related to inflammatory cell infiltration, around renal tubules in acute rejection episodes (ARE) of transplanted kidneys. However, there is little information on the lymph vessels after treatment of an ARE, particularly in relation to renal function and histological findings.

Patients and Methods. We investigated 13 cases of ARE diagnosed by kidney transplant biopsy performed from 1997 to 2005 within 3 years of transplantation. Treatment of the ARE lead to an improved serum creatinine level in all cases. There was neither an ABO-incompatible nor an acute humoral rejection case. Lymphatic vessels in re-biopsies were examined using immunohistochemical staining with D2-40 antibody that detected lymphatic endothelium. Re-biopsy cases in which the baseline creatinine had increased by more than 20% despite treatment were considered the severe group; the others, as the stable group. The relation between lymphatic vessel density (LVD) and renal function was examined using Banff scores.

Results. LVD was significantly higher in the severe than the stable group. The expression of lymph vessels versus the Banff score showed a direct relation: greater Banff scores showed higher expressions of lymph vessels.

Conclusions. The expression of lymph vessels in renal allograft specimens after treatment of an ARE was related to deterioration of renal function and inflammatory cell invasion. We plan a further examination of the relationship between the expression of lymph vessels and long-term prognosis.

SEVERAL NEW, RELIABLE antibodies have been produced to show lymphatic vessels. In the human kidney, lymph vessels are present around arterial branches at the level of the interlobular arteries, but not in peritubular spaces or around glomeruli.[1] It has been reported that lymphatic neoangiogenesis is associated with cellular infiltration. In renal allograft cases of acute rejection episodes (ARE), lymphatic neoangiogenesis is located in peritubular spaces and around glomeruli.[1] Lymphatic neoangiogenesis appears soon after transplantation.[2] It has been speculated that lymphatic neoangiogenesis contributes to cell drainage maintaining alloimmune responses to the graft.[1] In this study, we examined the clinicopathological significance of lymph vessel expression in renal allograft tissue biopsies after treatment of an ARE.

METHODS

Patients and Tissue Samples

We have performed 671 renal allograft biopsies from 1997 to 2005, including 39 in patients diagnosed with ARE greater than grade 1. Furthermore, among 39 patients who had ARE treated fewer than 3 years prior, 13 subjects underwent protocol biopsies. (Table 1).

Their mean serum creatinine (s-Cr) at ARE was 2.1 ± 0.6 mg/dL. Following ARE, treatment s-Cr improved in all cases and no case showed ARE upon a re-biopsy. Cases in which s-Cr increased more than 20% after treatment at re-biopsy were considered the severe group; whereas, other cases were the stable group. There was neither an ABO-incompatible nor an acute humoral rejection case. The study protocol was approved by our ethics committee. Written informed consent was obtained from each patient who underwent a biopsy. No patient suffered from diabetes mellitus.

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Table 1. Clinical Finding Profiles of Patients

Sex Male 7, female 6
Grade of AR AR-IA, 9; AR-IB, 1; AR-IIA, 3
Days after transplantation to AR onset 107 ± 128
Period from diagnosis of AR to re-biopsy (y) 1.9 ± 1.2

Abbreviation: AR, acute rejection.

Immunohistochemistry

Lymphatic vessels were detected in paraffin sections using an antibody against lymphatic endothelium—the mouse monoclonal anti-D2-40 immunoglobulin G1 (#17730-23, Signet Laboratories Inc.). This novel monoclonal antibody recognizes a Mr. 40,000 O-linked sialoglycoprotein that reacts with a fixation-resistant epitope in lymphatic endothelium. D2-40 has been described as a reliable marker for lymphatic endothelium in paraffin-embedded tissue. It binds to human podoplanin.

Immunohistochemical staining for D2-40 was performed as described previously: histological specimens fixed in 10% buffered-formalin were routinely processed for paraffin embedding, and serial 2-μm sections that were deparaffinized, were heated in an autoclave in 0.01 M citric acid buffer (pH 6.4; 121°C/15 min), incubated with 0.3% hydrogen peroxide in methanol for 15 minutes at room temperature to inhibit endogenous peroxidase activity, and washed in 0.05 M phosphate buffer (pH 7.6) 3 times for 3 minutes at room temperature before treatment with PBA for 5 minutes to block nonspecific staining. The sections were incubated with the mouse antihuman D2-40 monoclonal antibody (1:500) overnight at 4°C. After washing in 0.05 M phosphate buffer (pH 7.6) 3 times at room temperature for 3 minutes, they were processed using an avidin-biotinylated peroxidase complex method (Vectastain ABC kit, Vector Laboratories, Inc., Burlingame, California, United States) with diaminobenzidine as the chromogen and counter-stained with hematoxylin.

Quantitative Analysis of D2-40 Staining

The expression level of lymphatic vessels was evaluated based on the method previous reported by Yamamoto et al.[2] **The lymphatic vessel density (LVD) in biopsies was quantified as the number of D2-40 positive vascular profiles per unit area of the cortex using image analysis (MacScope). The analyses were performed by two of the authors (K.O., Y.N.) without prior knowledge of patients' characteristics.**

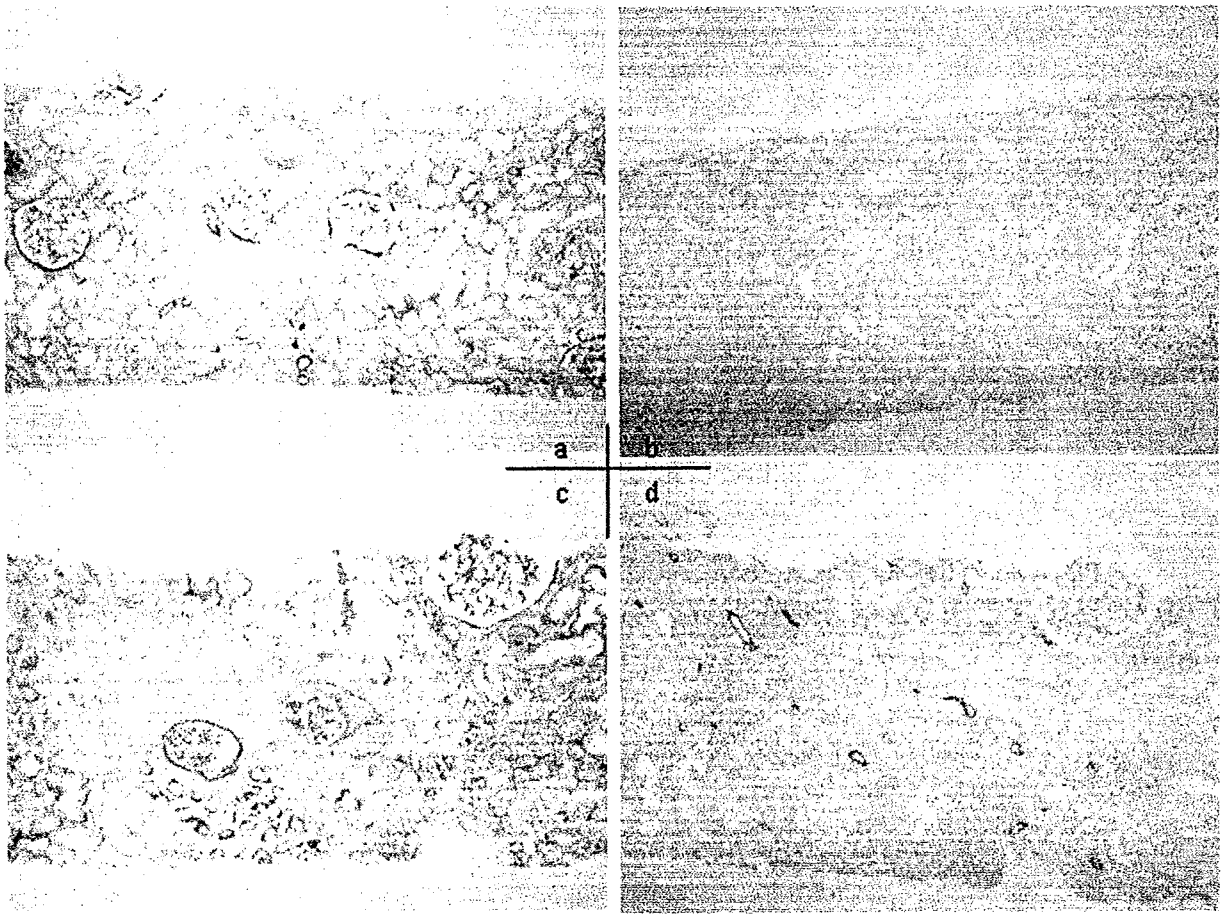

Fig 1. Stable group [**(A)** PAS staining × 100, **(B)** immunohistochemistry staining of D2-40]. Severe group [**(C)** PAS staining × 100, **(D)** immunohistochemistry staining of D2-40].

Histological Evaluation

We used the Banff97 system to evaluate renal allograft biopsies and to explore a relation to LVD and renal function.

Statistical Analysis

Results were expressed as mean values ± SEM. The Mann-Whitney *U* test was used to compare unpaired means of the 2-groups with differences evaluated using Stat View software (Abacus Concepts Inc., Berkeley, California, United States). *P* values <.05 were considered to be significant.

RESULTS

Among 13 patients, the stable group included 10 patients, and the severe group, 3 patients. The expression of lymph vessels in the stable group was mainly observed around blood vessels. In the severe group, it was recognized around cortical renal tubules, in addition to blood vessels (Figure 1). LVD was significantly higher among the severe compared with the stable group (Figure 2). Cases in which the Banff i score indicated higher degrees of mononuclear cell interstitial inflammation showed greater lymph vessels expression (Figure 3). Because all of the other Banff scores were 0, no comparisons could be performed for these examinations. Table 2 shows the real numbers or scores for lymphatic vessels or Banff scores.

DISCUSSION

We examined the expression of lymph vessels after treatment of an ARE, showing that patients in whom renal function showed a 20% increase of s-Cr displayed greater expression of lymph vessels upon re-biopsy. The expression of lymph vessels was related to interstitial cell infiltration in terms of the Banff i score. The cases in which renal function was stable after treatment displayed little expression of lymph vessels around renal tubules.

There are several reports of the relationship between interstitial cell infiltration and the expression of lymph vessels in renal allograft specimens during an ARE.[1–4] The expression of lymph vessels has been immediately recognized in inflammatory cell invasion.[2] Although it may be a speculation because examined specimen were only obtained after treatment, we considered that specimens from cases in which inflammation had disappeared would also show disappearance of lymph vessel, and that specimens showing persistence of inflammation would also show persistence of lymph vessel expression. Stuht et al.[4] reported that interstitial cell infiltration without lymphatic neoangiogenesis was related to renal function degeneration upon protocol biopsies. This observation contradicts our result; however, the difference may depend on the fact that we used episode biopsies, whereas they used protocol biopsies.

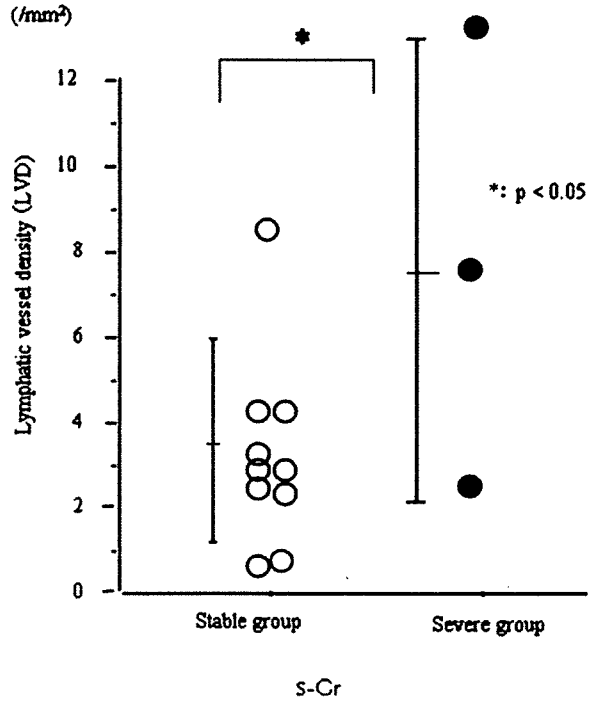

Fig 2. Comparison of lymph vessel density between the stable group and the severe group.

In conclusion, the expression of lymph vessels in renal allograft specimens after treatment of an ARE was related to deterioration of renal function and inflammatory cell invasion. We plan to examine the relation of lymph vessel expression to long-term prognosis.

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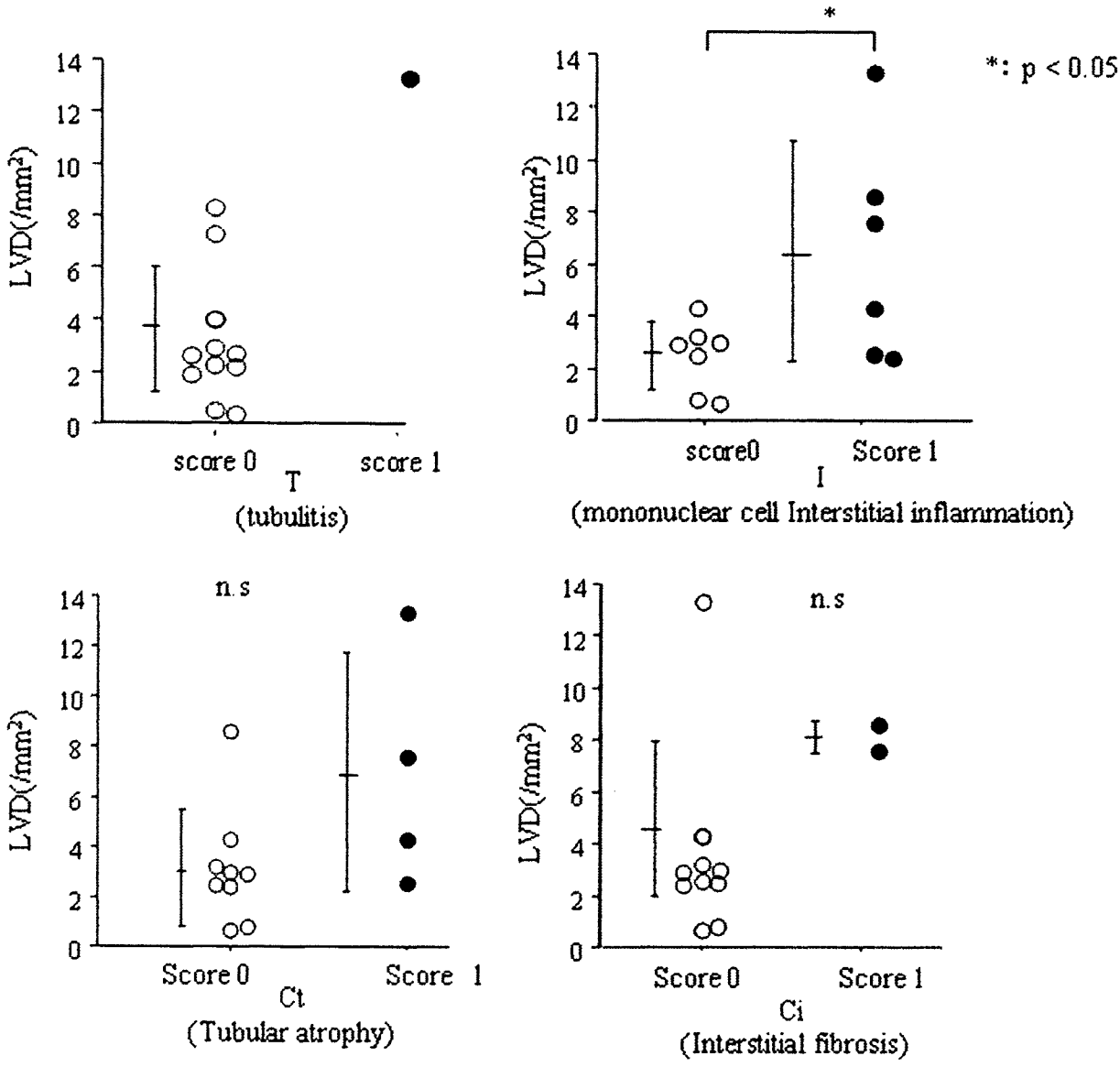

Fig 3. Relationship between lymph vessel density and Banff score.

Table 2. The Real Numbers or Scores for Lymphat Vessels or Banff Scores

	Group	LVD/mm^2	t	i	ct	ci
1	Severe	13.36	1	1	1	0
2	Severe	7.60	0	1	1	1
3	Severe	2.57	0	1	1	0
4	Stable	8.59	0	1	0	1
5	Stable	4.33	0	1	1	0
6	Stable	4.32	0	0	0	0
7	Stable	3.21	0	0	0	0
8	Stable	2.96	0	0	0	0
9	Stable	2.91	0	0	0	0
10	Stable	2.50	0	0	0	0
11	Stable	2.37	0	1	0	0
12	Stable	0.83	0	0	0	0
13	Stable	0.66	0	0	0	0

Abbreviations: ci, xxx; ct, xxx; i, xxx; LVD, lymphatic vessel density; t, xxx.

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特集

慢性腎臓病(CKD)対策の現状と今後
—CKD診療ガイドラインを中心に—

1．CKDの疫学

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はじめに

腎臓病の多くは慢性の経過をとり，その疾患スペクトラムは多岐にわたる．その分類には病変の主座によるもの(糸球体，尿細管，間質，血管)，また原発性と二次性があり，さらに腎生検によって得られる腎病理診断がここに盛り込まれることになる．このことは，腎臓病が非専門医にとってはなじみにくいものとなっている原因の1つである．腎機能低下が進行した場合には，原疾患に関わらず慢性腎不全として病態を把握し，治療/管理に当たってきた．CKD(chronic kidney disease：慢性腎臓病)という概念は，慢性に経過する腎臓病全体を，腎機能低下がない，もしくは軽度の段階から腎障害の存在(主に蛋白尿で検出される)および糸球体濾過量によって分類し，そこには従来腎臓病学が構築してきた精緻な疾病の体系を一切持ち込まないで，比較的軽度から末期腎不全に至るすべての腎臓病を把握し，治療・対策を考えていこうというものである．

米国腎臓財団(NKF)により，2002年にKidney Disease Outcome Quality Initiative(K/DOQI)からガイドラインが提示された[1]．一方，米国循環器学会は心血管疾患(CVD)のリスクとしてのCKDの重要性を踏まえて，2002年にはCVDリスクとしてのCKDに関するscientific statement[2]を，また2006年にはCVD患者におけるCKDの早期発見の重要性についてのscience advisory[3]を発表している．ここに米国での大規模疫学研究の結果を示すが[4]，腎機能低下が比較的軽度と考えられる段階から心血管事故，総死亡などのリスクが明らかに上昇する事実はインパクトをもって受け止められた(図1)．

このような国際的動向を背景として，日本腎臓学会においてもCKDの総合的対策を担う組織として，2005年に慢性腎臓病対策委員会が発足し，疫学調査，ガイドライン作成，企画推進，国際連携を担うワーキンググループが活動を開始した．その活動の成果の1つとして，2007年9月には「CKD診療ガイド」が上梓され，CKD対策に大いに資するものとして診療現場で広く活用されている[5]．本年3月，科学的根拠に基づくCKD診療ガイドラインが上梓され，CKD対策の一層の展開が期待されている[6]．

CKDの定義と診断基準

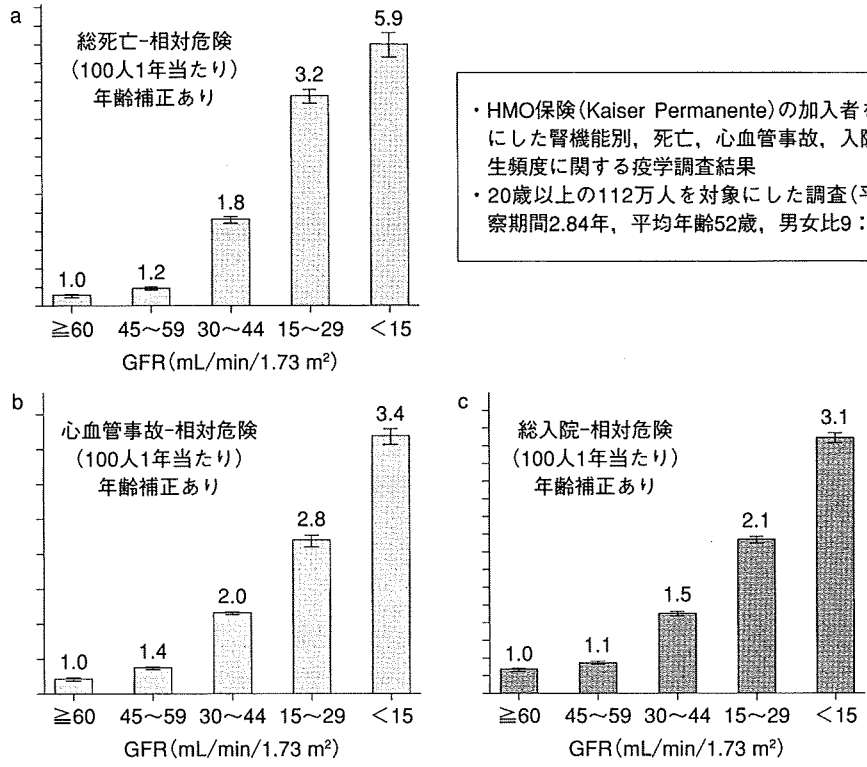
CKDは表1のように診断される．

わが国におけるCKDの日常診療において，血清クレアチニン，年齢，性別の3つのデータからeGFRを算出する下記の推算式を日本腎臓学会として作成し推奨している[7]．

$$eGFR(mL/min/1.73\ m^2) = 194 \times Cr^{-1.094} \times Age^{-0.287} \times 0.739(\text{女性の場合})$$

日本におけるCKDの頻度

わが国におけるCKDの頻度について，日本腎臓学会慢性腎臓病対策委員会疫学調査ワーキンググループが調査を行った．全国8カ所の20歳以上の健診データをもとに，日本人の年齢，性別人口構成比で補正して推



・HMO保険(Kaiser Permanente)の加入者を対象にした腎機能別，死亡，心血管事故，入院の発生頻度に関する疫学調査結果
・20歳以上の112万人を対象にした調査(平均観察期間2.84年，平均年齢52歳，男女比9：11)

図1　腎機能(GFR)別の死亡，心血管事故および入院の相対危険
(文献4より引用改変)

表1　CKDの診断基準

1．GFRの値に関わらず，腎障害を示唆する所見(検尿異常，画像異常，血液異常など)が3カ月以上存在すること
2．GFR 60 mL/min/1.73m²未満が3カ月以上持続すること
この片方または両方を満たす場合にCKDと診断される．

表2　ステージ別CKD推定患者数

病期ステージ	進行度による分類 GFR mL/min/1.73 m²	推定患者数
1	≧90	605,313
2	60～89	1,708,870
3	30～59	10,743,236
	[50～59]	[7,809,261]
	[40～49]	[2,363,987]
	[30～39]	[569,988]
4	15～29	191,045
5	<15	45,524

ステージ5には透析5D，腎移植5Tは含まれない．
CKDステージ1，2は尿蛋白陽性のみとして推計した．
(文献5より引用)

計したところ，CKD 3～5は成人人口の約10.4%，1,097万人，またCKD 1～2(腎機能低下なく，蛋白尿陽性)は2.3%，231万人となった(表2)[6]．CKDの診断基準を満たす患者は1,300万人を超えることになり，高血圧，糖尿病にも比肩されるようなcommon diseaseであるといえる．

CKDの診断を構成する蛋白尿と腎機能につきそれぞれ述べる．蛋白尿の頻度は年齢とともに上昇するが，女性ではその増加は60歳代以降で顕著となる．いずれの年齢層においても蛋白尿の頻度は男性＞女性である(図2)[8]．年齢別のeGFR分布からみたCKDの頻度も加齢とともに増加がみられる．男性女性ともに60歳を過ぎるとCKDが急速に増加する(図3)[9]．

わが国における末期腎不全(慢性維持透析)患者数[10]

わが国の慢性維持透析患者数は2008年末で282,622人であり，前年度より7,503人の増加がみられた(図4)．増加の内訳は新規導入37,671人，死亡26,901人であった．

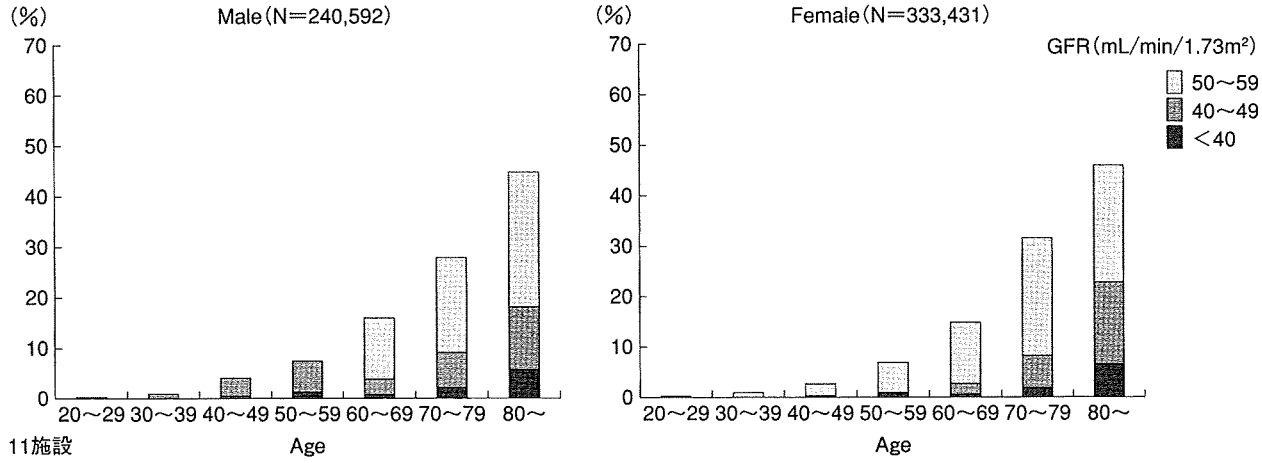


図3　加齢によるCKD患者の増加

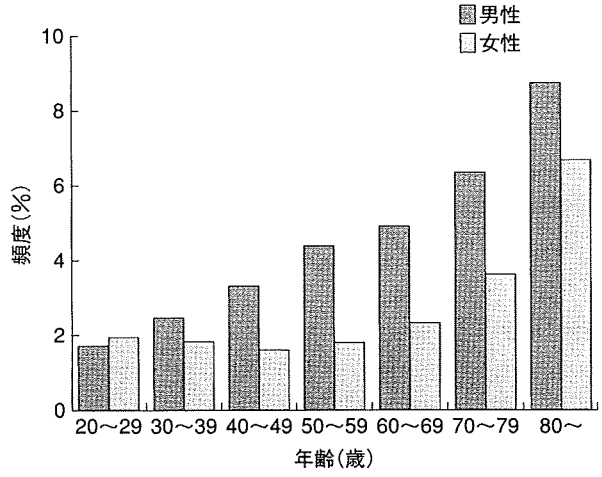


図2　蛋白尿の頻度

導入原疾患1位は糖尿病性腎症(16,126人，43.2%，平均年齢65.62歳)，2位は慢性糸球体腎炎(8,602人，23.0%，平均年齢66.86歳)，3位不明(3,976人，10.6%，平均年齢70.24歳)，4位腎硬化症(3,936人，10.5%，平均年齢73.99歳)であり，腎硬化症は増加傾向が続くが，糖尿病性腎症による導入の割合が前年度より減少した点が特記事項となっている．

わが国の疫学研究から示されたCKDのリスクファクター

茨城県の40歳以上の住民健診データ123,764人分を用いたYamagataらの報告に基づき，CKD発症および進展のリスクファクターにつき述べる[11]．10年間の観察期間中，新規に発症したCKDステージ1～2 (eGFR ≧60 mL/min/1.73 m²かつ尿蛋白定性≧＋1)は4,307人，ステージ3～5 (eGFR＜60 mL/min/1.73 m²)は19,411人であった．健診受診者で10年間の経過観察中にCKDステージ1～2となるリスクファクターは，年齢，血尿，高血圧，耐糖能異常(impaired glucose tolerance, IGT)，糖尿病，脂質代謝異常，肥満，喫煙であった(図5)．一方，10年間の経過で腎機能低下(eGFR＜60 mL/min/1.73 m²)を来した状態であるCKDステージ3に至るリスクファクター(≒進行のリスク)としては，年齢，腎機能(eGFR)，蛋白尿，血尿，高血圧，脂質代謝異常，糖尿病治療中，肥満(女性のみ有意)，喫煙であった(図6)．男性，女性で多少重みは異なるが，これらがCKD進行のリスク因子と考えられる．久山町における経年的な検討では，CKDの頻度は増加しており(図7)，その背景に肥満，メタボリックシンドロームの増加が関与すると推定されている[12]．

心血管イベントのリスクとしてのCKD

eGFR＜90 mL/min/1.73 m²の対象者27,998名を66カ月観察し，腎死とCVDによる死亡の発症率を追跡調査したKeithらの報告[13]では，CKDの病期分類2～4期の順におのおの，腎死が1.1%，1.3%，19.9%であったのに対して，死亡は19.5%，23.3%，45.7%であった．すなわち，多くのCKD患者(特にステージ2～3)では，腎代替療法が必要な末期腎不全に至ることなく死亡していることを意味する．この死亡の原因の多くがCVDと推定されており，本研究からCKD患者をCVDの高リスク群であることを認識することの重要性が実感される．わが国においてもCVDのリスクとしてのCKDに関する報告がなされているが，eGFR＜60 mL/min/1.73

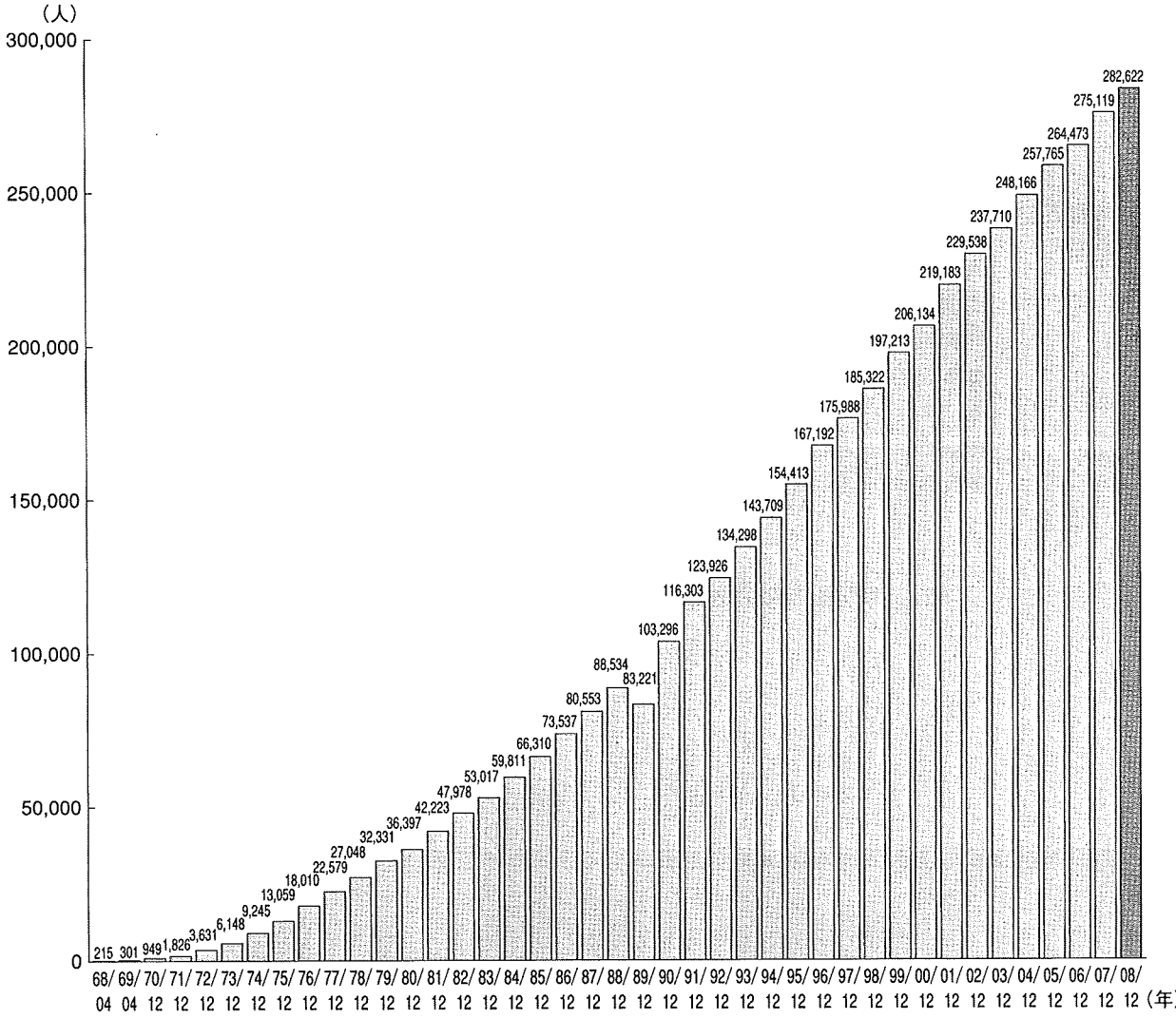


図4　慢性透析患者数の推移

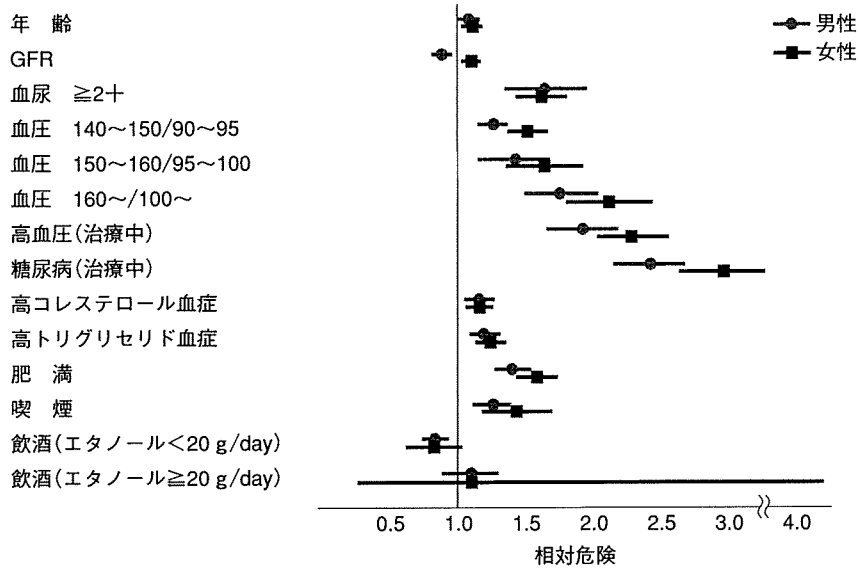


図5　10年間の経過観察中に蛋白尿(CKDステージ1 or 2)が出現するリスク
(文献11より引用改変)

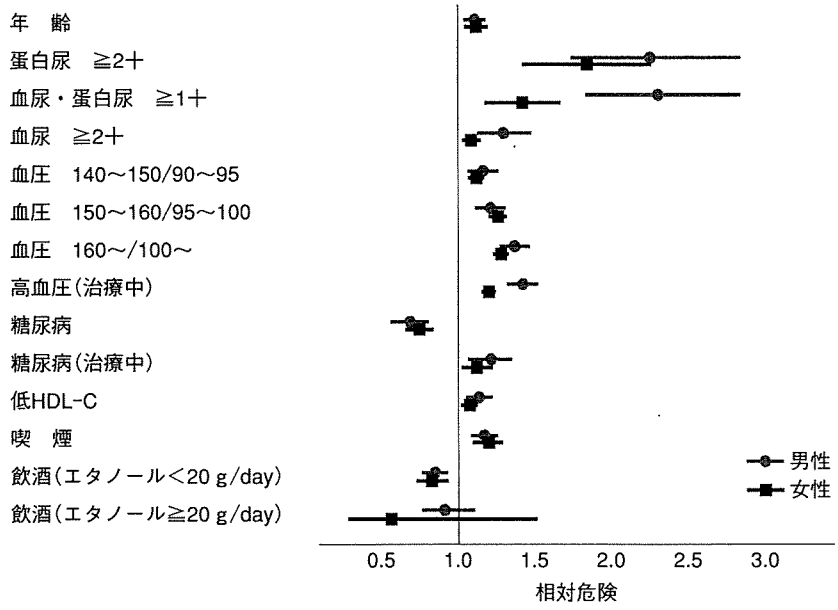


図6　10年間の経過観察中にCKDステージ3以上となるリスクファクター
(文献11より引用改変)

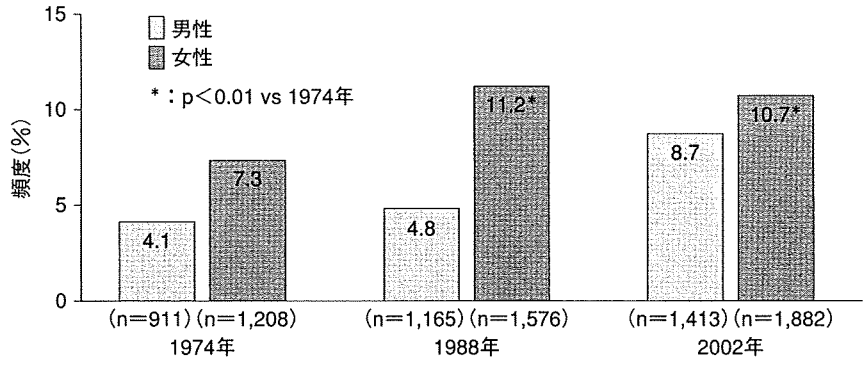


図7　CKD頻度の推移(久山町研究)
久山町第3集団の断面調査，40歳以上，年齢調査.

m^2から心血管イベントリスクが高まることは示されている[14-16]．一方，欧米の研究から，微量アルブミン尿の出現は糖尿病，非糖尿病において心血管イベントの予測因子となることが確立している[17,18]．山形県のTakahata研究によると，一般健診2,321人における微量アルブミン尿の頻度は13.7%であったが，心血管イベントとの関連については現時点で明らかではない[19]．わが国では腎機能およびアルブミン尿の細かい層別の心血管リスク増加に関する知見は十分とはいえず，これからの課題と考えられる．

末期腎不全に至るCKDの特徴

CKDという観点から末期腎不全のリスクを考察すると以下のごとくとなる．

日本人のGFRの低下速度は平均0.36 mL/min/1.73 m^2/年であり，40～69歳で50 mL/min/1.73 m^2以下，70～79歳では40 mL/min/1.73 m^2以下の腎機能の場合，腎機能低下速度が有意に速まる．その際，蛋白尿(試験紙法で＋1以上)の存在でどの年齢層でも腎機能低下速度はおよそ2倍となる(図8)[20]．進行した腎機能の低下(CKDステージ4，5)は末期腎不全のリスクであり，蛋白尿およびアルブミン尿陽性は末期腎不全のリスクである．蛋白尿，アルブミン尿の程度が増すごとにリスクが高くなる．糖尿病患者では微量アルブミン尿出現は末期腎不全のリスクとなる．また，治療による蛋白尿，アルブミン尿の減少の程度は，腎機能悪化抑制と相関がある．一方，尿潜血(試験紙法)は，男性

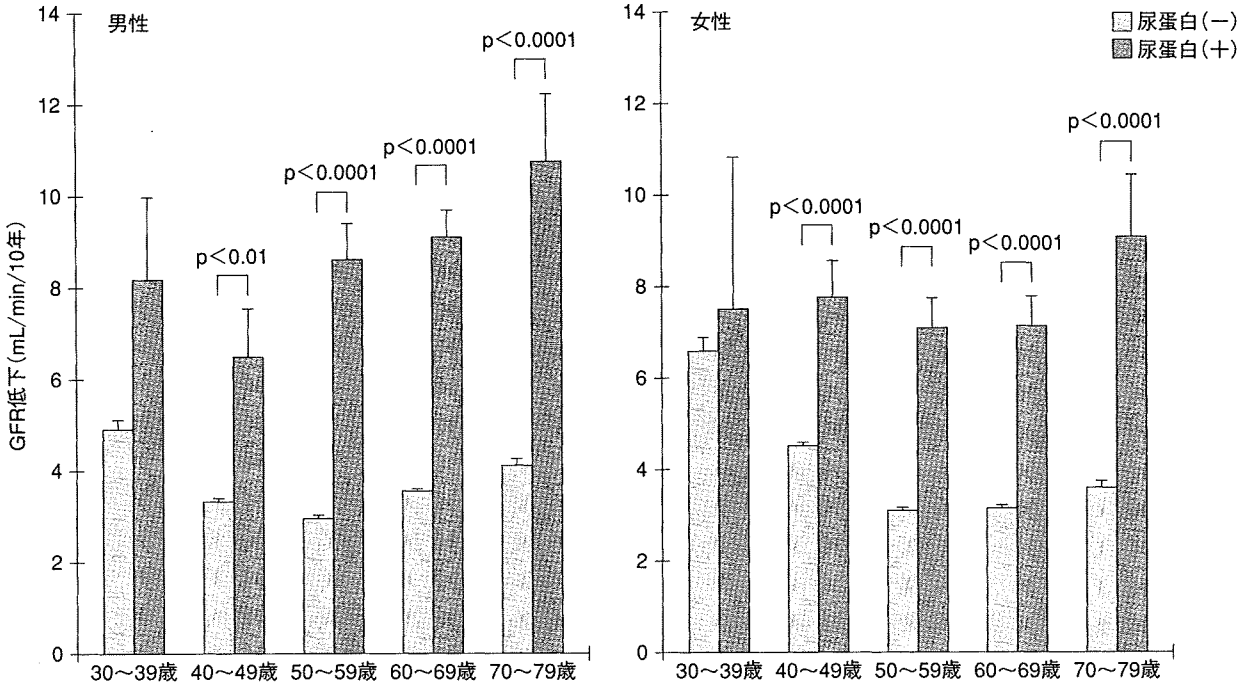


図8　尿蛋白と腎機能(GFR)低下速度

(文献20より引用)

において末期腎不全のリスクであるが，その関与度は蛋白尿に比べると弱い．また，尿潜血と尿蛋白の両方が陽性の場合，末期腎不全のリスクが高い．

先述のように，わが国の末期腎不全の原疾患としては，糖尿病，慢性糸球体腎炎，腎硬化症が三大疾患となっており，これらで約77%を占める．これらのうち前二者は1 g/日以上の蛋白尿(アルブミン尿)を呈することが多い．蛋白尿の存在は末期腎不全へと至る大きなリスクであり，Isekiら[21]によれば，蛋白尿がある場合の1,000人当たりの末期腎不全発症人数は，86.8人(Ccr<50.2)，13.6人(Ccr：50.2〜63.9)，8.3人(Ccr：64.0〜79.3)，7.9人(Ccr>79.4)であり，蛋白尿がない場合，1.2人(Ccr<50.2)，0.7人(Ccr：50.2〜63.9)，0.04人(Ccr：64.0〜79.3)，0.13人(Ccr>79.4)であり，蛋白尿がある場合，Ccrの低下は末期腎不全のリスクといえるが，蛋白尿がない場合，軽度腎機能低下患者の末期腎不全のリスクは高くないといえる．

おわりに

CKDの疫学について現時点での知見につき解説した．欧米の成績から，CKDにおける心血管イベントリスクは腎機能低下，尿中アルブミン排泄いずれの観点でも閾値は明らかでなく，軽度の異常を伴う時点からそのリスクの有意な上昇を認める．これに対して，末期腎不全へと至るリスクには閾値の存在が想定され，中等度以上の腎機能低下の存在もしくは顕性蛋白尿の持続によって明白に上昇する．わが国におけるCKDの心血管イベント，および末期腎不全発症リスクの定量的解析は未だ十分ではなく，特にこれら両者を同時に比較検討した研究はない．今後，腎機能と蛋白尿の両者の心血管イベント，末期腎不全発症に対する寄与度を定量的にとらえ，CKDという観点からの心血管イベントおよび末期腎不全のリスク評価を確立していくことが重要な課題と考えられる．

文　献

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2) Sarnak MJ, Levey AS, Schoolwerth AC, et al；American Heart Association Councils on Kidney in Cardiovascular Disease, High Blood Pressure Research, Clinical Cardiology, and Epidemiology and Prevention：Kidney disease as a risk factor for development of cardiovascular disease：a statement from the American Heart Association Councils on Kidney in Cardiovascular Disease, High Blood Pressure Research, Clinical