Table 4. Associations of grip strength with variables which showed significant associations with grip strength

Variables	OR	95% CI	*p*
Height	1.17	1.08-1.26	<0.001
Weight	1.09	1.03-1.15	0.003
Serum albumin	11.8	2.4-57.4	0.002

anaemic, grip strength[24] is weaker and functional status[25] is worse for those with low-normal haemoglobin (12.0-13.0 g/dL) than those with high-normal haemoglobin values (13.0-14.0 g/dL). These findings may be in line with our observation that grip strength was weaker for those in the median haemoglobin quintile (12.6-13.2 g/dL) than those in the highest haemoglobin quintile (≥13.8 g/dL) in older non-anaemic women. Further, in the present study, the relationship between grip strength and haemoglobin was not linear. Although anaemic women had lower grip strength than women in the highest and second highest haemoglobin quintiles, there was no significant difference in grip strength between anaemic women and those in non-anaemic women in the second lowest and median quintiles of haemoglobin. The failure to detect significant association between anaemia and grip strength (Table 4) may be due in part to a small sample size in anaemia or non-linear relationship between haemoglobin concentrations and grip strength in our sample.

Several biologically plausible mechanisms could explain the link between low haemoglobin and muscle strength in older adults as reviewed by Chaves.[26] Low haemoglobin levels can diminish the maximal capacity of the musculo-skeletal systems to consume oxygen, leading to decreased muscular conditioning. Synergistically, by decreasing oxygen-carrying capacity, low haemoglobin could lead to chronic tissue hypoxia, which in turn would promote further decline in physiologic reserve.

Studies have shown that higher levels of inflammatory markers are associated with lower grip strength.[27-33] Recent findings suggest possible contributions of obesity-associated inflammatory milieu, and impairment of muscle function/strength to adverse functional outcomes.[34] For example, the amount of body fat appears to be the best correlate of CRP[35,36] and randomized studies of weight reduction in older persons with knee osteoarthritis have found reductions in concentrations of CRP, IL-6 and soluble TNF-α receptor.[37] Most of these studies on the relationship between grip strength and inflammation have analyzed single biomarkers or biomarkers for a single pathway of inflammation. The present study examined associations between grip strength and a broad range of factors associated with declines in muscle strength including inflammatory markers and found a significant association of grip strength with haemoglobin independent of inflammatory markers. Another possible explanation is big differences in BMI and hence serum CRP levels. Mean BMI and CRP were 25.9 to 29.4 kg/m^2 and 3.2-3.76 mg/L, respectively, in the aforementioned studies.[27-33] They were 22.5 kg/m^2 and 0.85 mg/L, respectively, in the present study.

Body height was positively related to grip strength in the present study of community-dwelling Japanese elderly women, as previously reported in the elderly[38-40] as well as in the young population.[38] Greater heights would lead to longer arms, with greater lever arm for force generation, resulting in an efficient amount of force.[41]

Several limitations must be acknowledged. The cross-sectional design did not allow causal relationship. The recruitment procedure may also have some potential impact on the results. As the participation was voluntary, women who pay more attention to health may be more likely to participate. Biochemical parameters were measured only once. Information on nutritional supplements and drugs which they used to take was not available, so possible contribution of these supplements and drugs to grip strength or other parameters could not be completely excluded. Use of some drugs may or may not be associated with muscle strength.[42,43]

In conclusion, low-normal haemoglobin above the WHO cut-off may contribute to lower muscle strength independently of age, anthropometric, nutritional, and inflammatory markers in the elderly, and may represent important confounders of the association between grip strength and functional decline in community-living Japanese elderly women.

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AUTHOR DISCLOSURES

There were no conflicts of interest.

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Original Article

Low haemoglobin levels contribute to low grip strength independent of low-grade inflammation in Japanese elderly women

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日本老年妇女低血红蛋白水平是独立于轻度炎症影响低握力强度的因素

肌肉强度随年龄增长而下降。然而，导致这种下降的因素没有好的相关文献记录和广泛研究。该研究分析了 202 名社区居住的老年日本妇女握力强度相关因素与肌肉强度下降的关系。结果表明调整了年龄的影响后，握力强度与体重、身高、血清白蛋白、血红蛋白、高密度脂蛋白胆固醇（HDL-C）、血清铁、血清铜的倒数、高敏 C 反应蛋白（hsCRP）的对数呈正相关。多重线性回归分析显示：以握力强度作为因变量，依据增加的吻合度，握力强度的47%可以被身高、年龄和血红蛋白所解释。总之，在老年人群中，低血红蛋白是独立于年龄、人体测量学、营养和炎症标志物影响低肌肉强度的因素，对社区生活的日本老年妇女，低血红蛋白可能是握力强度和功能减退之间关系的一个重要混杂因子。

关键词：握力强度、血红蛋白、身高、年龄、老年妇女

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RESEARCH ARTICLE Open Access

Direct association of visit-to-visit HbA1c variation with annual decline in estimated glomerular filtration rate in patients with type 2 diabetes

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Abstract

Background/Aims: This study examined associations of visit-to-visit variability of glycemic control with annual decline in estimated glomerular filtration rate (eGFR) in patients with type 2 diabetes attending an outpatient clinic.

Methods: Intrapersonal mean and coefficient of variation (CV) of 8-12 measurements of HbA1c and those of 4-6 measurements of fasting and post-breakfast plasma glucose (FPG and PPG, respectively) during the first 12 months after enrollment were calculated in a cohort of 168 patients with type 2 diabetes. Annual changes in eGFR were computed using 52 (median) creatinine measurements obtained over a median follow-up of 6.0 years. Multivariate linear regressions assessed the independent correlates of changes in eGFR.

Results: CV-HbA1c (standardized β、 -0.257、 $p = 0.004$) were significantly and log urine albumin/creatinine ratio (standardized β、 -0.155、 $p = 0.085$) and smoking (standardized β、 -0.186、 $p = 0.062$) tended to be associated with annual eGFR decline independently of mean HbA1c, age, sex, BMI, waist circumference, diabetes duration and therapy, means and CVs of FPG, PPG and systolic blood pressure, baseline eGFR, and uses of anti-hypertensive and lipid-lowering medications. Association between HbA1c variability and renal function decline was stronger in patients with albumin/creatinine ratio ≧ 30 mg/g than in those with normoalbuminuria ($r = -0.400$, $p = 0.003$ and $r = -0.169$, $p = 0.07$, respectively).

Conclusions: Consistency of glycemic control is important to preserve kidney function in type 2 diabetic patients, in particular, in those with nephropathy.

Keywords: HbA1c, Standard deviation, Kidney function, eGFR

Background

Diabetes is an important cause of mortality and morbidity worldwide, through both direct clinical sequelae and increased mortality from cardiovascular and kidney diseases [1]. Long-term glycemic control, as expressed by hemoglobin (Hb) A1c levels, is the main risk factor for the development of microvascular complications including diabetic kidney disease [2, 3]. Among patients with diabetes mellitus, elevated blood pressure (BP) is associated with progression of microvascular complications such as nephropathy and retinopathy [4]. In addition to high BP and hyperglycemia, dyslipidemia has an important role in the progression of kidney disease in patients with diabetes [5].

There is emerging interest to examine the influence of glycemic and BP variance in diabetic vascular complications [6, 7]. Recently, variation of HbA1c, a reflection of long-term glycemic fluctuation, was found to increase the risk of renal and cardiovascular complications [8–17]. In all studies on renal complications (8–10, 12–17), researchers focused on the relation between HbA1c variability and development and/or progression of diabetic

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nephropathy. Direct association between HbA1c variability and changes in kidney function has hardly been investigated. We, therefore, asked the question whether HbA1c variability might directly associated with annual decline in estimated glomerular filtration rate (eGFR) in patients with type 2 diabetes attending a long-term follow-up in a single outpatient clinic.

Methods

The setting for this observational study was an outpatient diabetes clinic in a private hospital in Japan. Study protocol was consistent with the Japanese Government's Ethical Guidelines Regarding Epidemiological Studies in accordance with the Declaration of Helsinki. We examined a cohort of 168 patients with type 2 diabetes who had been regularly attending the clinic in 2004 and 2005. They were enrolled in the study at the first visit in 2005 and had at least 8 monthly visits with blood samplings during the first 12 months after enrollment. Of 168 patients, 153 patients (91 %) had 12 monthly visits with blood samplings. In the 153 patients, blood was withdrawn on 2 occasions; at 2 h after breakfast taken at home and after an overnight fasting. This was done every other month. In the remaining 15 patients, blood was obtained after an overnight fasting. The main clinical features of these subjects at baseline are reported in Table 1.

After the first visit in 2005 they were followed up in the subsequent at least 24 months through December

Table 1 Anthropometric, clinical and biochemical features of 168 patients with type 2 diabetes and correlation coefficients of annual changes in estimated glomerular filtration rate and coefficients of variation of HbA1c

	Mean ± SD or n, %			⊿eGFR		CV-HbA1c	
Male sex (n, %)	90	,	54	−0.013		−0.17	*
Smokers (n, %)	58	,	34	−0.159	*	0.111	
Age (years)	62.3	±	10	0.037		−0.145	
BMI (kg/m^2)	24.2	±	3.6	−0.048		0.045	
Waist circumference (cm)	86.9	±	9.9	−0.108		0.017	
Duration of diabetes (years)	9.9	±	7.3	−0.047		−0.009	
Treatment of							
diabetes; diet/OHA/insulin (%)	31/51/18			−0.078		0.201	**
hypertension; CCB/RASi/diuretics (%)	34/41/5			−0.076		−0.044	
HbA1c (%)	7.0	±	0.8	−0.050		0.343	***
Fasting PG (mg/dL)	125	±	22	−0.012		0.299	***
Post-breakfast PG (mg/dL)	154	±	49	0.047		0.229	**
CV-HbA1c (%)	7.0	±	6.4	−0.187	*	1	
CV-Fasting PG (%)	14.1	±	9.3	−0.127		0.473	***
CV-Post-breakfast PG (%)	21.9	±	11.0	−0.152		0.190	*
Total cholesterol (mg/dL)	188	±	21	0.048		0.025	
LDL cholesterol (mg/dL)	111	±	22	0.0004		0.096	
HDL cholesterol (mg/dL)	56	±	15	0.128		−0.202	**
Fasting TG (mg/dL)	115	±	51	−0.161	*	0.187	*
Post-breakfast TG (mg/dL)	145	±	64	−0.164	*	0.235	**
Serum creatinine (mg/dL)	0.75	±	0.2	−0.042		0.084	
eGFR (mL/min/1.73m^2)	76	±	16	−0.111		0.165	*
⊿eGFR (mL/min/1.73m^2/year)	−1.05	±	3.39	1		−0.187	*
Uric acid (mg/dL)	5.2	±	1.3	−0.125		0.033	
Systolic BP (mmHg)	128	±	12	−0.014		−0.051	
CV-Systolic BP (%)	8	±	22	−0.035		0.098	
Diastolic BP (mmHg)	72	±	1	0.003		0.112	
Urinary ACR (mg/g)	84	±	322	−0.208	**	0.067	
log ACR	1.30	±	0.6	−0.243	**	0.072	

OHA oral hypoglycemic agents, *CCB* calcium channel blockers, *RASi* renin-angiotensin system inhibitors, PG; plasma glucose, CV; coefficient of variation, *eGFR;* estimated glomerular filtration rate, ⊿eGFR; annual changes in eGFR, *BP* blood pressure, *ACR* albumin/creatinine ratio, *; $p < 0.05$,**; $p < 0.01$,***; $p < 0.001$

31, 2012 to assess kidney function with a median follow-up of 6.0 years (interquartile range; 4.1–6.5 years). Patients with hepatitis B surface antigen or antibodies against hepatitis C virus were excluded. Those who had aspartate aminotransferase and alanine aminotransferase of 100 U/L or greater, serum creatinine≧2.0 mg/dL were excluded as well. Information on smoking habits was collected through face-to-face interviews by TK. Smoking status was classified into one of three categories: current smokers, ex-smokers, and never smokers. Smokers in statistical analysis included current smokers ($n = 52$) and ex-smokers with the Brinkman index of 400 and higher ($n = 5$).

For each subject on each monthly visit, waist circumference, weight and BP were measured by registered nurses. BP using a sphygmomanometer after patients sat and rested for at least 5 min. Plasma glucose (PG), serum lipids and lipoproteins, creatinine, hepatic enzymes, uric acid and other blood tests were measured by standard methods using an autoanalyzer. HbA1C values were determined by high performance liquid chromatography and inter-assay CVs were between 2.0 and 3.0 %. LDL cholesterol was calculated using Friedewald's formula in samples taken after an overnight fasting. Complete blood cell count was analyzed using an automated blood cell counter.

Urinary albumin was measured once during the first 3–4 months after enrollment in random urine samples using a turbidimetric immunoassay and expressed as albumin/creatinine ratio (ACR). Serum and urinary creatinine were measured enzymatically and estimated glomerular filtration rate (eGFR) was determined using the equation recommended by the Japanese Society for Nephrology [18].

Intrapersonal mean and coefficient of variation (CV) of HbA1c, fasting and post-breakfast plasma glucose (FPG and PPG, respectively) and serum triglycerides (FTG and PTG, respectively) taken during the first 12 months after enrollment were calculated in 168 patients with type 2 diabetes; 153 patients (91 %) had 12 measurements of HbA1c, systolic BP and 6 measurements of FPG, PPG, FTG and PTG, respectively. Linear regression was used to estimate changes in eGFR using a median of 52 creatinine measurements (interquartile range; 31-60) over 6.0 years of follow-up in each patient. Baseline means of serum creatinine and eGFR in Table 1 were means of 2–4 measurements during the first 3–4 months after enrollment.

Data were presented as mean ± SD unless otherwise stated. Differences between 2 groups were analyzed by t test and frequencies of conditions by Chi-square tests. Differences among 3 groups were analyzed using analysis of variance. Correlations of annual eGFR decline and CV-HbA1c were evaluated by Pearson correlation analysis. Stepwise multiple linear regression analyses were performed to further identify the most significant variables contributing to annual eGFR decline and CV-HbA1c. Potential confounders were forced into the model and standardized β coefficients were calculated. The explanatory power of the model was expressed as adjusted R^2 values. A two-tailed $P < 0.05$ was considered statistically significant. All calculations were performed with SPSS system 15.0 (SPSS Inc., Chicago, IL).

Results

Table 1 shows means of the intrapersonal mean values during the first 12 months after enrollment, except for age, duration of diabetes, serum creatinine, eGFR, ⊿eGFR and ACR. Means of age and duration of diabetes were those on enrollment of patients in the study. Baseline means of serum creatinine and eGFR in Table 1 were means of 2–4 measurements during the first 3–4 months after enrollment. ACR was measured once during the first 3–4 months after enrollment.

Patients had relatively good glycemic, lipid and BP control with a mean HbA1c of 7.0 %. CVs of HbA1c, FPG and PPG were 7.0 %, 14.1 % and 21.9 % respectively (Table 1). Baseline eGFR averaged 76 ± 16 ml/min/1.73m^2 and eGFR change was linear and averaged -1.05 ± 3.39 ml/min/1.73m^2 per year. Among 168 patients, 27 (16.0 %) had eGFR < 60 ml/min/1.73m^2 and 53(31.5 %) had albuminuria (microalbuminuria 47, macroalbuminuria 6).

Changes in eGFR were inversely associated with CV-HbA1c (Fig. 1)、FTG、PTG、log ACR and smokers (Table 1). However, eGFR changes did not show significant associations with age, sex, duration of diabetes, baseline eGFR, treatment for diabetes, mean HbA1c and mean and CV of FPG, PPG and SBP.

Multiple linear regression analysis (Table 2) revealed that CV-HbA1c (standardized β、-0.257、$p = 0.004$) were associated with and log ACR (standardized β、-0.155、$p =$

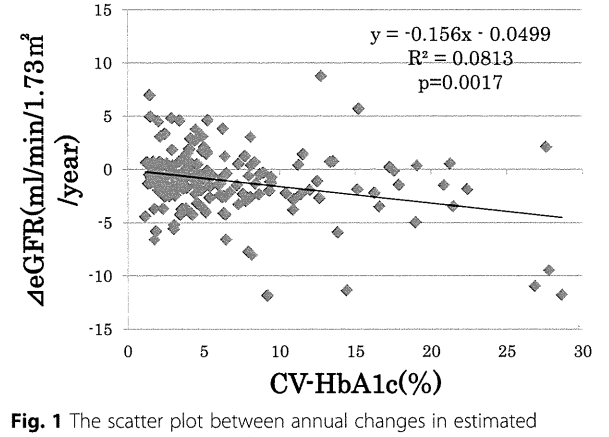

Fig. 1 The scatter plot between annual changes in estimated glomerular filtration rate (⊿eGFR) and coefficient of variations (CV) of HbA1c

Table 2 Multiple linear regression analysis for coefficient variation of HbA1c as a dependent variable

Independent variables	Standardized β	p values
sex	-.106	.267
age	-.101	.326
BMI	.061	.635
waist circumference	-.130	.316
duration of diabetes	-.047	.622
treatment for diabetes	-.121	.231
uses of anti-hypertensive medications	.002	.980
uses of lipid-lowering medications	.055	.537
smoking	-.186	.062
Fasting PG	.084	.488
Post-breakfast PG	.106	.376
HbA1c	.027	.812
CV-HbA1c	-.257	.004
Fasting TG	-.002	.989
Post-breakfast TG	-.072	.665
log ACR	-.155	.085
eGFR	-.074	.450

Abbreviations are the same as in Table

0.085) and smoking (standardized β、 -0.186、 $p = 0.062$) tended to be associated with annual eGFR decline independently of age, sex, BMI, waist circumference, duration of and treatment for diabetes, means and CVs of FPG and PPG, mean HbA1c, baseline eGFR, FTG、 PTG, and uses of anti-hypertensive and lipid-lowering medications.

Patients were divided into 3 groups according to tertiles of CV-HbA1c (Table 3). As CV-HbA1c increased, the percentage of smokers, mean HbA1c, means and CVs of FPG and PPG, and TG increased whereas HDL cholesterol decreased. Diabetic patients in the highest as compared to the lowest and median tertiles of CV-HbA1c had faster annual decline in eGFR. Other parameters including baseline eGFR were not different among 3 groups.

Association between HbA1c variability and renal function decline was significant in 53 patients with nephropathy (ACR≧30 mg/g) but did not reach statistical significance in 115 patients without nephropathy (r = -0.400, $p = 0.003$ and r = -0.169, $p = 0.07$, respectively). Compared with patients with normoalbuminuria, annual eGFR declines were significantly faster in patients with microalbuminuria (ACR≧30 mg/g) after controlling for confounders described above (-2.0 ± 0.4 (SE) vs. -0.6 ± 0.3 ml/min/1.73m^2 per year, $p = 0.01$).

Discussion

Variations of HbA1c, a reflection of long-term glycemic fluctuation, were found to increase the risk of chronic kidney disease defines as estimated GFR (eGFR) <60 ml/min/1.73 m^2 in some studies in patients with type 2 diabetes [14–16]. However, we are not aware of previous studies to determine whether HbA1c variability might directly associated with annual decline rate in eGFR in patients with type 2 as well as type 1 diabetes. The present study is the first to demonstrate a direct association between CV of HbA1c and annual eGFR decline in patients with diabetes independently of mean HbA1c and known predictors of GFR decline [19]. Further, association between HbA1c variability and renal function decline was stronger in patients with nephropathy (ACR≧30 mg/g) than in those with normoalbuminuria.

By comparison to short-term glucose variability, it has proven far less difficult to show an association between HbA1c variability and microvascular complication risk [20]. It has been shown that HbA1c variability predicted the development of chronic kidney disease in patients with type 2 diabetes [14, 16]. Further, Penno et al [15] have demonstrated that among 8260 patients with type 2 diabetes SD-HbA1c was associated with albuminuric chronic kidney disease independently of mean HbA1c and other known predictors of diabetic nephropathy, whereas mean HbA1c was not. These findings may be in line with our observation that albuminuria and CV-HbA1c were directly associated eGFR decline independently of mean HbA1c and other known predictors of GFR decline. However, among 4399 patients with type 2 diabetes in the intensive group of the ADVANCE trial [17], the association between SD of HbA1c and microvascular events did not reach statistical significance ($p = 0.06$ for trend) although there were significant linear associations of SD of HbA1c with combined macro—and microvascular events, major macrovascular events and all-cause mortality.

Although glycemic variability has been inconsistently associated with the risk of vascular complications in diabetes [21], several reasons may be involved in the association between visit-to-visit HbA1c variability and outcomes as suggested by Kilpatrick et al [20, 21]. They include 'metabolic memory' phenomenon [22]. They may be related to the fact that microvascular complication risk rises exponentially, rather than linearly, as HbA1c rises. They also may be related to the observation that acute improvement in HbA1c can lead to a short-term "early" worsening in retinopathy before subsequently resulting in a net long-term improvement. It is also possible that patient with HbA1c variability are those in whom the rest of their diabetes management is suboptimal.

Type 2 diabetic patients in the present study had annual eGFR decline which was even slower as compared with non-diabetic Japanese patients with early-stage chronic kidney disease (eGFR > 60 ml/min/1.73m^2) [23] (-1.05 vs. -1.64 ml/min/1.73m^2 per year). Further, annual eGFR

Table 3 Anthropometric, clinical and biochemical features of patients with type 2 diabetes according to tertiles of CV-HbA1c

	CV-HbA1c tertiles									
	Low			Median			High			
	(1.17-3.64)			(3.64-6.50)			(6.50-28.65)			p values
Smokers (n, %)	10	,	18.2	22	,	39.3	25	,	44.6	0.008
Age (years)	63.6	±	9.6	62.3	±	9.4	61.2	±	11.3	0.453
BMI (kg/m^2)	24.0	±	3.9	24.1	±	3.0	24.6	±	4.0	0.638
Waist circumference (cm)	87.2	±	9.4	86.6	±	8.7	87.0	±	11.5	0.959
Duration of diabetes (years)	9.2	±	7.7	10.6	±	6.7	9.8	±	7.5	0.622
Treatment of										
diabetes; diet/OHA/insulin (%)	43/50/7			32/45/23			20/ 57/ 23			0.026
hypertension; CCB/RASi/diuretics (%)	36/39/5			30/41/4			38/ 45/ 5			0.424
HbA1c (%)	6.6	±	0.6	7.1	±	0.7	7.4	±	1.0	<0.001
Fasting PG (mg/dL)	114	±	14	129	±	24	133	±	23	<0.001
Post-breakfast PG (mg/dL)	135	±	40	160	±	49	169	±	51	0.001
CV-HbA1c (%)	2.5	±	0.7	4.9	±	0.9	13.6	±	7.3	<0.001
CV-Fasting PG (%)	8.8	±	3.9	14.1	±	9.7	19.5	±	9.6	<0.001
CV-Post-breakfast PG (%)	18	±	10	23	±	11	25	±	11	0.007
Total cholesterol (mg/dL)	188	±	19	189	±	18	187	±	25	0.890
LDL cholesterol (mg/dL)	108	±	17	111	±	23	114	±	25	0.288
HDL cholesterol (mg/dL)	60	±	15	57	±	17	50	±	12	0.001
Fasting TG (mg/dL)	102	±	43	112	±	49	130	±	58	0.017
Post-breakfast TG (mg/dL)	131	±	59	144	±	65	137	±	65	0.047
Serum creatinine (mg/dL)	0.73	±	0.15	0.73	±	0.15	0.80	±	0.25	0.072
eGFR (mL/min/1.73m^2)	74	±	12	76	±	15	77	±	21	0.641
⊿eGFR (mL/min/1.73m^2/year)	−0.69	±	2.77	−0.63	±	2.29	−2.19	±	3.68	0.008
Uric acid (mg/dL)	5.3	±	1.5	5.0	±	1.4	5.2	±	1.1	0.495
Systolic BP (mmHg)	128	±	12	129	±	11	128	±	13	0.905
CV-Systolic BP (%)	8.2	±	2.3	7.6	±	2.1	8.3	±	2.2	0.254
Diastolic BP (mmHg)	72	±	6	72	±	7	72	±	7	0.787
Urinary ACR (mg/g)	21	±	24	69	±	152	162	±	532	0.066
log ACR	1.1	±	0.5	1.4	±	0.6	1.4	±	0.7	0.012

Mean ± SD or n, %. Abbreviations are the same as in Table 1

decline of our patients was much slower than the rate found in a previous study of Japanese type 2 diabetic patients without clinical albuminuria (-2.94 ml/min/1.73m^2 per year) [24] despite comparable baseline eGFR (76 and 75 ml/min/1.73m^2). These findings may be due in part to the fact that our patients had better glycemic (mean HbA1c; 7.0 vs. 8.4 %) and BP (128/72 vs. 135/81 mmHg) control. Slower eGFR decline associated with better diabetic control in our patients may be related to failure to detect association between mean HbA1c and annual eGFR decline in the present study.

In the present study, patients with microalbuminuria had faster decline of eGFR than those with normoalbuminuria. This finding may be in line with previous studies that urinary albumin, even in the microalbuminuric range, is a predictor of renal function impairment in the general population [25], type 2 diabetic patients with preserved kidney function [26, 27] and in CKD patients (GFR < 50 ml/min) [28]. In the last-cited longitudinal observational study [28], Lorenzo et al. compared the rate of renal decline in diabetic and non-diabetic CKD patients with comparable levels of albuminuria. They found that urinary ACR was a robust predictor of poor outcome. In addition, the mean slope of renal decline was similar in diabetic and non-diabetic patients when controlling for albuminuria.

The strength of the current study is that we used a 1-year period when mean HbA1c and HbA1c variability were calculated from 12 measurements in 91 % participants. In addition, we measured serum creatinine and

hence eGFR during follow-up period much more frequently than in previous studies [14–16]. This could contribute to the reliability of changes in kidney function. Such a testing frequency is routine in clinical settings in Japan. However, frequent measures of HbA1c may artificially inflate precision and decrease standard deviation, which may impact the results. Finally, BP control and variability and postprandial TG also have been taken into accounted. Major limitations are that study participants were small in number and from a single clinic in Japan. However, the characteristics of our study participants are similar to those reported in a previous large-scale study in Japan [29].

Conclusions

The current study has shown direct association between HbA1c variability and kidney function decline in type 2 diabetic patients and demonstrated stronger association in patients with microalbuminuria than in patients with normoalbuminuria. These findings suggest that more attention should be paid by clinicians in diabetes control, avoiding excessive oscillations in blood glucose levels in type 2 diabetic patients in general and in those with microalbuminuria in particular. Further studies are needed to confirm the association in other ethnic groups with more patients.

Abbreviations

ACR: Albumin /creatinine ratio; BP: Blood pressure; CV: Coefficient of variation; eGFR: Estimated glomerular filtration rate; FPG: Fasting plasma glucose; FTG: Fasting serum triglycerides; PPG: Post-breakfast plasma glucose; PTG: Post-breakfast serum triglycerides.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

AKT, AYK, MTT and MK have made substantial contributions to acquisition, analysis and interpretation of data. KF has been involved in drafting the manuscript. TK has been involved in revising it critically for important intellectual content; have given final approval of the version to be published; and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All authors read and approved the final manuscript.

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Original Article

Association of cystatin C with leptin and TNF-α in elderly Japanese women

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Background and Objectives: Determinants of cystatin C, a novel marker of mortality in the elderly, have not been extensively studied in Asian elderly population. **Methods and Study Design:** Associations of cystatin C with anthropometric, cardiometabolic, hematological, nutritional variables and inflammatory markers were examined in 159 community-living elderly Japanese women whose BMI averaged 22.6±2.9 (SD) kg/m^2. **Results:** Serum creatinine and cystatin C averaged 0.73±0.16 mg/dL and 0.85±0.20 mg/L, respectively. Creatinine-based estimated glomerular filtration rate (standardized β, -0.538, p<0.001), age (standardized β, 0.274, p<0.001), serum leptin (standardized β, 0.218, p<0.001) and tumour necrosis factor-α (TNF-α, standardized β, 0.165, p=0.002) emerged as significant predictors of serum cystatin C independent of percentage body fat, homeostasis model assessment of insulin resistance, high-sensitivity C-reactive protein, systolic blood pressure and HDL cholesterol (cumulative R^2=0.674). **Conclusions:** Elevated serum levels of leptin and TNF-α contributed to elevated cystatin C independent of kidney function, fat mass, insulin resistance and inflammation in community-living elderly women and may represent confounders of associations between cystatin C and mortality in this population.

Key Words: cystatin C, leptin, TNF-α, kidney function, elderly

INTRODUCTION

Cystatin C is a novel measure of kidney function that appears to be more sensitive than creatinine for determining changes in glomerular filtration rate (GFR).[1] Serum cystatin C as compared to creatinine was less influenced by diet and reduced muscle mass and hence, cystatin C-based estimated GFR appeared to be more accurate in assessing kidney function than creatinine-based GFR in the elderly population.[2] Cystatin C has recently been found to be a predictor of cardiovascular disease in elderly Western populations.[3,4] The impact of elevated cystatin C on cardiovascular disease risk was larger in the elderly versus the general population.[2] However, the relationship between cystatin C and traditional and non-traditional CV risk factors has not been well characterized, particularly in community-dwelling elderly persons of Asian origin.

Recently, cystatin C has been shown to be associated with insulin resistance, components of metabolic syndrome and inflammation.[5-8] However, many of those studies were performed in patients with type 2 diabetes, dyslipidemia and hypertension,[6-8] and data are limited in the general population, specifically in elderly population.[9] Therefore, we evaluated associations of cystatin C with anthropometric, cardiometabolic, hematological, nutritional variables and inflammatory markers in community-living elderly Japanese people.

PARTICIPANTS AND METHODS

We examined 159 free-living elderly women whose details have been reported elsewhere.[10] They were residents in Nishinomiya City and were recruited as volunteers by local welfare commissioners from the city of Nishinomiya, Hyogo, Japan. Although 8 men participated in the study, we reported data on women only. Although 43, 9 and 58 women (27.0%, 5.7%, and 36.5%, respectively) reported to be receiving statins, anti-diabetic and anti-hypertensive drugs, respectively, detailed drug information was not available. Subjects with clinical diagnosed acute or chronic inflammatory diseases, cancer, cardiovascular, hepatic and renal diseases, unusual dietary habits were excluded from the study. This research followed the tenets of the Declaration of Helsinki. The design of this study was approved by the Ethical Committees of Mukogawa Women's' University (No. 11-7) and written informed consents were obtained from all partici-

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pants.

Anthropometric indices and blood pressure were measured after an overnight fasting. Thereafter, blood samples were obtained from the cubital vein. Fat mass was measured using an impedance method (InBody 430, Biospace, Tokyo, Japan).

We evaluated routine chemical parameters, including plasma glucose, insulin, serum lipids and lipoproteins as previously reported.[11] Insulin resistance was evaluated using homeostasis model assessment (HOMA-IR).[12]

Metabolic syndrome was defined using the modified criteria of the National Cholesterol Education Program Adult Treatment Panel III (NCEP) guidelines.[13] Because waist circumference was not available in all participants, obesity (a cardinal feature of the metabolic syndrome) was defined using Asian criteria as BMI ≥25.0 kg/m^2.[14] Elevated blood pressure was defined as systolic/diastolic blood pressures of 130/85 mmHg or greater and/or current use of antihypertensive medicine. Hypertriglyceridemia was defined as a serum triglyceride level of 150 mg/dL or greater. Low HDL cholesterol level was defined as less than 50 mg/dL for all participants are women. Elevated blood glucose level was defined as fasting blood glucose level of 100 mg/dL or greater and/or current use of anti-diabetic medicine. Metabolic syndrome was defined as the presence of 3 or more of these components.

High-sensitivity C-reactive protein (hsCRP) was measured by an immunoturbidometric assay with the use of reagents and calibrators from Dade Behring Marburg GmbH (Marburg, Germany; interassay CV <5%). Tumor necrosis factor-α (TNF-α) were measured by immunoassays (R&D Systems, Inc., Minneapolis, MN, interassay CV <6%). Interleukin-6 (IL-6) was measured by a commercially available kit (IL-6, Human, ELISA Kit, QuantiGlo, 2nd Generation, Funakoshi Co, Ltd, Tokyo, Japan). Plasminogen activator-inhibitor-1 (PAI-1) was measured by an ELISA method (Mitsubishi Chemicals, interassay CV <8%). Complete blood cell count, total and differential peripheral leukocytes was analyzed using an automated blood cell counter (Sysmex XE-2100, Sysmex, Kobe, Japan).

Serum creatinine concentrations were measured enzymatically using an autoanalyzer (AU 5200, Olympus, Tokyo, Japan) and cystatin C was measured by latex immunoassay using a commercially available kit (IatroCys-C, Mitsubishi Chemical Medience, Tokyo, Japan). The eGFR was calculated using the equation recommended by the Japanese Society for Nephrology.[15,16]

Data are presented as mean±SD unless otherwise stated. Due to deviation from normal distribution, hsCRP, insulin and HOMA-IR were logarithmically transformed for analysis. Differences between 2 groups were analyzed by t test and frequencies of conditions by chi-square tests. Differences among 3 groups were analyzed using analysis of variance. When p values in analysis of variance were $p<0.05$, Bonferroni's multiple comparison procedure was performed. Correlations of cystatin C were evaluated by Pearson correlation analysis. Stepwise multiple regression analyses were performed to further indentify the most significant variables contributing to the variation of cystatin C. Potential confounders were forced into the model and standardized β coefficients were calculated. The explanatory power of the model was expressed as adjusted R^2 values. A two-tailed $p<0.05$ was considered statistically significant. All calculations were performed with SPSS system 15.0 (SPSS Inc, Chicago, IL).

RESULTS

As previously reported,[10] participants were relatively healthy, community-living, ambulatory elderly women (Table 1). Cystatin C and creatinine averaged 0.85±0.20 mg/L and 0.73±0.16 mg/dL, respectively (Table 1). Creatinine-based and cystatin C-based eGFR averaged 62±14 and 80±20 mL/min/1.73m^2, respectively. The prevalence of renal dysfunction (eGFR <60 mL/min/1.73m^2) was 46.5% (74 women) and 12.6% (13 women) when diagnosed using creatinine-based and cystatin C-based eGFR, respectively.

Of 159 elderly women, only 18 (11.3 %) had metabolic syndrome. There was no difference in serum cystatin C between women with and without the syndrome (0.95±0.32 and 0.84±0.18 mg/L, p=0.183). However, cystatin C increased as the number of components of metabolic syndrome increased [0 (n=21); 0.76±0.15, 1 (n=80); 0.85±0.17, 2 (n=40); 0.87±0.20, 3-5 (n=18); 0.95±0.32 mg/L, $p<0.01$ for trend].

In univariate analysis (Table 1), age, percentage body fat and serum leptin correlated positively with cystatin C. In addition, cystatin C showed a positive association with systolic blood pressure, pulse pressure, fasting glucose and insulin, and hence HOMA-IR. Further, cystatin C showed positive associations with uric acid and transthyretin and a negative association with HDL cholesterol. Finally, cystatin C was correlated positively with TNF-α and leukocyte count, but not with log hsCRP and IL-6. After adjustment for creatinine-based eGFR, an association with triglyceride became significant and associations with age, TNF-α, leptin, systolic blood pressure, HDL cholesterol and HOMA-IR remained significant.

In women with renal dysfunction (eGFR <60 mL/min/1.73m^2) diagnosed using creatinine-based eGFR (Table 2), associations of cystatin C with HOMA-IR, triglyceride, HDL cholesterol and leptin were stronger than in women without renal dysfunction. Associations with age, creatinine-based eGFR and TNF-α were consistent in women with and without renal dysfunction.

We have done multiple regression analysis with cystatin C as a dependent variable (Table 3). The model included creatinine-based eGFR and variables which showed significant associations with cystatin C after adjustment for eGFR (Table 1) as independent variables. Creatinine-based eGFR, age, serum TNF-α and leptin emerged as significant determinants of cystatin C independently of systolic blood pressure, pulse pressure, HOMA-IR, HDL cholesterol and serum TG. These 4 variables explained 68.2% of variability of serum cystatin C.

Because participants with high cystatin C (≥1.0 mg/dL)[5] were small in number (n=28), we decided to divide participants according to cystatin C tertiles in order to confirm associations of cystatin C with cardiometabolic and inflammatory variables (Table 4). Higher concentrations of cystatin C were associated with older age and higher leptin. In addition, higher cystatin C concentrations were associated with higher log TNF-α and leu-

Table 1. Anthropometric and biochemical features of 159 elderly women studied and correlation coefficients of serum cystatin C before (simple) and after (partial) adjustment for creatinine-based estimated glomerular filtration rate.

	Mean±SD	Serum cystatin C			
		Simple		Partial	
Age (years)	75.6±8.0	0.527	***	0.354	***
BMI (kg/m^2)	22.6±2.9	0.076		0.103	
Body fat percentage (%)	33.0±6.9	0.165	*	0.159	
SBP (mmHg)	143±19.0	0.249	**	0.230	**
DBP (mmHg)	84.4±10.4	0.095		0.134	
Pulse pressure (mmHg)	59.0±12.7	0.295	***	0.236	**
Albumin (g/dL)	4.4±0.3	-0.299	***	-0.191	*
Transthyretin (mg/dL)	28.2±4.8	-0.163	*	-0.211	*
Plasma glucose (mg/dL)	100±29	0.176	*	0.103	
Insulin (μU/mL)	8.3±7.5	0.220	**	0.127	
HOMA-IR	1.23±1.08	0.270	***	0.164	*
Total cholesterol (mg/dL)	219±31	-0.153		-0.038	
HDL-cholesterol (mg/dL)	64±14	-0.208	**	-0.234	**
Non-HDL-cholesterol (mg/dL)	155±33	-0.056		0.079	
Triglyceride (mg/dL)	119±64.9	0.019		0.169	*
Serum uric acid (mg/dL)	4.8±1.0	0.343	***	0.098	
Serum creatinine (mg/dL)	0.73±0.16	0.770	***	0.343	***
Cystatin C (mg/L)	0.85±0.20	1.000	***	1.000	***
eGFRcreat (mL/min/1.73m^2)	62±14	-0.742	***	adjusted	
Leptin (ng/mL)	7.7±4.7	0.293	***	0.267	**
Adiponectin (μg/mL)	14.1±7.8	0.097		0.021	
hsCRP (μg/dL)	85±109	0.075		0.049	
TNF-α (pg/mL)	1.6±1.0	0.481	***	0.310	***
PAI-1 (ng/mL)	26.5±16.5	-0.004		0.129	
IL-6 (pg/mL)	5.5±12.0	0.134		0.043	
Leukocytes (×10^3/μL)	6.1±1.6	0.161	*	0.135	

BMI: body mass index; SBP: Systolic blood pressure; DBP: diastolic blood pressure; HOMA-IR: homeostasis model insulin resistance; eGFRcreat: creatinine-based estimated glomerular filtration rate; hsCRP: high-sensitivity C-reactive protein; TNF: tumour necrosis factor; PAI-1: plasminogen activator inhibitor-1; IL-6: interleukin-6.
$^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$

Table 2. Associations of cystatin C in women with and without renal dysfunction (estimated glomerular filtration rate <60 mL/min/1.73m^2) diagnosed using creatinine-based equation

	Renal dysfunction			
	No (n=85)		Yes (n=74)	
	r	*p*	r	*p*
Age (years)	0.552	**	0.456	**
BMI (kg/m^2)	0.026		0.074	
Body fat percentage (%)	0.138		0.170	
SBP (mmHg)	0.176		0.216	
DBP (mmHg)	0.092		0.105	
Pulse pressure (mmHg)	0.204		0.223	
Albumin (g/dL)	-0.412	**	-0.259	*
Transthyretin (mg/dL)	-0.240	*	-0.146	
Plasma glucose (mg/dL)	-0.097		0.228	
Insulin (μU/mL)	-0.120		0.249	*
HOMA-IR	-0.131		0.309	**
Total cholesterol (mg/dL)	-0.169		-0.121	
HDL-cholesterol (mg/dL)	-0.044		-0.295	*
Non-HDL-cholesterol (mg/dL)	-0.157		0.014	
Triglyceride (mg/dL)	-0.113		0.246	*
Serum uric acid (mg/dL)	0.674		-0.271	
Serum creatinine (mg/dL)	0.392	**	0.714	**
Cystatin C (mg/L)	1		1	
eGFRcreat (mL/min/1.73m^2)	-0.964	**	-0.947	**
Leptin (ng/mL)	0.159		0.296	*
Adiponectin (μg/mL)	0.210		0.102	
hsCRP (μg/dL)	0.262	*	0.005	
TNF-α (pg/mL)	0.323	**	0.456	**
PAI-1 (ng/mL)	-0.074		0.103	
IL-6 (pg/mL)	0.259	*	0.086	
Leukocytes (×103/μL)	0.117		0.123	

Abbreviations are the same as in Table 1. $^*p<0.05$, $^{**}p<0.01$

Table 3. Stepwise multiple regression analysis with serum cystatin C as a dependent variable in community-dwelling elderly Japanese women

	Standardized β	*p* values	Cumulative R^2
eGFRcreat	-0.538	<0.001	0.545
Age	0.274	<0.001	0.602
Leptin	0.218	<0.001	0.654
TNF-α	0.165	0.002	0.674

The model included eGFRcreat, age, serum leptin, TNF-α, systolic blood pressure, pulse pressure, homeostasis model insulin resistance, HDL cholesterol and triglycerides as independent variables. Abbreviations are the same as in Table 1.

Table 4. Anthropometric and biochemical characteristics of elderly women stratified by tertiles of serum cystatin C.

	Low (n=50)	Medium (n=55)	High (n=54)
Age (years)	70.0±7.4[a]	75.3±7.1[b]	81.1±5.5[c]
BMI (kg/m^2)	22.6±2.8	22.5±2.4	22.6±3.5
Body fat percentage (%)	32.3±7.3	32.4±5.8	34.1±7.4
SBP (mmHg)	136±17.2[a]	144±17.1[ab]	149±20.6[b]
DBP (mmHg)	82.1±10.5	85.9±9.7	84.9±10.8
Pulse pressure (mmHg)	54.1±10.1[a]	57.6±10.8[a]	64.3±14.5[b]
Albumin (g/dL)	4.5±0.2[a]	4.4±0.2[b]	4.3±0.3[c]
Transthyretin (mg/dL)	29.4±4.3[a]	28.4±4.4[ab]	26.8±5.5[b]
Plasma glucose (mg/dL)	88±10	87±12	88±15
Insulin (μU/mL)	5.3±3.3	5.3±3.9	5.9±4.8
HOMA-IR	1.17±0.82	1.15±0.88	1.37±1.44
Total cholesterol (mg/dL)	228±33	219±34	216±31
HDL-cholesterol (mg/dL)	69±17	65±16	65±14
Non-HDL-cholesterol (mg/dL)	158±30	154±33	151±31
Triglyceride (mg/dL)	128±80	122±65	107±46
Serum uric acid (mg/dL)	4.4±0.7[a]	4.7±1.2[a]	5.2±1.0[b]
Serum creatinine (mg/dL)	0.62±0.09[a]	0.70±0.09[b]	0.86±0.18[c]
Cystatin C (mg/L)	0.67±0.06[a]	0.82±0.03[b]	1.06±0.20[c]
eGFRcreat (mL/min/1.73m^2)	73.3±11.1[a]	63.0±9.5[b]	50.4±10.4[c]
Leptin (ng/mL)	8.1±5.0[a]	8.8±5.1[ab]	10.6±7.9[b]
Adiponectin (μg/mL)	14.3±7.5	16.0±7.0	17.0±7.6
hsCRP (μg/dL)	158±232	182±316	297±493
TNF-α (pg/mL)	1.8±0.9[a]	2.1±0.8[a]	3.0±1.6[b]
PAI-1 (ng/mL)	30.7±13.1	28.6±8.9	27.8±10.5
IL-6 (pg/mL)	4.0±6.5	4.1±4.7	8.4±18.9
Leukocytes (×10^3/μL)	5.5±1.1	5.8±1.4	6.1±1.7

Data are mean±SD. Abbreviations are the same as in Table 1. Means not sharing common alphabetical letters are statistically significant each other at $p<0.05$ or less.

kocyte count. Further, higher cystatin C concentrations were associated with higher systolic blood pressure, pulse pressure and uric acid. After controlling for creatinine-based eGFR, associations with TNF-α, systolic blood pressure, pulse pressure and age remained significant (data not shown).

DISCUSSION

The current study demonstrates that higher serum levels of leptin and TNF-α are associated with higher cystatin C in community-living elderly women. These associations remained significant after adjustment for age, creatinine-based eGFR, a conventional measure of renal function, percentage body fat measured using an impedance method and hsCRP, a marker of systemic low-grade inflammation. We confirmed the previous findings that high cystatin C is associated with some components of metabolic syndrome in elderly women, specifically in women with renal dysfunction, as previously reported in patients at higher risk,[6-8] and demonstrated that high cystatin C is associated with high TNF-α and leptin in community-living elderly women independent of age, fat mass, systemic low-grade inflammation and kidney function.

Naour et al[17] reported that serum cystatin C is elevated in obese subjects and that adipose tissue expression of the protein is increased in obesity. Although several prior studies have reported the relationship between cystatin C and BMI,[18-20] a crude marker of fat mass, the present study is the first to demonstrate an independent relationship between serum cystatin C and leptin, a sensitive marker of fat mass.[21] However, this association was independent of percentage body fat measured using bioelectrical impedance method, which is a good alternative for dual-energy X ray absorptiometry, a gold standard for estimating percentage body fat, when subjects are within a normal body fat range.[22] In this context, it has recently been shown even in the elderly that high levels of leptin are associated with arterial stiffness, hypertension and low endothelial-dependent vasodilation,[23] all of which are known CV risk factors. These associations were attenuated after adjusting for body mass index suggesting that leptin may be the mediator between obesity and im-

paired vascular function.[23]

Although TNF-α is an inflammatory cytokine produced mainly by monocytes and macrophages, TNF-α produced by adipose tissue may play an important role in obesity-associated insulin resistance and diabetes.[24] In the Insulin Resistance Atherosclerosis Study,[25] increased TNF-α levels were predominantly associated with insulin resistance.[18] In our study of elderly women, TNF-α was correlated with cystatin C independently of hsCRP and IL-6, hallmarks of systemic inflammation. Taken together, these findings suggest that TNF-α- may be a biomarker of insulin resistance rather than systemic inflammation in our elderly women. It is probable that locally produced TNF-α may act synergistically with circulating TNF-α on fatty and muscular tissues to induce insulin resistance although the serum levels of TNF-α found in the present study were relatively low and circulating TNF-α may not be biologically active at such low concentrations.

Previous studies have reported positive associations between cystatin C and CRP,[6,21,22,26,27] but most of these studies analyzed a single biomarker. The present study examined associations between cystatin C and a broad range of inflammatory markers and found a significant association of cystatin C with serum TNF-α independent of hsCRP and IL-6. Although it is known that TNF-α stimulates the synthesis of CRP in the liver, correlation coefficient between circulating levels of TNF-α and CRP was 0.173 in the present study and 0.27 in a large, multi-ethnic population of the Insulin Resistance Atherosclerosis Study.[18] As mentioned above, TNF-α may be a biomarker of insulin resistance rather than systemic inflammation in the present study. Associations between serum cystatin C and TNF-α independent of eGFR and CRP were also reported in well-functioning older population of white and black subjects[28] and in patients with essential hypertension[29] or acute heart failure.[30]

Cystatin C levels progressively increased in association with the number of MS components in community-living elderly women in the present study as previously reported in patients with type 2 diabetes, hypertension and dyslipidemia.[6-8] Although cystatin C was higher in patients with MS than in patients free of MS,[6-8] there was no significant difference in cystatin C between elderly women with and without MS in the present study. This may be due in part to small sample size in women with MS (n=18, 11.3%).

Our study has several limitations. We are unable to determine either the direction of association or the causal pathway given the cross-sectional design of our study. The recruitment procedure may also have had an impact on the results. As participation was voluntary, women who pay more attention to their health may have been more likely to participate. Biochemical parameters, including cystatin levels, were measured only once. Although 43, 9 and 58 women (27.0%, 5.7%, and 36.5%, respectively) reported to be receiving statins, anti-diabetic and anti-hypertensive drugs, respectively, detailed drug information was not available. These drugs may have effects on serum cystatin C levels.[31] In addition, a more direct measurement of GFR, such as inulin clearance, was not used in this study as a gold standard for comparison. We also lacked measures of urine albumin excretion, clinical outcomes and fat distribution. Finally, participants were relatively small in number and were all females.

In conclusion, elevated levels of serum leptin, a mediator between obesity and impaired vascular function,[23] and TNF-α, a marker of insulin resistance,[25] were associated with higher cystatin C in ambulatory elderly women. Higher body fat and insulin resistance may represent important confounders of the relationship between serum cystatin C and mortality in the elderly population.

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AUTHOR DISCLOSURES

No potential conflicts of interest were disclosed.

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Original Article

Association of cystatin C with leptin and TNF-α in elderly Japanese women

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日本老年女性胱蛋白 C 與瘦體素及 TNF-α 的相關性

背景與目的：胱蛋白 C 為一個老年死亡的新標記，其決定因子在亞洲老年族群尚未被大規模的探討。**方法與研究設計：**評估 159 名 BMI 平均值為 22.6±2.9 (SD) kg/m^2 的日本社區老年女性，其胱蛋白 C 與體位測量值、心血管代謝、血液資料、營養狀況及發炎標記之相關。**結果：**血清肌酸酐與胱蛋白 C 平均值分別為 0.73±0.16 mg/dL 及 0.85±0.20 mg/L。肌酸酐評估腎絲球過濾率（標準化迴歸係數-0.538，p<0.001）、年齡（標準化迴歸係數 0.274，p<0.001）、血清瘦體素（標準化迴歸係數 0.218，p<0.001）及腫瘤壞死因子-α（TNF-α 標準化迴歸係數 0.165，p=0.0021）顯示為血清胱蛋白 C 的顯著預測因子，且此結果獨立於體脂肪百分比、胰島素抗性之恆定模式評估、高敏感度 C 反應蛋白、收縮壓及高密度脂蛋白膽固醇（累積 R^2=0.674）。**結論：**社區老年女性血清瘦體素及 TNF-α 的增加導致胱蛋白 C 的提高，此相關獨立於腎功能、體脂質量、胰島素阻抗性及發炎反應。這個族群之胱蛋白 C 與死亡率的相關，可能是干擾因子造成的相關。

關鍵字：胱蛋白 C、瘦體素、腫瘤壞死因子-α、腎功能、老人

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RESEARCH ARTICLE

Dietary Yeasts Reduce Inflammation in Central Nerve System via Microflora

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Abstract

Objectives: The intestinal microflora affects the pathogenesis of several autoimmune diseases by influencing immune system function. Some bacteria, such as lactic acid bacteria, have been reported to have beneficial effects on immune function. However, little is known about the effects of yeasts. Here, we aimed to investigate the effects of various dietary yeasts contained in fermented foods on experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis (MS), and to elucidate the mechanisms underlying these effects. **Methods**: The effects of eight yeasts selected from 18 types of yeasts contained in fermented foods were examined using an EAE model. Of these, *Candida kefyr* was investigated by analyzing the intestinal microflora and its effects on intestinal and systemic immune states. **Results**: Administration of *C. kefyr* ameliorated the severity of EAE. Reduced numbers of Th17 cells, suppressed interleukin (IL)-6 production by intestinal explants, and increased Tregs and CD103-positive regulatory dendritic cells in mesenteric lymph nodes (MLNs) were observed. Analysis of 16s-rDNA from feces of *C. kefyr*-treated mice demonstrated increased *Lactobacillales* and decreased *Bacteroides* compared to control flora. Transfer of intestinal microbiota also resulted in decreased *Bacteroides* and ameliorated symptoms of EAE. Thus, oral administration of *C. kefyr* ameliorated EAE by altering the microflora, accompanied by increased Tregs and CD103-positive regulatory dendritic cells in MLNs and decreased Th17 cells in the intestinal lamina propria. **Interpretation**: Oral ingestion of *C. kefyr* may have beneficial effects on MS by modifying microflora. In addition, our findings also suggested the potential health benefits of dietary yeasts.

Introduction

Food habits and intestinal microflora have been shown to modulate the intestinal and systemic immune states, thereby affecting human health.[1,2] Th17 cells are induced by intestinal segmented filamentous bacteria and have been implicated in the pathogenesis of autoimmune diseases, including experimental autoimmune encephalomyelitis (EAE).[3–5] On the other hand, certain groups of commensal bacteria and their metabolites play critical roles in the induction of Foxp3-positive regulatory T cells in the colon.[6] Furthermore, the intestine itself has a mechanism to control excessive inflammation by eliminating or suppressing pro-inflammatory Th17 cells.[7]

These data highlight the importance of immune responses in the intestine.

Indeed, intestinal microflora and related intestinal immune mechanisms affect the susceptibility of humans and animals to inflammatory autoimmune diseases. For example, fermented foods and lactic acid bacteria are thought to have healthful effects, and recent studies have shown that modification of intestinal microflora ameliorates clinical symptoms of experimental disease models such as EAE and inflammatory bowel disease.[8,9] Although the effects of lactic acid bacteria on various autoimmune diseases have been reported,[10,11] few studies have investigated the effects of yeasts, such as *Saccharomyces*, *Candida*, and *Aspergillus* species, which are found in fermented foods.

Kefir is an acidic, mildly alcoholic fermented milk originating from the Caucasus mountains. Kefir grains represent a natural symbiosis of yeasts and lactic acid bacteria.[12] Importantly, in a mouse model of bronchial asthma, kefir has been reported to have anti-inflammatory and anti-allergic effects.[13]

In the current study, we sought to determine whether yeasts found in fermented foods have beneficial effects on EAE. Our results suggested that ingestion of *Candida kefyr*, one of the yeasts examined in this study, is a novel therapeutic strategy for overcoming autoimmune disease.

Materials and Methods

Reagents and animals

All yeasts (Table S1) were purchased from the National Institute of Technology and Evaluation (NITE) Biological Resource Center (NBRC, Chiba, Japan). They were cultured according to the manufacturer's protocols. The use of viable yeast is restricted in our animal facility because of the requirement for maintenance of specific pathogen-free conditions, yeasts were dissolved in 0.2 g/mL double distilled water (DDW), and all yeasts were heat-killed at 120°C for 15 min and stored at −80°C. C57BL/6 mice were administered water containing 8 mg/mL heat-killed yeasts in water bottles beginning at 14 days before immunization.

Induction of EAE

All experimental procedures were approved by the Animal Care and Use Committee of Osaka University Graduate School of Medicine. C57BL/6 mice were obtained from Oriental Yeast Corp. (Tokyo, Japan). EAE was induced as described previously.[10] In brief, after administration of heat-killed yeasts for 14 days, as described above, C57BL/6 mice were subcutaneously injected with 100 μg myelin oligodendrocyte glycoprotein (MOG) 35–55 (MEV GWYRSPFSPVVHLYRNGK) peptide (MOG_{35-55}) emulsified in complete Freund's adjuvant (CFA) containing 200 μg of *Mycobacterium tuberculosis* H37Ra (Difco Laboratories, Detroit, MI). Mice were concurrently injected twice with 200 ng of pertussis toxin (List Laboratories, Campbell, CA) on days 0 and 2. All mice were monitored daily for clinical signs and were scored as described previously.[10]

Histology and semiquantification of data

Mice were sacrificed on day 22 postimmunization followed by transcardiac perfusion with 4% paraformaldehyde in PBS. Spinal cords were fixed in 4% paraformaldehyde in PBS and prepared for histological analysis. Cryosections (10-μm thick) were stained with hematoxylin and eosin (H&E). Semiquantitative histological analysis of inflammatory cellular infiltration was performed as previously described.[14]

Isolation of MNCs and lymphocytes

MLNs, inguinal lymph nodes (ILNs), and cervical lymph nodes (CLNs) were harvested and homogenized. Cells were centrifuged and the resulting pellets were used as lymphocytes. Lamina propria (LP) lymphocytes were isolated as previously described.[10] The detailed method to isolate LP lymphocytes is described in the Data S1.

Cytokine assay

For the assessment of antigen-specific cytokine production, mononuclear cells (MNCs) were isolated from draining ILNs and cervical LNs of mice on day 8 after immunization with MOG_{35-55}. Cells were restimulated with the peptide for 72 h, and interleukin (IL)-17, interferon (IFN)-γ, and IL-10 were assayed by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (R&D Systems, Minneapolis, MN).

Intracellular cytokine staining

Intracellular expression of IL-17 and IFN-γ in $CD4^+$ T cells was analyzed using an Intracellular Fixation and Permeabilization Buffer Set (eBioscience, San Diego, CA) according to the manufacturer's instructions. Surface staining was performed with anti-CD4-APC-H7 antibodies (BD Biosciences, Franklin Lakes, NJ, USA). The cells were then stained with Fixable Viability Dye eFluor 450, fixed with fixation solution, and then washed with permeabilization diluent. Intracellular cytokine staining was performed with anti-IL-17A Alexa Fluor 647 (BD Biosciences), anti-IL-10-PE (BD Biosciences), and anti-IFN-γ-FITC (fluorescein isothiocyanate) (BioLegend,

