TABLE 4. Age- or Multivariate-Adjusted HRs for Development of CVD, CHD, and Stroke According to the Number of the MetS Components in 2452 Subjects During a 14-Year Follow Up

	Population at Risk	No. of Events	Age- and Sex-Adjusted			Multivariate-Adjusted*		
			HR	(95% CI)	*P* Value	HR	(95% CI)	*P* Value
Cardiovascular disease								
No. of MetS components								
0	436	30	1.00	(reference)		1.00	(reference)	
1	756	84	1.49	(0.98–2.26)	0.06	1.45	(0.95–2.20)	0.08
2	625	72	1.47	(0.96–2.26)	0.08	1.39	(0.91–2.15)	0.15
3	394	65	2.12	(1.37–3.28)	<0.01	1.95	(1.25–3.04)	<0.01
≥4	241	56	3.19	(2.03–5.02)	<0.01	2.99	(1.89–4.73)	<0.01
Coronary heart disease								
No. of MetS components								
0	436	13	1.00	(reference)		1.00	(reference)	
1	756	35	1.41	(0.75–2.67)	0.29	1.38	(0.72–2.62)	0.33
2	625	22	1.05	(0.53–2.09)	0.89	0.95	(0.47–1.90)	0.88
3	394	32	2.55	(1.33–4.89)	<0.01	2.29	(1.18–4.47)	0.01
≥4	241	23	3.36	(1.68–6.72)	<0.01	2.96	(1.45–6.01)	<0.01
Stroke								
No. of MetS components								
0	436	20	1.00	(reference)		1.00	(reference)	
1	756	58	1.52	(0.91–2.53)	0.11	1.48	(0.89–2.47)	0.14
2	625	50	1.50	(0.89–2.53)	0.13	1.45	(0.86–2.46)	0.16
3	394	41	1.89	(1.10–3.25)	0.02	1.78	(1.03–3.09)	0.04
≥4	241	40	3.16	(1.83–5.46)	<0.01	3.05	(1.75–5.31)	<0.01

*Adjusted for age, sex, proteinuria, electrocardiogram abnormalities, serum total cholesterol, smoking habits, alcohol intake, and regular exercise.

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TABLE 5. Age- and Sex-Adjusted or Multivariate-Adjusted HRs of the MetS for Development of CVD According to the Presence or Absence of Hypertension or Diabetes in 2452 Subjects During a 14-Year Follow Up

	Population at Risk	No. of Events	Age- and Sex-Adjusted		Multivariate-Adjusted*	
			HR	(95% CI)	HR	(95% CI)
Hypertension						
HT (−)+MetS (−)	1269	89	1.00	(reference)	1.00	(reference)
HT (−)+MetS (+)	200	25	1.79	(1.14–2.79)*	1.75	(1.12–2.75)*
HT (+)+MetS (−)	548	97	1.81	(1.35–2.43)†	1.75	(1.29–2.37)†
HT (+)+MetS (+)	435	96	2.59	(1.93–3.48)†‡	2.45	(1.81–3.32)†‡
Diabetes						
DM (−)+MetS (−)	1732	171	1.00	(reference)	1.00	(reference)
DM (−)+MetS (+)	498	84	1.60	(1.23–2.09)†	1.54	(1.17–2.02)†
DM (+)+MetS (−)	85	15	1.35	(0.80–2.30)	1.38	(0.81–2.34)
DM (+)+MetS (+)	137	37	2.75	(1.93–3.93)†‡	2.60	(1.81–3.74)†‡

*Adjusted for age, sex, proteinuria, electrocardiogram abnormalities, serum total cholesterol, smoking habits, alcohol intake, and regular exercise.

*$P<0.05$, †$P<0.01$ vs reference.

‡$P<0.05$ vs HT(+)+MetS (−) or DM (+)+MetS (−).

HT indicates hypertension; DM, diabetes mellitus.

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Disclosures

None.

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Liver Enzymes as a Predictor for Incident Diabetes in a Japanese Population: The Hisayama Study

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Abstract

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Objective: We studied the relationship between liver enzymes and the development of diabetes in a general Japanese population.

Research Methods and Procedures: A total of 1804 nondiabetic subjects 40 to 79 years of age were followed-up prospectively for a mean of 9.0 years.

Results: During the follow-up, 135 subjects developed diabetes. In both sexes, the age-adjusted cumulative incidence of diabetes increased significantly with elevating quartiles of serum γ-glutamyltransferase (GGT) and alanine aminotransferase (ALT) levels. This pattern was also observed in aspartate aminotransferase (AST) quartiles for men but not for women. In multivariate analyses after adjusting for comprehensive risk factors and other liver enzymes, the risk of developing diabetes was significantly higher in the highest GGT quartile than in the lowest quartile [odds ratio (OR), 2.54; 95% confidence interval (CI), 1.03 to 6.26 for men; OR, 5.73; 95% CI, 1.62 to 20.19 for women]. Similar results were observed in ALT quartiles (OR, 2.32; 95% CI, 0.91 to 5.92 for men; OR, 4.40; 95% CI, 1.38 to 14.06 for women) but not in AST quartiles in either sex. Significant positive associations of GGT and ALT with diabetes were seen within each stratified category of risk factors, namely fasting insulin, BMI, waist-to-hip ratio, high-sensitivity C-reactive protein, and alcohol consumption. In receiver operating characteristic analyses, the areas under the receiver operating characteristic curve of GGT and ALT were significantly larger than that of AST, fasting insulin, waist-to-hip ratio, or C-reactive protein.

Discussion: Our findings suggest that serum GGT and ALT concentrations are strong predictors of diabetes in the general population, independent of known risk factors.

Key words: liver, longitudinal, C-reactive protein, diabetes, visceral obesity

Introduction

The liver, a major site of insulin clearance, plays an important role in maintaining normal glucose concentrations during fasting and postprandially (1). Recently, several cohort studies have shown that serum γ-glutamyltransferase (GGT)[1] (2–6), alanine aminotransferase (ALT) (7–9), and aspartate aminotransferase (AST) (10) levels are predictors of diabetes. In one of these reports, a study on Pima Indians (8) found that high serum ALT levels were a significant risk factor for diabetes, although no clear association between serum GGT and diabetes was seen. On the other hand, serum GGT levels, but not AST levels, have been identified as an independent predictor of incident diabetes in British men selected from lists of general practitioners (2). Moreover, the Mexico City Diabetes Study

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[1] Nonstandard abbreviations: GGT, γ-glutamyltransferase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HS-CRP, high-sensitivity C-reactive protein; HDL-C, high-density lipoprotein cholesterol; OR, odds ratio; CI, confidence interval; ROC, receiver operating characteristic.

found that serum AST is an independent risk factor for future diabetes in multivariable adjustment, whereas no association was observed between serum GGT or ALT and the development of diabetes (10). These reports suggest that the liver is associated with the development of diabetes; however, to the best of our knowledge, there have been no studies to date to determine which of these three enzymes is the best marker for incident diabetes. Furthermore, it also remains unknown whether liver enzyme markers are stronger predictors of future diabetes than well-known risk factors for diabetes, such as adiposity, insulin resistance, and inflammation. The purpose of this study is to examine the effects of serum liver enzymes, i.e., GGT, ALT, and AST, on the development of diabetes in a prospective study of a defined Japanese population, taking into account comprehensive risk factors, including BMI, waist-to-hip ratio, fasting insulin, and high-sensitivity C-reactive protein (HS-CRP) levels.

Research Methods and Procedures

Study Population and Follow-up Survey

A population-based prospective study of cardiovascular disease has been underway since 1961 in the town of Hisayama, a suburb in the Fukuoka metropolitan area on Kyushu Island in Japan. The age and occupational distributions of the town population were almost identical to those of Japan as a whole from 1961 to the present based on data from the national census. A screening survey for this study was performed in 1988. A detailed description of this survey has been published previously (11,12). Briefly, of all 3227 residents 40 to 79 years of age listed in the town registry, 2587 (80.2%) consented to take part in a comprehensive assessment, including an interview covering medical history (including diabetes, hypertension, and other chronic diseases) and current medical treatment with insulin and oral anti-diabetic agents. The baseline classification of subjects as either having or not having diabetes was based on the fasting criteria of the American Diabetes Association (13): subjects with a fasting plasma glucose level of ≥7.0 mM or those who were taking anti-diabetic medications were defined as having diabetes. A total of 2274 subjects (963 men and 1311 women) were enrolled in the baseline examination after the exclusion of 1 subject for whom no blood sample was obtained, 75 subjects who had already taken breakfast before the examination, 233 subjects with diabetes, and 4 subjects who had died before starting our follow-up.

After the initial screening in 1988, fasting glucose levels were again measured between 1993 and 1998. Of the 2274 subjects, 1804 (719 men and 1085 women) underwent a follow-up examination (follow-up rate, 79.3%). We considered a subject to have developed diabetes when his/her fasting glucose level met the above-mentioned American Diabetes Association criteria or if the subject started taking anti-diabetic medication during the follow-up period. During this period, 135 subjects (71 men and 64 women) developed diabetes.

Clinical Evaluation and Laboratory Measurements

Blood samples were collected after at least 12 hours of fasting for the determination of serum liver enzymes, plasma glucose, and other parameters. Serum GGT concentrations were measured using a modified version of the method of Orlowski and Meister (14). Both serum ALT and AST concentrations were determined by a kinetic ultraviolet ray method based on the rate of reduced nicotinamide adenine dinucleotide oxidation. Plasma glucose levels were determined by a glucose-oxidase method, and serum insulin levels were measured by double-antibody, solid-phase radioimmunoassay. Hemoglobin A_{1c} levels were measured by high-pressure liquid chromatography. Total cholesterol, high-density lipoprotein cholesterol (HDL-C) and triglycerides were determined enzymatically. HS-CRP concentrations were analyzed using a modified latex-enhanced HS-CRP assay (Behring Diagnostics, Westwood, MA). Serum hepatitis B surface antigen was detected by an immunoprecipitation method (Shino-test, Tokyo, Japan), and presence of hepatitis C virus antibody was assessed by both particle agglutination assay (Serodia-HCV; Fujirebio, Tokyo, Japan) and recombinant immunoblot assay (RIBA 2.0; Ortho Diagnostic Systems, Raritan, NJ).

Blood pressure was obtained three times using a mercury sphygmomanometer with the subject in a sitting position; the averages of the three values were used in this analysis. Hypertension was defined as a systolic blood pressure of ≥140 mm Hg and/or a diastolic blood pressure of ≥90 mm Hg and/or current treatment with anti-hypertensive agents. The height and weight of each subject were recorded with the subject wearing light clothes but no shoes, and BMI (kg/m^2) was calculated. Abdominal girth at the umbilical level and hip circumference at 5 cm below the spina iliaca anterior superior were measured and used to calculate the waist-to-hip ratio.

On baseline examination, each participant completed a self-administered questionnaire covering medical history, anti-hypertensive treatment, alcohol intake, and smoking habits, and the questionnaire was checked by trained interviewers at the screening. Diabetes in first- or second-degree relatives was taken to indicate a family history of diabetes. Subjects engaging in sports at least three times per week during their leisure time were defined as the regular exercise group. Alcohol intake and smoking habits were used to classify subjects as having current habits or not.

Statistical Analysis

Because the distributions of GGT, ALT, AST, fasting insulin, HS-CRP, and triglycerides were skewed, these variables were natural log-transformed for statistical analyses.

Table 1. Characteristics of subjects by sex

	Men (n = 719)	Women (n = 1085)
Age (yrs)	58 ± 10	58 ± 10
GGT (units/L)	22 (11 to 95)	13 (8 to 35)
ALT (units/L)	14 (7 to 38)	11 (6 to 24)
AST (units/L)	22 (14 to 45)	19 (12 to 33)
Fasting plasma glucose (mM)	5.6 ± 0.5	5.5 ± 0.5
Hemoglobin A_{1c} (%)	5.5 ± 0.5	5.4 ± 0.5
Family history of diabetes (%)	9.2	7.2
Fasting insulin (pM)	30.0 (18.0 to 72.0)	36.0 (18.0 to 72.0)
BMI (kg/m^2)	22.9 ± 2.9	23.0 ± 3.1
Waist-to-hip ratio	0.92 ± 0.05	0.91 ± 0.07
Total cholesterol (mM)	5.07 ± 1.03	5.54 ± 1.04
HDL-cholesterol (mM)	1.25 ± 0.30	1.34 ± 0.29
Triglycerides (mM)	1.24 (0.57 to 3.49)	1.02 (0.49 to 2.32)
HS-CRP (mg/L)	0.49 (0.07 to 7.14)	0.36 (0.06 to 3.22)
Systolic blood pressure (mm Hg)	131 ± 17	130 ± 20
Diastolic blood pressure (mm Hg)	82 ± 11	76 ± 11
Hypertension (%)	42.8	33.2
Current drinking (%)	60.8	8.6
Current smoking (%)	47.3	5.5
Regular exercise (%)	15.9	4.9

HDL, high-density lipoprotein; HS-CRP, high-sensitivity C-reactive protein; GGT, γ-glutamyltransferase; AST, aspartate aminotransferase; ALT, alanine aminotransferase. Variables of GGT, AST, ALT, fasting insulin, triglycerides, and CRP are median values (95% confidence intervals). All other values are given as mean ± standard deviation or as a percentage.

To analyze liver enzyme levels as categorical variables, these levels were divided into four groups on the basis of quartiles by sex: GGT, men, 6 to 16, 17 to 22, 23 to 37, and 38 to 529 U/L; GGT, women, 6 to 10, 11 to 13, 14 to 17, and 18 to 261 U/L; ALT, men, 5 to 10, 11 to 13, 14 to 18, and 19 to 354 U/L; ALT, women, 5 to 8, 9 to 11, 12 to 14, and 15 to 153 U/L; AST, men, 8 to 17, 18 to 21, 22 to 27, and 28 to 424 U/L; AST, women, 7 to 16, 17 to 18, 19 to 22, and 23 to 273 U/L. The age-adjusted cumulative incidences of diabetes were calculated by the direct method using all subjects, and the results were compared by the Mantel-Haenszel χ^2 test using 10-year age-groupings. Age- and multivariate-adjusted odds ratios (ORs) and 95% confidence intervals (CIs) were calculated by logistic regression analysis. The sensitivity of cut-off points was defined as their ability to correctly identify individuals who later developed diabetes, and their specificity was defined as their ability to correctly identify individuals who did not develop diabetes. To compare the prognostic abilities of risk factors including liver enzymes and to detect the presence or absence of future diabetes across a range of the values for each risk factor, we plotted receiver operating characteristic (ROC) curves and compared the areas under them (15,16). The diagnostic properties of specific cut-off levels of each risk factor were defined by maximizing the sensitivity and specificity to identify future diabetes. A value of $p < 0.05$ was considered statistically significant in all analyses.

This study was conducted with the approval of the Ethics Committee of the Faculty of Medicine, Kyushu University, and written informed consent was obtained from all participants.

Results

The clinical characteristics of all subjects by sex are shown in Table 1. The mean age was 58 years for both sexes. The mean values of GGT, ALT, AST, fasting plasma glucose, hemoglobin A_{1c}, waist-to-hip ratio, triglycerides, HS-CRP, systolic and diastolic blood pressures, frequency of hypertension, alcohol intake, smoking habits, and regular exercise were higher in men than in women, whereas women had higher concentrations of fasting insulin, total cholesterol, and HDL-C. The frequency of family history of diabetes and mean BMI levels did not differ between the sexes.

The age-adjusted cumulative incidence of diabetes was 9.6% for men and 5.9% for women, giving a statistically significant difference (p = 0.002). Figure 1 shows the age-adjusted cumulative incidence of diabetes according to quartiles of each liver enzyme level by sex. The cumulative incidence in the third and forth GGT quartiles was significantly higher compared with that of the first quartile in both sexes. A similar tendency was observed for ALT quartiles: there were significant differences between the first and fourth quartiles in both sexes. This pattern was also found in AST quartiles for men but not for women.

The age-adjusted OR for the development of diabetes increased significantly with elevating quartiles of each liver enzyme concentrations in both sexes (Table 2, Model 1). In the multivariate analyses after adjustment for age, family history of diabetes, fasting insulin, BMI, waist-to-hip ratio, total cholesterol, HDL-C, triglycerides, HS-CRP, hypertension, current drinking, current smoking, and physical activity, the ORs of future diabetes increased significantly with elevating quartiles of serum GGT and ALT (Model 2). These trends were also observed in AST quartiles for men but not for women. As shown in Model 3 of Table 2, after additional adjustment for the other liver enzymes, these relationships remained substantially unchanged in both GGT and ALT quartiles but not in AST in either sex.

To examine the influence of insulin resistance-related factors, inflammation and alcohol intake on the development of diabetes, we estimated the age- and sex-adjusted ORs and 95% CIs of diabetes by increments of 1 log in each liver enzyme in men and women together in accordance with other risk factor levels (Table 3). Analyses were performed dividing the subjects into three groups according to tertiles of fasting insulin, BMI, waist-to-hip ratio, and HS-CRP or to alcohol intake levels (0, 1 to 30, and ≥30 g/d). Significant positive associations of GGT and ALT with diabetes were observed in all stratified categories of each risk factor; however, we found no significant associations between AST and diabetes in the third tertile of BMI, in the third tertile of waist-to-hip ratio, or in the second level of alcohol intake.

To compare the ability of each risk factor to predict future diabetes over a mean of 9 years of follow-up, we plotted ROC curves and calculated optimal cut-off points, sensitivities, specificities, and the area under the ROC curves (Table 4). Both maximum sensitivity and specificity exceeded 60% only for GGT and ALT, and the areas under the ROC curve of GGT and ALT were significantly larger than that of AST, fasting insulin, waist-to-hip ratio, or HS-CRP and were slightly but not significantly larger than that of BMI. The difference in the area under the ROC curve between GGT and ALT was not significant.

Viral hepatitis infection can increase liver enzyme levels without liver fat accumulation. Thus, hepatitis B and C virus markers were examined in 1583 of the 1804 subjects in 1998. We found 13 viral hepatitis subjects (3 subjects with hepatitis B virus and 10 with C virus; 10.7%) in 122 subjects of the group developing diabetes and 104 viral hepatitis subjects (25 subjects with hepatitis B virus and 79 with C virus; 7.1%) in 1461 subjects of the group that did not develop diabetes: the difference was not significant (χ^2 = 2.1; p = 0.15).

Discussion

We have shown, in a prospective study of a general Japanese population, that elevated levels of GGT and ALT, but not AST, are independent predictors of diabetes for both sexes after adjustment for age, family history of diabetes, fasting insulin, BMI, waist-to-hip ratio, total cholesterol, HDL-C, triglycerides, HS-CRP, hypertension, current drinking, current smoking, physical activity, and the other liver enzymes. In our stratified analyses, associations of both GGT and ALT with the development of diabetes were observed in all layers of other risk factors, such as fasting insulin, BMI, waist-to-hip ratio, HS-CRP, and alcohol intake. ROC analyses showed that the predictive power of GGT and ALT was similar to that of BMI but stronger than that of AST, fasting insulin, waist-to-hip ratio, and HS-CRP. To the best of our knowledge, this study is the first report to indicate that liver enzymes are independent risk factors for developing diabetes in a general Japanese population in either sex, taking into account comprehensive risk factors for diabetes. Several prospective studies have found that high levels of hepatic enzymes, including GGT (2–6), ALT (7–9), and AST levels (10), are associated with later development of diabetes. These findings, together with these results, strongly suggest that the liver plays an important role in the development of diabetes in relatively lean Asian populations who may have smaller fat content in the liver, as it does in Western populations.

A recent study using a fatless mouse model has shown that ectopic fat accumulation in the liver is associated with severe insulin resistance (17). In normal weight and moderately overweight subjects, directly determined liver fat content has also been shown to correlate with several features of insulin resistance, independent of BMI and intra-abdominal or overall obesity (18). These findings indicate that hepatic fat accumulation is a critical manifestation of insulin resistance. However, direct measurement of liver fat requires ultrasound, computed tomography, or proton spectroscopy, and such techniques are unlikely to be recommended in routine clinical practice. Some circulating variables, including serum ALT, GGT, and AST, provide insight into the extent of liver fat accumulation. Among these, ALT is found primarily in the liver, whereas AST and GGT are also found in other tissues and are, therefore, less specific markers of liver function. Therefore, ALT is the most specific marker of liver pathology and seems to be the best marker for liver fat accumulation: serum ALT is cor-

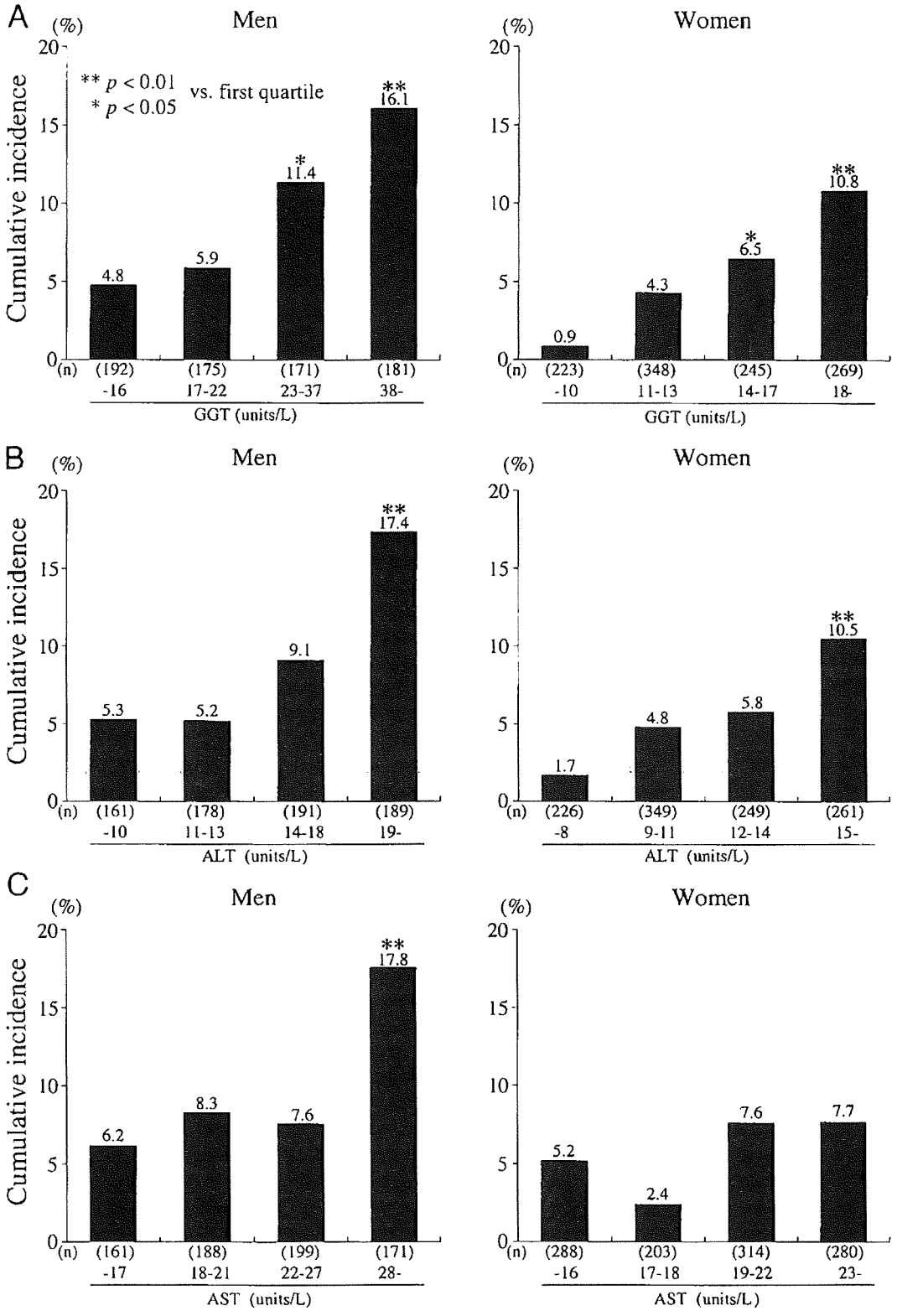

Figure 1: The age-adjusted cumulative incidences of diabetes according to quartiles of serum liver enzymes. (A) GGT, γ-glutamyltransferase; (B) ALT, alanine aminotransferase; (C) AST, aspartate aminotransferase.

Table 2. Age- and multivariate-adjusted ORs and 95% CIs for the development of diabetes according to quartiles of each liver enzyme by sex during mean 9 years of follow-up

	Range	Population at risk (*n*)	Number of events (*n*)	Model 1: OR (95% CI)	*p* for trend	Model 2: OR (95% CI)	*p* for trend	Model 3: OR (95% CI)	*p* for trend
GGT (U/L)									
Men	0 to 16	192	10	1 (referent)		1 (referent)		1 (referent)	
	17 to 22	175	10	1.10 (0.45 to 2.71)		0.85 (0.32 to 2.28)		0.85 (0.31 to 2.27)	
	23 to 37	171	20	2.39 (1.09 to 5.28)		2.02 (0.84 to 4.88)		1.99 (0.82 to 4.80)	
	38 to	181	31	3.71 (1.75 to 7.87)	0.0001	2.71 (1.13 to 6.52)	0.0040	2.54 (1.03 to 6.26)	0.0088
Women	0 to 10	223	3	1 (referent)		1 (referent)		1 (referent)	
	11 to 13	348	15	3.29 (0.94 to 11.48)		2.65 (0.74 to 9.47)		2.64 (0.74 to 9.42)	
	14 to 17	245	16	5.10 (1.46 to 17.74)		3.72 (1.04 to 13.29)		3.66 (1.02 to 13.09)	
	18 to	269	30	8.98 (2.70 to 29.87)	0.0001	5.80 (1.67 to 20.12)	0.0011	5.73 (1.62 to 20.19)	0.0017
ALT (U/L)									
Men	0 to 10	161	9	1 (referent)		1 (referent)		1 (referent)	
	11 to 13	178	9	0.90 (0.35 to 2.33)		0.71 (0.25 to 1.98)		0.68 (0.24 to 1.92)	
	14 to 18	191	17	1.65 (0.71 to 3.83)		1.31 (0.52 to 3.32)		1.18 (0.46 to 3.03)	
	19 to	189	36	3.98 (1.83 to 8.64)	0.0001	2.85 (1.17 to 6.92)	0.0017	2.32 (0.91 to 5.92)	0.016
Women	0 to 8	226	5	1 (referent)		1 (referent)		1 (referent)	
	9 to 11	349	17	2.18 (0.79 to 6.00)		2.28 (0.74 to 7.02)		2.26 (0.73 to 6.99)	
	12 to 14	249	14	2.60 (0.92 to 7.34)		2.83 (0.90 to 8.92)		2.86 (0.90 to 9.07)	
	15 to	261	28	5.15 (1.95 to 13.59)	0.0001	4.53 (1.50 to 13.64)	0.0027	4.40 (1.38 to 14.06)	0.0077
AST (U/L)									
Men	0 to 17	161	10	1 (referent)		1 (referent)		1 (referent)	
	18 to 21	188	16	1.43 (0.63 to 3.24)		0.96 (0.40 to 2.31)		0.91 (0.38 to 2.19)	
	22 to 27	199	15	1.26 (0.55 to 2.89)		0.88 (0.37 to 2.10)		0.81 (0.33 to 1.95)	
	28 to	171	30	3.27 (1.54 to 6.94)	0.0016	2.30 (1.01 to 5.21)	0.030	1.87 (0.77 to 4.53)	0.17
Women	0 to 16	288	12	1 (referent)		1 (referent)		1 (referent)	
	17 to 18	203	5	0.56 (0.19 to 1.62)		0.40 (0.12 to 1.29)		0.40 (0.12 to 1.28)	
	19 to 22	314	24	1.79 (0.86 to 3.73)		1.69 (0.80 to 3.58)		1.70 (0.79 to 3.62)	
	23 to	280	23	1.91 (0.91 to 4.04)	0.019	1.49 (0.68 to 3.24)	0.073	1.26 (0.55 to 2.92)	0.17

OR, odds ratio; CI, confidence interval; GGT, γ-glutamyltransferase; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

Model 1: adjustment was made for age.

Model 2: adjustment was made for age, family history of diabetes, fasting insulin, BMI, waist-to-hip ratio, total cholesterol, high-density lipoprotein-cholesterol, triglycerides, high-sensitivity C-reactive protein, hypertension, current drinking, current smoking, and physical activity.

Model 3: adjustment was made for the variables used in Model 2 and for the other liver enzymes

Table 3. Age- and sex-adjusted ORs and 95% CIs for the occurrence of diabetes by increments of 1 log in each liver enzyme according to risk factor levels during mean of 9 years of follow-up

	Range	Population at risk (*n*)	Number of events (*n*)	Age- and sex-adjusted [OR (95% CI) for GGT]	*p*	Age- and sex-adjusted [OR (95% CI) for ALT]	*p*	Age- and sex-adjusted [OR (95% CI) for AST]	*p*
Fasting insulin (pM)	0 to 24.0	605	32	2.93 (1.87 to 4.59)	0.0001	2.78 (1.45 to 5.35)	0.0022	2.26 (1.08 to 4.74)	0.031
	24.1 to 36.0	547	36	2.21 (1.38 to 3.52)	0.0009	2.53 (1.39 to 4.63)	0.0025	2.50 (1.26 to 4.96)	0.0087
	36.1 to	651	67	1.86 (1.22 to 2.82)	0.0039	2.07 (1.31 to 3.26)	0.0018	1.93 (1.09 to 3.44)	0.025
BMI (kg/m^2)	0 to 21.5	601	29	2.91 (1.82 to 4.64)	0.0001	2.87 (1.50 to 5.48)	0.0014	3.32 (1.57 to 7.04)	0.0017
	21.6 to 24.2	602	36	2.09 (1.30 to 3.38)	0.0025	3.43 (1.81 to 6.49)	0.0002	2.54 (1.26 to 5.13)	0.0090
	24.3 to	601	70	1.99 (1.32 to 3.00)	0.0010	1.71 (1.10 to 2.65)	0.016	1.72 (0.97 to 3.03)	0.063
Waist-to-hip ratio	0 to 0.88	586	24	3.71 (2.10 to 6.54)	0.0001	2.19 (1.15 to 4.17)	0.017	2.38 (1.09 to 5.22)	0.030
	0.89 to 0.94	583	59	2.28 (1.55 to 3.35)	0.0001	3.19 (1.86 to 5.49)	0.0001	2.83 (1.53 to 5.23)	0.0009
	0.95 to	590	50	1.78 (1.15 to 2.75)	0.01	2.24 (1.37 to 3.67)	0.0014	1.73 (0.91 to 3.30)	0.093
HS-CRP (mg/L)	0 to 0.25	586	21	2.12 (1.21 to 3.73)	0.009	2.49 (1.31 to 4.74)	0.0056	2.68 (1.29 to 5.54)	0.0081
	0.26 to 0.64	587	46	2.62 (1.60 to 4.27)	0.0001	2.78 (1.50 to 5.15)	0.0011	2.45 (1.11 to 5.40)	0.026
	0.65 to	586	64	2.09 (1.45 to 3.02)	0.0001	2.51 (1.59 to 3.96)	0.0001	2.35 (1.32 to 4.17)	0.0038
Alcohol intake (g/day)	0	1,274	79	2.81 (1.90 to 4.15)	0.0001	2.56 (1.73 to 3.79)	0.0001	2.19 (1.29 to 3.72)	0.0036
	1 to 29	289	24	2.86 (1.50 to 5.44)	0.001	3.04 (1.45 to 6.35)	0.0032	2.20 (0.91 to 5.31)	0.079
	30 to	241	32	1.69 (1.08 to 2.64)	0.02	2.49 (1.24 to 5.00)	0.011	2.31 (1.12 to 4.74)	0.023

OR, odds ratio; CI, confidence interval; GGT, γ-glutamyltransferase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HS-CRP, high-sensitivity C-reactive protein.

Table 4. Optimal cut-off points of risk factors defined by maximizing sensitivity and specificity to predict future diabetes and their ROC curve areas

	GGT (units/L)	ALT (units/L)	AST (units/L)	Fasting insulin (pM)	BMI (kg/m^2)	Waist-to-hip ratio	HS-CRP (mg/L)
Cut-off point	18	13	19	30.0	24.1	0.91	0.44
Sensitivity (%)	63.3	63.4	48.6	50.7	66.2	47.8	54.8
Specificity (%)	63.7	65.9	71.1	65.2	56.3	69.9	67.9
ROC curve area (%)	67.9	67.4	62.3*†	61.1*†	64.6	60.0*†	61.6*†
(95% CI)	(63.1 to 72.3)	(62.4 to 72.1)	(57.0 to 67.4)	(56.0 to 65.9)	(59.5 to 69.6)	(55.4 to 64.5)	(56.9 to 66.2)

ROC, receiver operating characteristic; GGT, γ-glutamyltransferase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HS-CRP, high-sensitivity C-reactive protein; CI, confidence interval.
* $p < 0.05$ vs. GGT.
† $p < 0.05$ vs. ALT.

related with liver fat measured by proton spectroscopy and, after weight loss, the change in serum ALT correlates with that in liver fat (19).

In our multivariate analysis, serum GGT and ALT were mutually independent in predicting incident diabetes. It is known that serum GGT is not only a marker of liver fat amount but also a marker of oxidative stress (20–22). GGT presenting at the outer side of the cell membrane is thought to maintain cellular glutathione levels, which are the major intracellular defense against free radicals (23). Increased oxidative stress impairs insulin secretion from the islets of Langerhans and insulin action in target tissues by damaging DNA, membranes, enzymes, etc. (24). Decreased insulin secretion and insulin sensitivity are major features of the pathophysiology of type 2 diabetes (25). This may be the reason why GGT and ALT has a highly predictive value for the development of diabetes. On the other hand, several epidemiologic studies examined which of these enzymes was the best marker for incident diabetes. Lee et al. (3,4) reported the dose–response relationship between GGT levels and incidence of diabetes in both Korean male workers and young black and white Americans with ALT or AST levels within the reference interval. Furthermore, in their other study, GGT levels within normal range predicted incidence of chronic elevation of ALT (26). These findings indicate the possibility that GGT is a more powerful predictor of incident diabetes than other liver enzymes. However, we showed in the ROC analysis that ALT and GGT but not AST have equally predictive value for the development of diabetes. These findings should be confirmed in other populations, having different BMI levels and lifestyles.

Some experimental studies have shown that selective deletion of the insulin receptor from muscle results in a slight increase in serum free fatty acid and triglycerides but not in glucose intolerance or diabetes (27), whereas a similar maneuver in the liver leads to marked glucose tolerance (28), suggesting that maintaining normal glucose concentrations is related to the liver rather than to peripheral tissue. Our stratified analysis showed that the associations of both GGT and ALT levels with the occurrence of diabetes were independent of markers of systemic insulin resistance, such as fasting insulin, BMI, waist-to-hip ratio, and HS-CRP. In our subjects, the areas under the ROC curve of GGT and ALT were also significantly larger than that of fasting insulin, waist-to-hip ratio, or HS-CRP. Insulin resistance in the liver through fat accumulation may offer a better explanation of the cause of diabetes than peripheral insulin resistance or systemic inflammation.

Alcohol intake causes fatty change of the liver. In alcoholic fatty liver, serum ALT tends to be depressed relative to serum AST, and serum GGT has the specificity to detect alcohol abuse (29), whereas liver fat accumulation caused by overeating predominantly increases ALT but not AST or GGT (19). However, these findings indicated that both GGT and ALT predict future diabetes, independent of current drinking habits. Additionally, in our stratified analyses, the associations of both GGT and ALT with diabetes were unrelated to alcohol intake levels. These observations suggest that elevated serum levels of GGT and ALT, irrespective of the causes of fatty liver, are associated with incident diabetes.

Viral hepatitis infection often increases liver enzyme levels without hepatic fat accumulation, and several clinical studies have shown that chronic hepatitis C virus infection is linked to type 2 diabetes (30,31). In our study, however, the distribution of hepatitis B and C virus positive markers

did not differ between subjects who developed diabetes and those who did not, indicating that viral hepatitis infection did not affect our findings.

A limitation of our study is that a diagnosis of diabetes was not based on a 75-gram oral glucose tolerance test, but on a single reading of fasting glucose level, as has been the case in other epidemiologic studies (3–5,9). Thus, subjects with diabetes having normal fasting glucose levels were misdiagnosed in our study. In addition, some of the participants who were classified as having worsening fasting glucose status may not have been so categorized after repeated testing. These misclassifications may have weakened the associations found in this study, and the true associations may, in fact, be stronger than those shown in our data.

In conclusion, we have shown that elevated serum GGT and ALT levels, even in the normal range, are better predictors of diabetes than the known risk factors except for BMI in a general Japanese population. The association between these enzymes and diabetes was found to be independent of insulin resistance, inflammation markers, and alcohol consumption levels. These results support the hypothesis that the liver is more important than previously thought in the pathogenesis of type 2 diabetes.

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Albuminuria and Chronic Kidney Disease in Association With the Metabolic Syndrome

Chronic kidney disease is a worldwide public health problem because it is an important risk factor for cardiovascular disease and premature death. The metabolic syndrome, which is characterized by abdominal obesity, high blood pressure, impaired glucose tolerance, and dyslipidemia, is also an increasingly common disorder and a major risk factor for diabetes and cardiovascular disease. A close association has been found between the metabolic syndrome and the risk for developing renal impairment, clinically expressed in the form of microalbuminuria or chronic kidney disease. Several potential mechanisms, including insulin resistance, renal atherosclerosis, and inflammation, induce the deterioration of renal function. Despite the close association between the metabolic syndrome and renal impairment, it is still unclear whether and to what extent treating the metabolic syndrome will prevent renal impairment. A clinical trial is needed to clarify whether the effect of preventing and treating metabolic syndrome components will result in improved renal prognosis. (JCMS. 2007;2:104–107) ©*2007 Le Jacq*

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Chronic kidney disease (CKD) is a worldwide public health problem. CKD is a major risk factor for end-stage renal and cardiovascular disease and causes premature death.[1–5] CKD is also increasingly common. According to data from the Third National Health and Nutrition Examination Survey (NHANES III), approximately 8 million US adults 20 years of age and older currently have CKD. Therefore, identifying and treating risk factors for early CKD may be the best approach to prevent and delay advanced outcomes.[2]

The metabolic syndrome (MetS), previously known as syndrome X, the deadly quartet, and the insulin-resistant syndrome, is characterized by the cluster of atherogenic metabolic disorders, including abdominal obesity, high blood pressure, impaired glucose tolerance, hypertriglyceridemia, and reduced high-density lipoprotein cholesterol.[6] The MetS is present in approximately 47 million US adults 20 years of age or older, according to data from NHANES III.[7] With the continuous increase in the prevalence of obesity in the United States, the MetS is expected to be even more common in the future.[8] In addition, the MetS has been associated with increased risk of developing diabetes mellitus and cardiovascular disease as well as increased mortality from cardiovascular disease and all causes.[9,10] Recent epidemiologic surveys have found that patients with the MetS are at high risk for microalbuminuria or CKD. The aim of this article is to review the MetS as a risk factor for the development of microalbuminuria or CKD.

The MetS and Risk for Developing Renal Impairment

As shown in the Table, several epidemiologic studies examined the association between the MetS and the risk of microalbuminuria or CKD.[11–18] In these studies, microalbuminuria was defined as a urinary albumin/creatinine ratio of 30 to 300 mg/g, and CKD was determined by estimated glomerular filtration rate (GFR) <60 mL/min/1.73 m^2. GFR was estimated by the Modification of Diet in Renal Disease study equation.[19]

In a cross-sectional study of nondiabetic Native Americans, Hoehner and colleagues[11] found a significant association between MetS profile and microalbuminuria: subjects with 3 or more MetS components had a 2.3-fold higher prevalence of microalbuminuria than did those with no components after adjustment for social, demographic, and comorbid factors. Palaniappan and associates[12] and Chen and colleagues[13] extracted data from NHANES III, which contained detailed clinical information on approximately 6000 subjects. Both of these cross-sectional studies showed significant associations between the MetS, defined by the National Cholesterol Education Program Adult Treatment Panel III (NCEP-ATP III) criteria, and microalbuminuria. Chen and colleagues also demonstrated that subjects with the MetS had a 2.6-fold higher prevalence of CKD compared with patients without the MetS, and the prevalence of CKD increased with elevating number of MetS components.[13] In Asian populations, a few studies also examined this issue.[14,15] In a cross-sectional survey of Korean adults who participated in a health examination program, the multivariate-adjusted odds ratio of microalbuminuria in subjects with the MetS was 1.5 compared with patients without the MetS.[14] The similar finding was observed in the results of health checks in Okinawa, a southern island in Japan.[15] Although the results of these studies suggest a close association

Table. Epidemiologic Studies Concerning the Association Between the MetS and CKD

Authors	Country	No. of Subjects	Design	MetS Definition	End Point	Multivariate-Adjusted OR (95% CI)
Hoehner et al[11]	United States	2068	Cross-sectional	Original[a]	MAU	2.3 (1.1–4.9)
Palaniappan et al[12]	United States	5659	Cross-sectional	NCEP	MAU	Men: 4.1 (2.5–6.7)[b] Women: 2.2 (1.4–3.3)[b]
Chen et al[13]	United States	6217	Cross-sectional	NCEP	MAU	CKD MAU: 1.9 (1.3–2.7) CKD: 2.6 (1.7–4.0)
Choi et al[14]	Korea	6588	Cross-sectional	NCEP[c]	MAU	1.5 (1.1–2.1)
Tanaka et al[15]	Japan	6980	Cross-sectional	NCEP[d]	CKD	1.5 (1.3–1.9)
Kurella et al[16]	United States	10,096	Prospective	NCEP	CKD	1.4 (1.2–1.7)
Ninomiya et al[17]	Japan	1440	Prospective	NCEP[c]	CKD	2.1 (1.2–3.5)
Bonnet et al[18]	France	2738	Prospective	NCEP IDF	MAU	NCEP: Men: 1.2 (0.7–2.2) Women: 1.9 (0.9–3.8) IDF: Men: 1.9 (1.3–2.8) Women: 1.4 (0.8–2.5)

Abbreviations: NCEP, National Cholesterol Education Program; IDF, International Diabetes Federation. [a]The metabolic syndrome (MetS) was defined by hypertension, impaired fasting glucose, high fasting insulin, low high-density lipoprotein cholesterolemia, and hypertriglyceridemia. [b]Age-adjusted odds ratio (OR) and 95% confidence intervals (CIs). [c]Waist circumference was modified by the Asian criteria of waist circumference (men >90 cm, women >80 cm). [d]Waist circumference was modified by the Japanese criteria of waist circumference (men >85 cm, women >90 cm). Microalbuminuria (MAU) was defined as a urinary albumin/creatinine ratio of 30 to 300 mg/g. Chronic kidney disease (CKD) was defined as glomerular filtration rate estimated by Modification of Diet in Renal Disease study equation <60 mL/min/1.73 m^2.

between the MetS and renal impairment, it is difficult to draw any definitive conclusion concerning a cause-and-effect relationship because these studies had a cross-sectional design.

Recent longitudinal epidemiologic studies provide further evidence to support the premise that the MetS independently contributes to the development of microalbuminuria or CKD.[16–18] Kurella and colleagues[16] showed in a 9-year follow-up survey of the Atherosclerosis Risk in Communities (ARIC) study that the MetS was independently associated with increased risk of incident CKD in nondiabetic adults; the risk for the development of CKD was 1.4-fold higher in subjects with the MetS than in patients without the MetS after adjustment for potential confounding factors. We also found that the MetS was an independent risk factor for incident CKD in a 5-year follow-up survey of the Hisayama Study,[17] a prospective cohort study of cardiovascular disease and its risk factors in a general Japanese population. Subjects with the MetS had a 2.1-fold increased risk for incident CKD compared with patients without the MetS after multivariate adjustment. Even after exclusion of subjects with diabetes at entry or adjustment for blood pressure at end of follow-up, the risk for incident CKD in subjects with the MetS remains significant. In a 6-year follow-up survey of the Data from an Epidemiological Study on the Insulin Resistance Syndrome (DESIR) trial, Bonnet and colleagues[18] showed that in men, the MetS was a significant risk factor for developing microalbuminuria according to the International Diabetes Federation (but not the NCEP-ATP III) definition of MetS. The presence of the MetS according to either definition was not related to the development of microalbuminuria in women.

These studies suggest the strong evidence that the MetS is an independent risk factor for developing microalbuminuria or CKD. A further clinical trial is needed to clarify whether the effect of preventing and treating the MetS will result in improved renal prognosis.

Mechanism of Developing Renal Impairment in the MetS

Several possible explanations have been proposed for the mechanism underlying the association between the MetS and the risk of renal impairment: (1) the adverse effects of insulin resistance, (2) the accumulation of atherogenic risk factors, (3) the adverse effects of inflammatory mediators, and (4) obesity-related glomerulopathy.

Insulin Resistance. Insulin resistance and compensatory hyperinsulinemia, which are considered to be fundamental pathogenetic factors of the MetS, directly contribute to the development of renal injury by worsening renal hemodynamics through multiple mechanisms, including sodium retention,[20] activation of the sympathetic nervous system,[21] decreased Na^+-K^+-ATPase activity,[22] and elevation of the glomerular filtration fraction.[20] In addition, on the basis of in vitro observations, hyperinsulinemia could induce glomerular hypertrophy either directly or by stimulating the insulin-like growth factor 1 (IGF-1) receptor.[23] Moreover, treatment of mesangial cells with insulin resulted in a marked increase in rate of protein synthesis as well as in an alteration in the type of interstitial and basement membrane collagen excreted by these cells.[24,25] Interestingly, insulin also seemed to evoke a phenotypic

change in mesangial cells, and these cells failed to synthesize a normal collagen pattern, namely, predominant synthesis of collagen type I and III, instead of collagen IV.[24,25] IGF-1 was found to inhibit apoptosis of mesangial cells in a dose-dependent manner, thereby promoting their survival.[26] IGF-1 also decreased the activity of matrix metalloproteinase-2, an enzyme normally responsible for extracellular matrix degradation; its inhibition led to extracellular matrix expansion and renal fibrosis.[27] Hyperinsulinemia could further interact with elevated intrarenal angiotensin II levels to augment angiotensin II concentration of glomerular mesangial cells.[20]

Atherogenic Risk Factors. Another possible explanation for the relationship between the MetS and incident CKD is that MetS components directly damage the kidneys through atherosclerosis. The mechanisms of hypertensive and diabetic renal injuries leading to CKD have been well described.[28–30] In the ARIC study, however, the association between the MetS and CKD in nondiabetic subjects remained robust even after adjustment for the subsequent development of diabetes and hypertension, suggesting that the risk for CKD is not solely attributable to the conditions of diabetes or hypertension.[16] In NHANES III, Chen and colleagues observed that increased abdominal obesity was significantly correlated with microalbuminuria and GFR decline, suggesting that obesity may be an independent risk factor for CKD.[13] Observational data and a recent meta-analysis suggest that elevated triglycerides and reduced high-density lipoprotein cholesterol are independent risk factors for the development of CKD and that the use of statins may slow CKD progression.[30–32] In our autopsy-based study of Hisayama residents,[33] hypertension, impaired glucose tolerance, and hypercholesterolemia were also significant risk factors for renal arteriosclerosis. Although data from the ARIC study also showed that each MetS component was significantly associated with increased risk for incident CKD, the cluster of these risk factors had a stronger impact on the development of CKD than did individual risk factors.[16] Furthermore, we observed in the Hisayama Study that the accumulation of 3 or more of the metabolic disorders promoted the development of CKD or progression of GFR decline independent of serum fasting insulin levels. This suggests that the risk of cluster of MetS components for the development of CKD is not solely attributable to the insulin resistance and compensatory hyperinsulinemia.[17] These findings support the hypothesis that the accumulation of atherogenic metabolic disorders induces renal vessel injury, resulting in the deterioration of renal function.

Inflammatory Mediators and Obesity-Related Glomerulopathy. Another potential mechanism includes direct effects of inflammatory mediators resulting from obesity and insulin resistance.[34] Adipose tissue is recognized as an immune organ that secretes numerous immunomodulatory factors, including leptin, interleukin 6, tumor necrosis factor α, adiponectin, and acylation-stimulation protein.[34] These adipose tissue cytokines may be crucial to the enhancement of insulin resistance or systemic atherosclerosis, resulting in the progression of CKD. Furthermore, a large renal biopsy-based clinicopathologic study demonstrated that the frequency of obesity-related glomerulopathy, which is characterized by focal segmental glomerulosclerosis and glomerulomegaly, increased from 0.2% to 2% in the patients who received renal biopsy during 15 years of the study period.[35] This glomerulopathy may also contribute to the renal dysfunction.

Potential Strategies for Preventing Renal Impairment in the MetS

The observed association between the MetS and the risk of developing CKD raises the question of whether correcting 1 or more of the syndrome's features may effectively prevent CKD. It has been shown that intensive blood pressure and blood glucose control effectively prevents the development of microalbuminuria and CKD in diabetic patients.[36] Furthermore, the nephroprotective effects of angiotensin-converting enzyme inhibitors have been suggested by a number of clinical trials involving diabetic and nondiabetic patients.[37,38] In addition, recent studies showed the effectiveness of lipid-lowering treatments in decreasing albuminuria and slowing the rate of decline in GFR in patients with CKD.[39,40] There are no clinical trials, however, that address whether treating MetS components will reduce the risk of development or progression of renal impairment; neither are there trials that define optimum target levels of treated components in these settings. Finally, although there is no doubt that all obese subjects should be encouraged to undertake physical activity and change their eating habits, further studies should address whether weight reduction, exercise, and other measures to increase insulin sensitivity as well as interventions that directly target biochemical MetS components, may reduce the risk for microalbuminuria or CKD. Until such trials are conducted, the only available preventive strategy consists of considering patients with the MetS as a subset of patients who are at high risk for developing microalbuminuria or CKD and who therefore require close monitoring to ensure the early recognition of subsequent renal impairment and aggressive treatment of all metabolic alterations.

Conclusions

A close association has been found between the MetS and the risk of developing renal impairment, clinically expressed in the form of microalbuminuria or CKD. This finding raises a major clinical and public health concern because both the MetS and CKD are increasingly common disorders. Several potential mechanisms, including insulin resistance, renal atherosclerosis, or inflammatory mediators, induce renal injury, resulting in the deterioration of renal function.

Despite the close association between the MetS and renal impairment, it is still unclear whether and to what extent treating patients with the MetS will prevent the development and progression of CKD. A clinical trial is needed to clarify whether the effect of preventing and treating MetS components will result in improved renal prognosis.

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Altered Expression of COX-2 in Subdivisions of the Hippocampus during Aging and in Alzheimer's Disease: The Hisayama Study

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Key Words
Cyclooxygenase · Alzheimer's disease · Hippocampus

Abstract
Background: It has been reported that nonsteroidal anti-inflammatory drugs may delay the onset of Alzheimer's disease (AD). Since nonsteroidal anti-inflammatory drugs inhibit cyclooxygenase (COX), COX-2, an inducible form of COX, may be involved in the pathology of AD in association with the arachidonic acid cascade. In addition, it has been suggested that alterations in the balance of polyunsaturated fatty acids are associated with brain dysfunctions such as neurodegerative pathologies of the aging brain. ***Method:*** To explore COX-2 expression in the hippocampus, we analyzed 45 consecutive autopsy subjects without dementia and 25 AD patients derived from the town of Hisayama, Japan. ***Results:*** The neuronal expression of COX-2 in the CA3 subdivision of the hippocampus, subiculum, entorhinal cortex and transentorhinal cortex were consistently observed in both nondemented and AD brains, and COX-2 immunoreactivity correlated with age in nondemented brains. In AD patients, neurons of CA1 exhibited increased COX-2 immunoreactivity which correlated with the severity of AD pathology. This correlation was not apparent in nondemented subjects. ***Conclusion:*** These results suggest that COX-2 expression may be differentially regulated among subdivisions of the hippocampus and that elevated COX-2 expression in the CA1 of AD brains may be associated with AD pathology and thus cognitive dysfunction.

Introduction

Many epidemiological studies suggest that the use of nonsteroidal anti-inflammatory drugs delays or slows the clinical expression of Alzheimer's disease (AD) [1, 2]. The mechanism by which these drugs might affect pathophysiological processes relevant to AD remains unclear. Most nonsteroidal anti-inflammatory drugs have an inhibitory effect on cyclooxygenase (COX), an enzyme involved in the metabolism of arachidonic acid into prostanoids. There are two major known COX isoforms, the constitutively expressed COX-1 and the mitogen-inducible COX-2 [3]. While COX-1 is mainly expressed in microglia and some neuronal cells throughout the brain, COX-2 is expressed in neurons [4, 5]. It has been suggested that alterations in the balance of polyunsaturated fatty acids, including arachidonic acid and its metabolites, in the central nervous system are associated with brain dysfunction, such as in neurodegenerative pathologies of the aging

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brain [6]. Following on these epidemiological reports, several histological analyses of COX-2 expression in AD brains have been conducted [4, 7–11], but have produced conflicting results. Several studies reported increased neuronal COX-2 immunoreactivity compared to control brain tissues [5, 7]. However, in other studies, in which COX-2 expression was related to specific hallmarks of the disease, such as clinical dementia rating and Braak stage of disease, the number of COX-2-positive neurons decreased with the severity of dementia, and in the end-stage AD, COX-2-positive neurons were significantly fewer than in nondemented controls [4, 11].

Although many studies have been conducted concerning COX-2 expression not only in AD brains, but also in Parkinson disease model mice brains [12], amyotrophic lateral sclerosis brains [13] and schizophrenia brains [14], the histological analyses concerning COX-2 expression in nondemented brains are few. Now that we know that COX-2 expresses constitutively in the brain even under normal conditions [15], it is important to explore the normal COX-2 expression pattern in the brain. Without this basic knowledge, it is difficult to interpret the conflicting results of COX-2 expression in AD brains.

In this study, we investigated the neuronal expression of COX-2 in some subdivisions of hippocampi of consecutive autopsy cases without dementia. In addition, we quantified senile plaque (SP) and neurofibrillary tangle (NFT) density to assess the influence of AD pathology on neuronal COX-2 expression, and explored any differences in the pattern of COX-2 expression in these regions between nondemented subjects and AD patients.

Materials and Methods

Subjects

To minimize the selection bias of the nondemented subjects, we collected the nondemented subjects from the series of consecutive autopsy cases in the Hisayama study. The Hisayama study is a prospective population-based study in the subrural community of Hisayama, which is adjacent to the metropolitan area of Fukuoka on Kyushu Island, Japan, and it started in 1961 [16–19]. We have carried out autopsies on most deceased subjects from this region in order to confirm the cause of death and to examine brain pathology. This paradigm has allowed a reliable recruitment of nondemented subjects in which to analyze the neuronal expression of COX-2. The diagnosis of dementia was based on the guidelines of the Diagnostic and Statistical Manual of Mental Disorders, Revised Third Edition [20]. For the clinical diagnosis of AD, we used the guidelines of the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association [21].

From October 1, 1998, to March 31, 2001, 148 Hisayama residents of varying initial ages died, 105 of whom (70.5%) underwent a postmortem examination. Consent to autopsy was unobtainable from 29.5% of the residents due to refusal, mainly on religious grounds. Of those 105 cases, 103 subjects received autopsies at the Departments of Pathophysiological and Experimental Pathology, Anatomic Pathology and Neuropathology of Kyushu University. In order to collect consecutive autopsy cases without dementia, we excluded 58 cases that were clinically diagnosed as exhibiting dementia or had some disease or condition that might influence the expression of COX-2 in the brain, such as severe chronic hepatic failure, autoimmune disease, disseminated intravascular coagulation, systemic inflammatory response syndrome, acute brain infarction, brain infection or a brain tumor. In total, 45 cases were analyzed in study A as nondemented subjects. In study B, in order to compare the nondemented subjects with AD patients, we examined all of the nondemented subjects aged 76 years or more at death of the nondemented subjects of study A and age- and sex-matched AD autopsy cases derived from Hisayama Town as the comparison group. In total, 25 nondemented subjects and 25 AD patients were analyzed in study B. We examined only cases aged 76 or more because there were few AD patients younger than 75 in the Hisayama study.

Neuropathological Assessment

Brains were weighed, evaluated for gross detectable lesions, abnormalities of the blood vessels and were fixed with 10% buffered formalin for at least 2 weeks. Brain specimens were taken following the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) guidelines and the consensus guidelines for dementia with Lewy bodies [22, 23]. Thus, the specimens in each case included the middle frontal gyrus, superior and middle temporal gyri, inferior parietal lobule, anterior cingulate gyrus, hippocampus with entorhinal cortex and transentorhinal cortex (at the level of the lateral geniculate body), calcarine cortex, basal ganglia, thalamus, substantia nigra, locus coeruleus and dorsal vagal nucleus. Samples were embedded in paraffin and cut into sections which were routinely stained using hematoxylin-eosin and a modified Bielschowsky method. Each case was also immunostained with anti-tau (polyclonal, rabbit, 1:200, Dako, Denmark), anti-ubiquitin (polyclonal, rabbit, 1:100, Dako) and anti-α-synuclein (LB509: monoclonal, mouse, 1:100, provided by Dr. Iwatubo) [24]. Immunolabeling was detected using a standard indirect immunoperoxidase method and visualized with diaminobenzidine (Dojindo, Japan). The sections were lightly counterstained with hematoxylin.

Assessment of AD Pathology

The presence of SPs was estimated by a modified Bielschowsky method. NFT presence was assessed by tau immunohistochemistry. In each case, the frequency of SPs and NFTs were evaluated and converted to a plaque score according to CERAD criteria and Braak stage for tau pathology as established by Braak and Braak [22, 25]. The CERAD score and Braak stage were combined to estimate the likelihood of AD according to the NIA-RI criteria [26]. A diagnosis of AD was made when 'definite AD' as defined by the CERAD criteria and/or a 'high-likelihood' as defined by the NIA-RI criteria were found.

In addition, SP and NFT levels in the CA1 subdivision of the hippocampus were assessed. The semiquantitative density of SPs in

CA1 was determined as being either none, sparse, moderate, or frequent, according to the guidelines established by CERAD. NFTs in CA1 were counted in 100× fields at each of three locations and the average was expressed as the NFT number per 100× field.

Immunohistochemistry and Assessment of COX-2

Immunohistochemistry was performed on 7-μm paraffin-embedded sections encompassing the hippocampus, entorhinal cortex and transentorhinal cortex (at the level of the lateral geniculate body). Sections were deparaffinized in xylene, hydrated in an ascending ethanol series and incubated in 0.3% hydrogen peroxide in absolute methanol for 30 min at room temperature to inhibit endogenous peroxidase activity. After rinsing with tap water, the sections were pretreated with 90% formic acid for 10 min and autoclaved at 121°C for 10 min in 0.01 M citrate buffer, pH 6.0, in order to enhance immunoreactivity. After washing with Tris-HCl buffer (50 mM Tris-HCl, pH 7.6), an anti-human COX-2 (polyclonal, rabbit, 1:100, Cayman Chemical Co.) was applied. The slides were incubated overnight at 4°C and then sequentially incubated for 1 h with a biotinylated secondary antibody diluted 1:200, and a peroxidase-conjugated streptavidin-biotin complex diluted 1:100 sequentially (Amersham, UK). The colored reaction product was developed with 3,3′-diaminobenzidine tetrahydrochloride solution. The sections were then lightly counterstained with hematoxylin.

For analysis of neuronal COX-2 immunoreactivity, the mean gray values of a random-selected 5 neurons and 5 neuropil backgrounds were quantified using ImageJ 1.36b (National Institute of Health, USA) and the average was calculated. Then, we converted the mean gray value into a density using the following equation; uncalibrated density = $\log_{10}$ (255/the mean gray value). Finally, we calculated the index that the neuronal density was divided by the neuropil background density, and we considered this index as the neuronal immunostaining density of COX-2. This index was calculated in CA1, CA3, subiculum entorhinal cortex and transentorhinal cortex.

The investigator was blind to the diagnosis of each case until analysis was completed and values were assigned to each specimen.

Statistical Methods

The quantitative data obtained were compared between the groups by Mann-Whitney's U test. Statistical significance was defined as $p < 0.05$. Correlation analysis was done using the Pearson parametric and Spearman nonparametric methods.

Results

Study A

Clinico-Neuropathological Information of Nondemented Subjects

The number of the nondemented subjects was 45 (M/F: 28/17). The ages at death were between 40 and 95 years and mean age at death was 76.4 ± 12.1 years. The brain weight was 1,264.0 ± 166.8 g (mean ± SD) and postmortem time was 13.1 ± 9.3 h (mean ± SD).

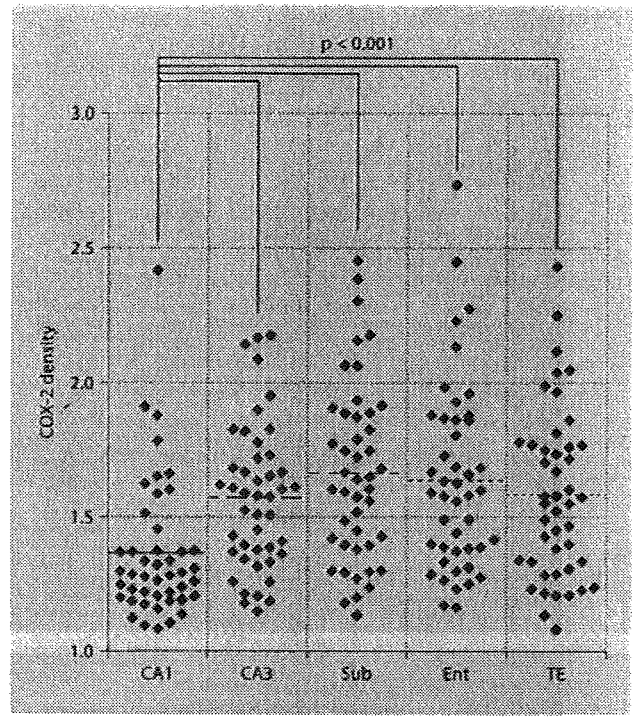

Fig. 1. Degrees of COX-2 immunoreactivity in different hippocampal subdivisions of nondemented subjects. Immunoreactivity is weak in CA1 as compared to all of the other fields examined with high statistical significance (Mann-Whitney U test, $p < 0.001$). Bars represent the mean density of neurons in each area. Sub = Subiculum; Ent = entorhinal cortex; TE = transentorhinal cortex.

COX-2 Immunoreactivity in the Hippocampus

The degree of COX-2 immunoreactivity in different hippocampal subdivisions of nondemented subjects is shown in figure 1. In nondemented subjects, the neuronal COX-2 immunoreactivity in CA3, subiculum, entorhinal cortex and transentorhinal cortex were strong (fig. 2a, d). On the other hand, the neuronal COX-2 immunoreactivity in CA1 was weak (fig. 2a, c) compared to all of the other fields examined, with high statistical significance (Mann-Whitney U test, $p < 0.001$). From these results, the constitutive expressions of COX-2 in CA3, subiculum, entorhinal cortex and transentorhinal cortex were thought to be strong, while weak in CA1.

COX-2 Immunoreactivity Correlates with Age in Nondemented Subjects

The correlation between neuronal COX-2 immunoreactivity and age is shown in figure 3. In the CA3 subdivi-

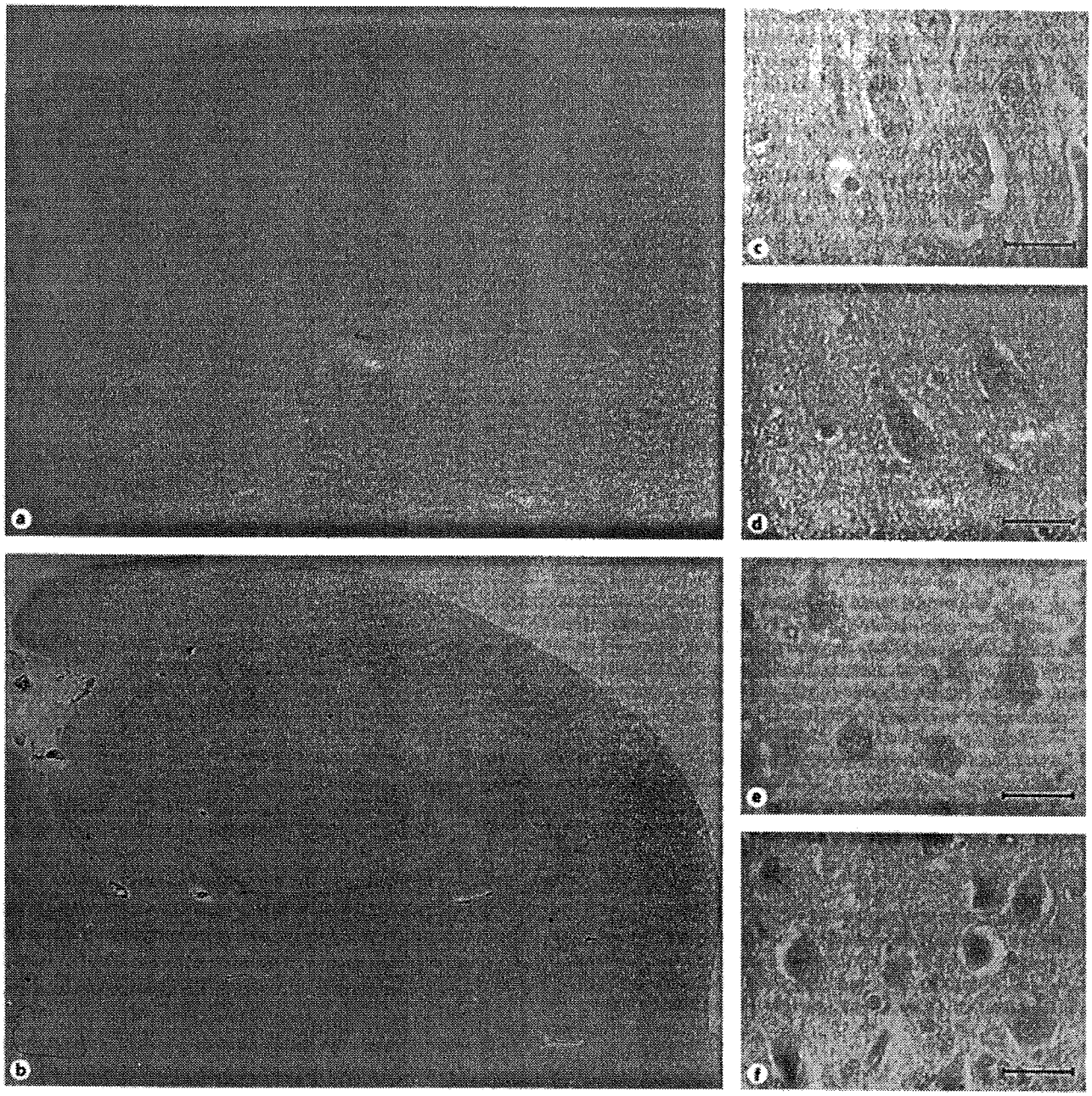

Fig. 2. COX-2 immunostaining in pyramidal neurons of the hippocampal formation in the brains of nondemented subjects (**a**, **c**, **d**) and in AD patients (**b**, **e**, **f**). **a**, **b** CA1–CA4 fields under low power. **c**, **e** Immunostaining within the CA1 field. **d**, **f** Immunostaining within CA3. Neuronal expression of COX-2 in CA3 is widely seen among both nondemented subjects and AD patients. Although neuronal expression of COX-2 in CA1 is widespread in AD patients, it is less detectable among nondemented subjects. **c–f** Bars are 30 μm.