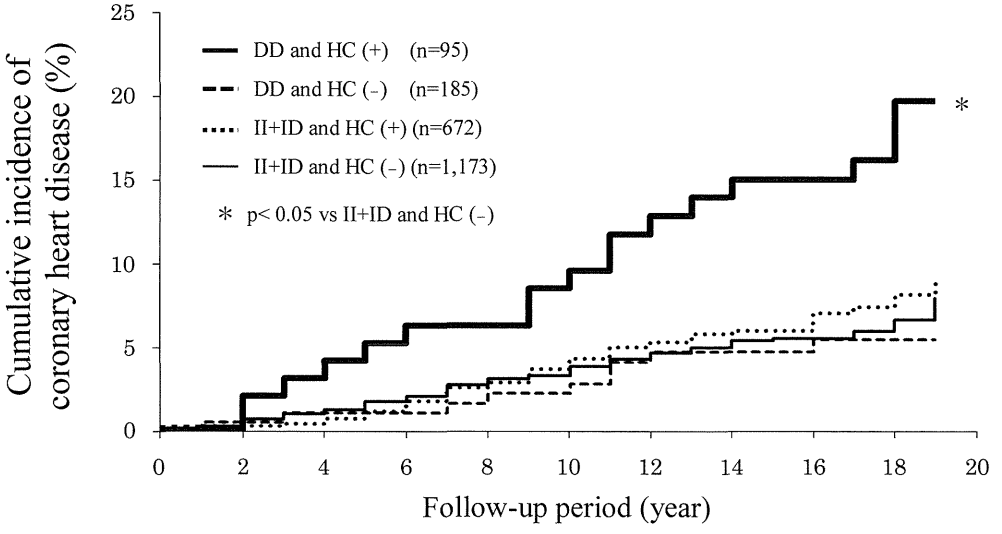

Fig. 2. Cumulative incidence of coronary heart disease according to the ACE genotype and cholesterol levels in the 2,125 subjects during the 19-year follow-up period.

ACE, angiotensin I-converting enzyme; HC, hypercholesterolemia
*$p < 0.05$ vs. II + ID and HC(−)

quently, the findings were not altered substantially (**Supplementary Table 1** and **Supplementary Fig. 3**).

Discussion

In the present study, there was no evidence of a significant association between the I/D polymorphism of the ACE gene and the risk of CHD. However, the magnitude of the effect of hypercholesterolemia on the risk of incident CHD was greater in the subjects with the DD genotype than those with the II + ID genotypes. To our knowledge, this is the first population-based prospective study to assess the interaction between the ACE polymorphism and hypercholesterolemia in patients with incident CHD.

Since the first report by Cambien *et al.* published in 1992[7], the DD genotype has been investigated as a potential CHD risk factor[8]. However, large cohort studies[9, 10] and subsequent meta-analyses have failed to confirm this association[28, 29]. In addition, both studies supporting these findings as well as questioning the veracity of the association have been published, resulting in uncertainty regarding the importance of this polymorphism. The present study also failed to confirm a significant association between the DD genotype of the ACE gene and an increased risk of incident CHD. Therefore, it may be said that there is no strong evidence to date to indicate that the DD genotype of the ACE gene is a significant risk factor for the development of CHD. This finding may reflect the relatively modest influence of the I/D polymorphism of the ACE gene itself on the risk of CHD.

In the present study, the observed differences in the magnitude of the association between hypercholesterolemia and the risk of CHD between the ACE genotypes were unexpected and may have been due to chance. However, we believe that these results represent a real difference, since similar heterogeneity of influence was observed in the analysis using the serum cholesterol level as a continuous variable. Several epidemiological studies have also demonstrated an interaction between the I/D polymorphism of the ACE gene and metabolic risk factors on the risk of CHD[11-14]. In addition, the results of a clinical trial conducted among 429 patients with coronary atherosclerotic lesions and hypercholesterolemia showed that the administration of cholesterol-lowering therapy with statins achieved a greater reduction in the plasma cholesterol levels and exhibited more significant preventive effects against the progression of coronary atherosclerosis in the subjects with the DD genotype than in those with the II and ID genotypes, suggesting that the I/D polymorphism of the ACE gene modifies the effectiveness of cholesterol-lowering therapy in ameliorating the risk of CHD[15]. On the other hand, recent findings concerning the influence of the ACE genotype with respect to the effects of cholesterol-lowering therapy on the risk of CHD are diverse[30, 31]. Therefore, further validation studies are needed to clarify this issue.

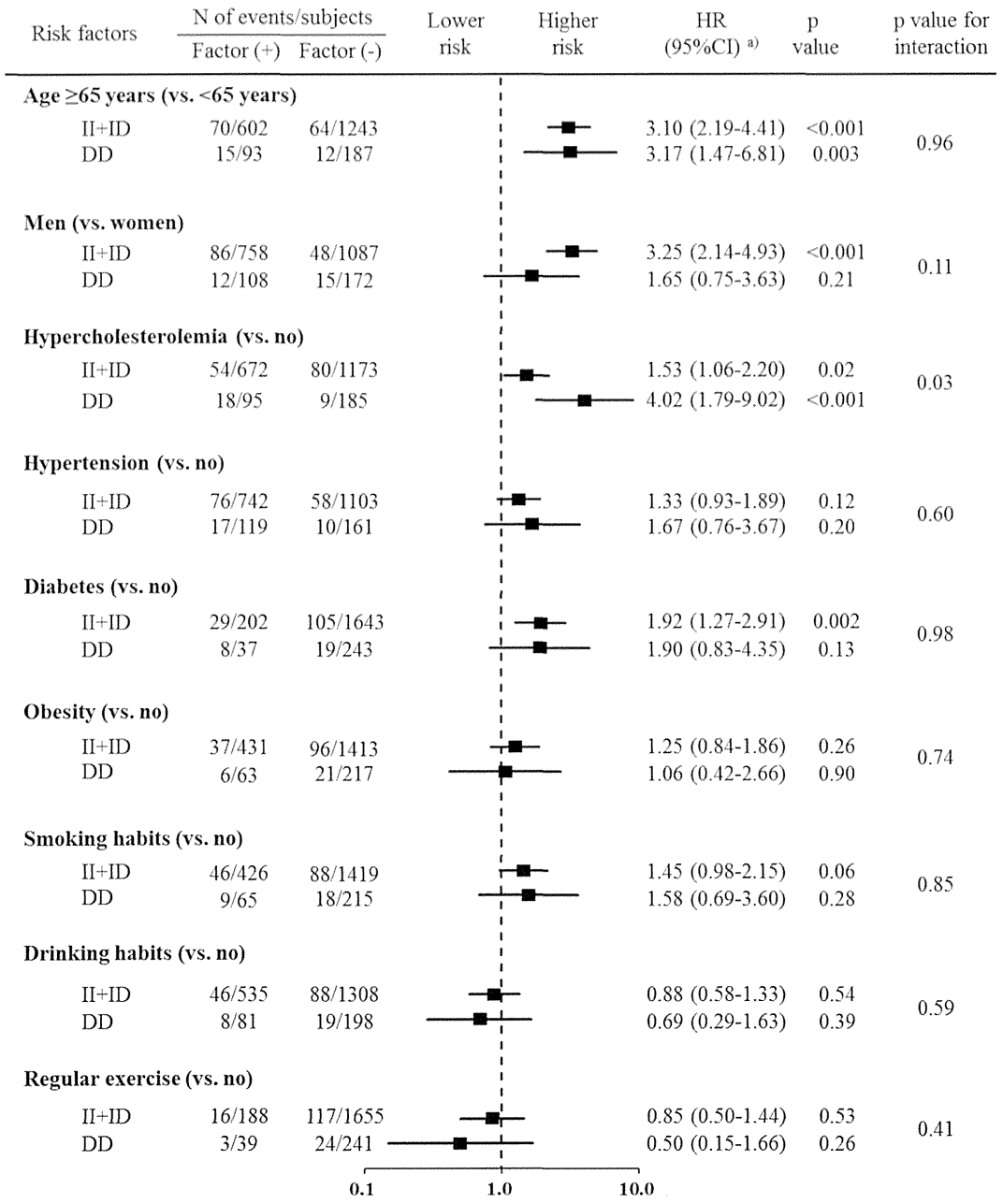

Fig. 3. Comparison of the effects of traditional cardiovascular risk factors on the development of coronary heart disease between the subgroups of the ACE DD/ID + II genotypes.

a) The risk estimates were adjusted for age, sex, hypertension, diabetes, hypercholesterolemia, obesity, electrocardiogram abnormalities, smoking, alcohol intake and regular exercise. The variable relevant to the subgroup was excluded from each model.
ACE, angiotensin I-converting enzyme; CI, confidence interval

Existing evidence does not indicate a clear biologically plausible mechanism explaining the association observed in this study. However, several experimental studies have indicated that there is an interaction between the products of the ACE enzymatic activity (e.g., angiotensin II and bradykinin) and low-density lipoprotein (LDL) cholesterol peroxidation[32-35]. Under hyperlipidemic conditions, angiotensin II has been found to promote the oxidation of LDL cholesterol and upregulation of its receptor and lectin-like

oxidized LDL receptor-1 (LOX-1) in human coronary artery endothelial cells[32]. At the same time, oxidized LDL cholesterol increases the angiotensin II expression[33] and angiotensin II type 1 receptor expression[34] and decreases the vasodilator reactivity of bradykinin *in vitro*[35]. This vicious feedback cycle may contribute to the initiation and progression of atherosclerosis. Since ACE is a key factor in the regulation of vasoconstrictor angiotensin II and vasodilator bradykinin and the D polymorphism of the ACE gene increases the ACE activity in the plasma[6] and inflammatory cells[36], it is reasonable to suppose that the differences in the plasma and local tissue concentrations of the products of the ACE activity among ACE genotypes affect LDL cholesterol peroxidation, endothelial-cell LDL uptake[37], the foam cell activity[38] and thus the subsequent severity of coronary atherosclerosis[39]. Therefore, the D polymorphism may enhance the effect of hypercholesterolemia on the risk of incident CHD. The strengths of this study include the longitudinal population-based design, long duration of follow-up and accuracy of the diagnosis of incident CHD. However, some limitations should also be noted. First, there may have been selection bias in the study population. Compared with the 2,125 study subjects for whom genotype information was available, the 509 subjects excluded from the study for whom no DNA samples were available were younger (56 versus 60 years of age, $p<0.01$) and included a greater proportion of men (45.0% versus 40.8%, $p=0.02$). When adjustments were made for age and sex, the subjects without genomic information had a greater incidence of prevalent diabetes (17.1% versus 11.2%, $p=0.03$) and hypertension (49.8% versus 40.5%, $p<0.01$). However, no significant differences were observed between the two groups in terms of the other risk factors, including the serum total cholesterol levels (5.29 versus 5.36, $p=0.6$) and incidence of CHD (5.1 versus 5.7 per 1,000 person-years, $p=0.7$). Second, the present analysis lacked information on medications, such as statins and ACE inhibitors, that affect cholesterol metabolism and the ACE activity. Importantly, these medications were rarely used in Japan in the 1980's and the early 1990's, and this limitation thus does not appear to have the potential to alter our findings.

In conclusion, in this prospective population-based study, we found that the DD genotype increases the effects of hypercholesterolemia on the occurrence of CHD. This knowledge may lead to the application of non-invasive screening for individuals at high risk for CHD, an additional means of preventing the premature development of CHD, and development of targeted interventional strategies based on each subject's ACE genotype, thereby dramatically reducing both the incidence and lethality of this disease. The current findings indicate that strict and immediate management of hypercholesterolemia should be considered, especially in subjects with the DD genotype, as an effective means of preventing the development of CHD in Japan, where the prevalence of hypercholesterolemia has increased rapidly in recent years.

Acknowledgements

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Disclosures

There are no conflicts of interest to declare with respect to this study.

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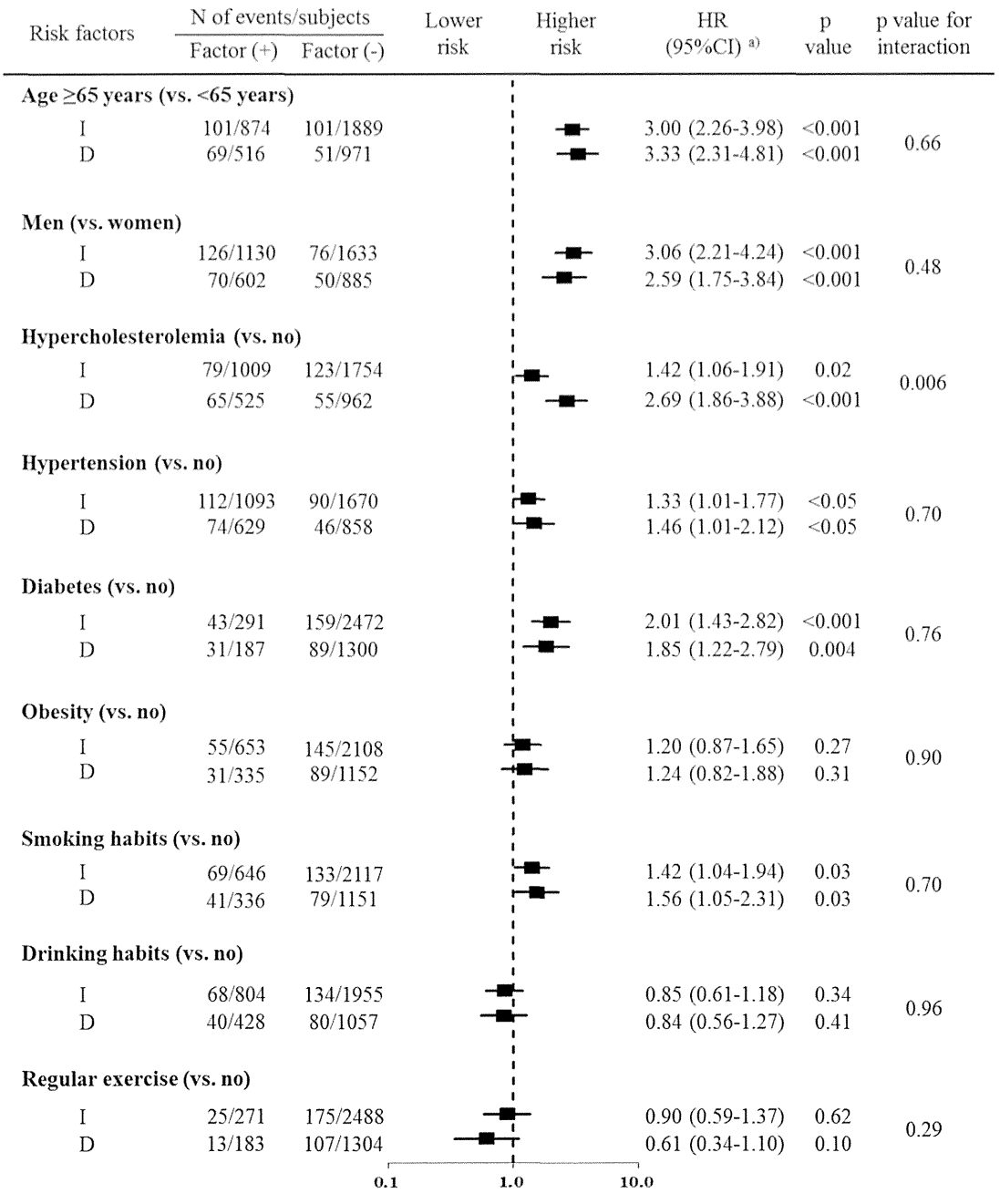
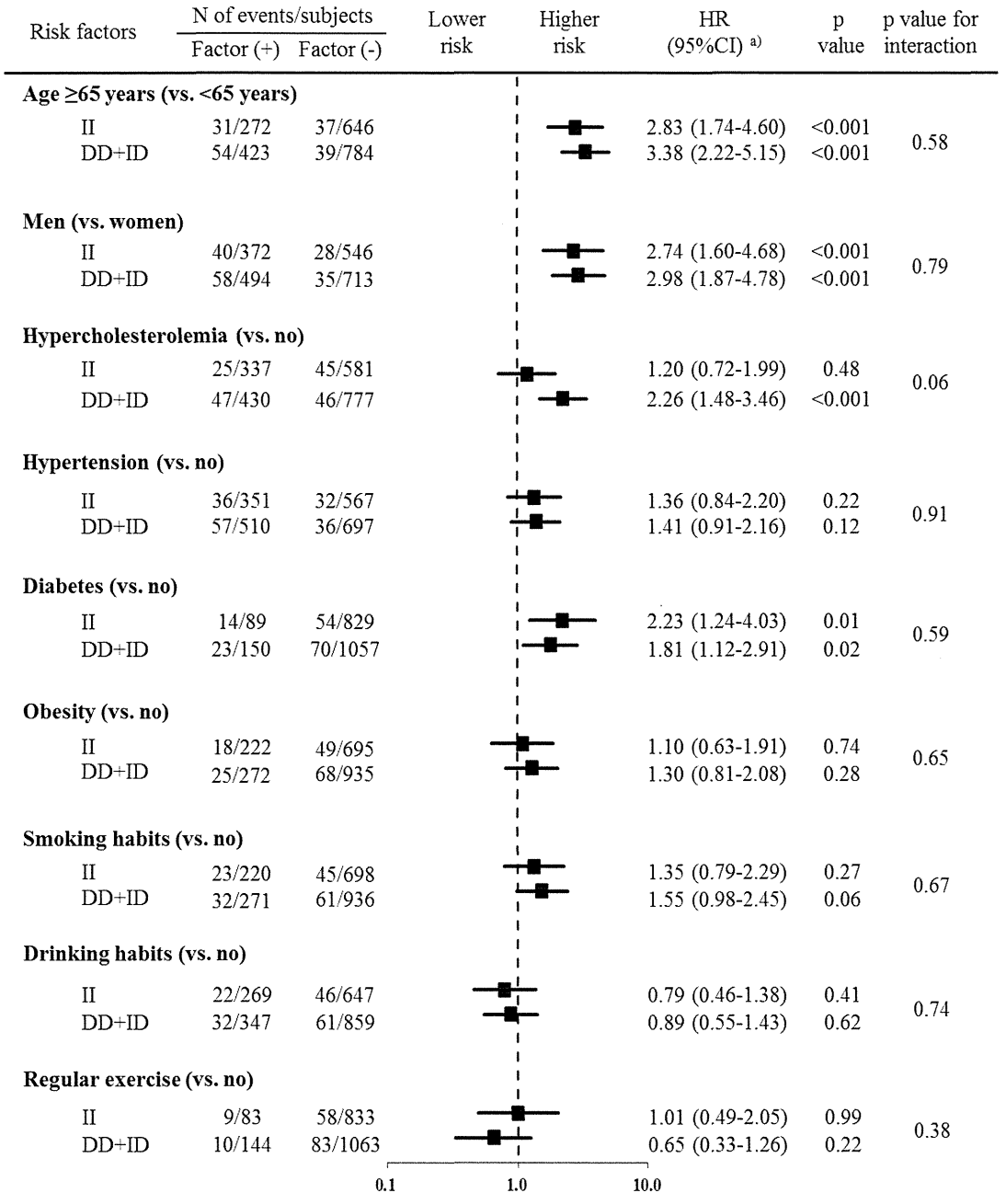


Supplementary Fig. 1.
Comparison of the effects of traditional cardiovascular risk factors on the development of coronary heart disease between the subgroups of the ACE D/I allele.

In the analysis of the I/D allele, the number of subjects and events was counted twice.
a) The risk estimates were adjusted for age, sex, hypertension, diabetes, hypercholesterolemia, obesity, electrocardiogram abnormalities, smoking, alcohol intake and regular exercise. The variable relevant to the subgroup was excluded from each model.
ACE, angiotensin I-converting enzyme; CI, confidence interval



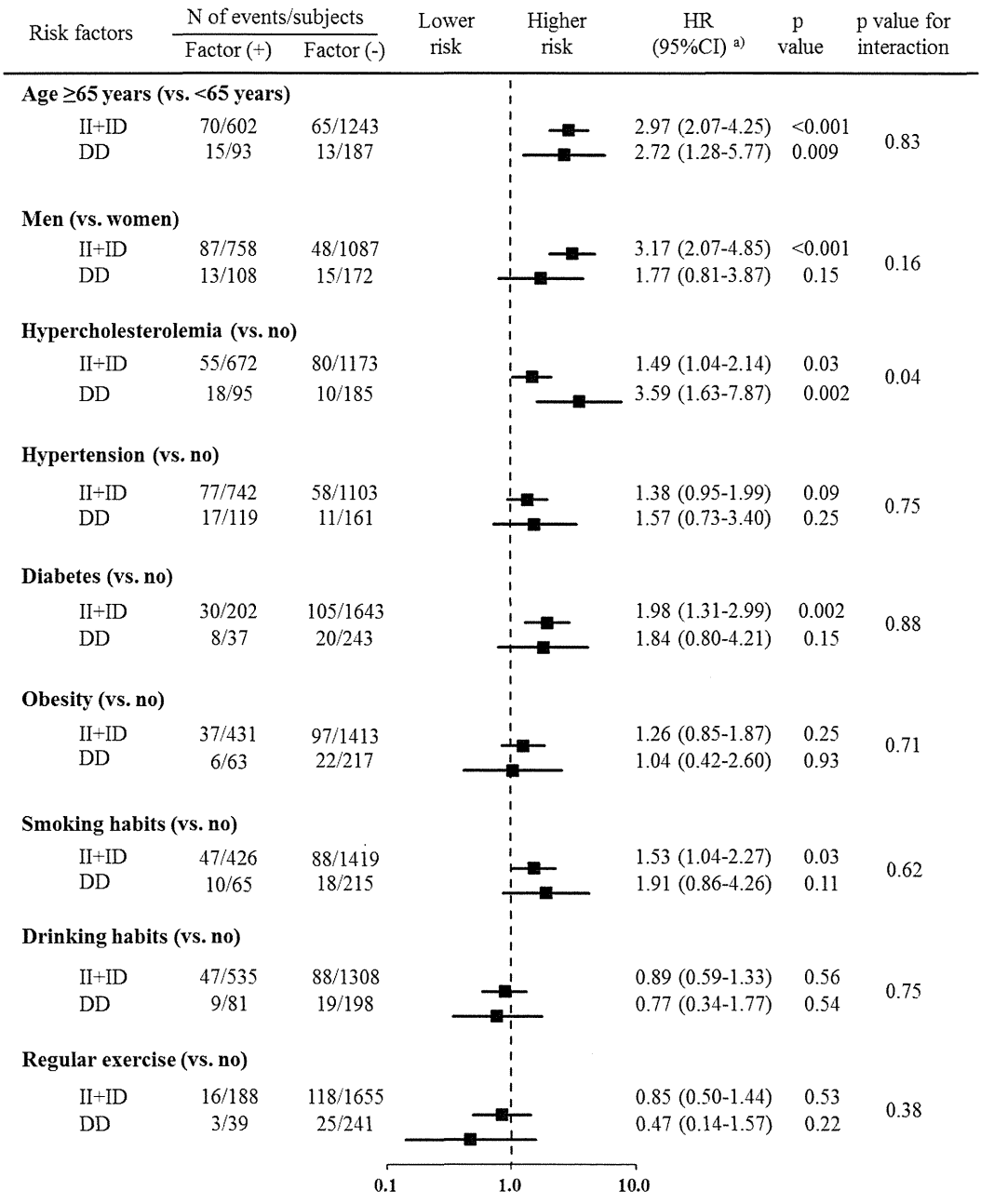
Supplementary Fig. 2.
Comparison of the effects of traditional cardiovascular risk factors on the development of coronary heart disease between the subgroups of the ACE DD + ID/II genotypes.

a) The risk estimates were adjusted for age, sex, hypertension, diabetes, hypercholesterolemia, obesity, electrocardiogram abnormalities, smoking, alcohol intake and regular exercise. The variable relevant to the subgroup was excluded from each model.
ACE, angiotensin I-converting enzyme; CI, confidence interval

Supplementary Table 1.
Age- and sex-adjusted incidence and adjusted HR for coronary heart disease according to the ACE genotype in the 2,125 subjects during the 19-year follow-up period

ACE genotype	Person-years at risk	No. of events	Age- and sex-adjusted incidence (per 1,000 PYs)	Age- and sex-adjusted		Multivariable-adjusted[a)]		ACE genotype and interaction	Multivariable-adjusted[a)]	
				HR (95% CI)	*p* value	HR (95% CI)	*p* value		HR (95% CI)	*p* value
II	14,890	69	5.9	1.00 (reference)		1.00 (reference)		II	1.00 (reference)	
ID	14,963	66	5.2	0.84 (0.60-1.18)	0.31	0.83 (0.59-1.17)	0.29	ID	0.70 (0.45-1.10)	0.12
DD	4,507	28	7.1	1.21 (0.78-1.88)	0.39	1.18 (0.76-1.84)	0.46	DD	0.68 (0.34-1.36)	0.28
								INT (ID x HC)	1.50 (0.75-2.99)	0.25
								INT (DD x HC)	2.95 (1.18-7.37)	0.03
II + ID	29,853	135	5.5	1.00 (reference)		1.00 (reference)		II + ID	1.00 (reference)	
DD	4,507	28	7.1	1.33 (0.88-1.99)	0.17	1.30 (0.87-1.96)	0.21	DD	0.82 (0.42-1.58)	0.55
								INT (DD x HC)	2.40 (1.03-5.61)	0.04
II	14,890	69	5.9	1.00 (reference)		1.00 (reference)		II	1.00 (reference)	
DD + ID	19,470	94	5.6	0.92 (0.68-1.26)	0.62	0.91 (0.67-1.25)	0.57	DD + ID	0.70 (0.46-1.06)	0.09
								INT (DD + ID x HC)	1.84 (0.98-3.48)	0.06
I	44,743	204	5.6	1.00 (reference)		1.00 (reference)		I	1.00 (reference)	
D	23,977	122	5.9	1.04 (0.83-1.30)	0.75	1.03 (0.82-1.29)	0.81	D	0.79 (0.57-1.08)	0.13
								INT (D x HC)	1.79 (1.14-2.82)	0.02

ACE, angiotensin I-converting enzyme; PYs, person-years; HR, hazard ratio; CI, confidence interval; HC, hypercholesterolemia; INT, interaction term
In the analysis of the I/D allele, the number of subjects and events was counted twice.
a) The risk estimates were adjusted for age, sex, hypertension, diabetes, hypercholesterolemia, obesity, electrocardiogram abnormalities, smoking, alcohol intake and regular exercise.



Supplementary Fig. 3.
Sensitivity analysis of the data for sudden cardiac death within 24 hours to compare the effects of traditional cardiovascular risk factors on the development of coronary heart disease between the subgroups of the ACE DD/ID + II genotypes.

a) The risk estimates were adjusted for age, sex, hypertension, diabetes, hypercholesterolemia, obesity, electrocardiogram abnormalities, smoking, alcohol intake and regular exercise. The variable relevant to the subgroup was excluded from each model.
ACE, angiotensin I-converting enzyme; CI, confidence interval

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ORIGINAL ARTICLE

Prognostic impact of serum bilirubin level on long-term renal survival in IgA nephropathy

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Abstract
Background Serum bilirubin has been recognized as a novel endogenous antioxidant. The aim of our study was to evaluate the impact of serum bilirubin on kidney prognosis in IgA nephropathy (IgAN).
Methods We followed retrospectively 694 patients with IgAN diagnosed by renal biopsy between 1982 and 2010. The risk factors for developing end-stage renal disease (ESRD) were estimated using a Cox proportional hazard model. Predictive performance between models with or without serum bilirubin was evaluated by calculating the net reclassification improvement (NRI) and integrated discrimination improvement (IDI).
Results Seventy-seven patients developed ESRD during the median 4.9 years of follow-up. Estimated glomerular filtration rate, proteinuria and histological severity were inversely related to bilirubin levels. In multivariate analysis, serum bilirubin was an independent risk factor for ESRD (hazard ratio for every 0.1 mg/dL decrease in serum bilirubin, 1.18; 95 % CI, 1.04–1.33). The incidence rate of ESRD decreased linearly with the increases in bilirubin levels (P for trend <0.01). When bilirubin was incorporated into a model with conventional ESRD risk factors, the NRI and IDI were 0.281 ($P = 0.02$) and 0.019 ($P = 0.01$), respectively.
Conclusions We demonstrated that lower bilirubin levels were significantly associated with higher risk of ESRD in IgAN. In addition, bilirubin provided incremental predictive value in the risk assessment for progression of IgAN beyond that provided by standard risk factors.

Keywords IgA nephropathy · Bilirubin · Oxidative stress · Risk reclassification

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Introduction

The long-term kidney prognosis in patients with immunoglobulin A nephropathy (IgAN) is thought to be poor [1]. Therefore, the identification of predictive factors for future end-stage renal disease (ESRD) at the time of biopsy remains relevant in patients with IgAN today. Numerous epidemiological studies have identified independent risk factors for the development of ESRD, such as blood pressure [2], urinary protein excretion [3], reduced kidney function [4], and histopathological findings [5, 6] in patients with IgAN. However, such conditions as heavy proteinuria, reduced estimated glomerular filtration rate (eGFR) and severe histological grade might merely reflect the advanced kidney injury already existing at presentation rather than underlying active inflammatory insults to the kidney. To detect the progression of the disease in the earlier stages of glomerular damage, the identification of prognostic factors relevant to the pathophysiological mechanism is of great interest in predicting the kidney outcome subsequent from the time of biopsy.

Recently, serum bilirubin has been recognized as a novel endogenous anti-inflammatory and antioxidant molecule [7]. Much recent research has focused on the inverse relationships between serum bilirubin level and

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various atherosclerotic diseases such as coronary artery disease [8], peripheral arterial disease [9], stroke [10] and diabetic complications [11, 12]. These previous findings suggest that a higher serum bilirubin level may protect against atherosclerosis via antioxidant and anti-inflammatory effects. Oxidative stress and inflammation may also play important roles in the progression of glomerulonephritis and glomerulosclerosis in patients with IgAN [13]. Recent studies have provided several lines of evidence that increased oxidative stress is present in the renal tissue of patients with IgAN as well as in their sera and/or erythrocytes, implying an imbalance between increased oxidant levels and reduced antioxidant activity [14]. In addition, atherosclerotic metabolic factors such as obesity [15], high triglycerides [16], insulin resistance [17] and high uric acid levels [16] have also been associated with kidney prognosis of IgAN. Thus, analogous pathophysiological mechanisms have been proposed to participate in both glomerulosclerosis and atherosclerosis [18]. Although oxidative stress-mediated inflammation and subsequent glomerulosclerosis would play a key role in the pathophysiological mechanism for progression of IgAN [13], it remains unclear whether high serum bilirubin has an endogenous protective impact on the progression of IgAN. We hypothesized that lower serum bilirubin at the time of biopsy would be associated with higher severity of kidney injury and more incidence of ESRD independently from major established confounders for the development of ESRD. Herein, we examined the possible association of serum bilirubin with the long-term kidney outcome in a cohort of 694 patients with IgAN. We also examined whether serum bilirubin provides additional predictive information in the risk assessment beyond the established risk factors by calculating the net reclassification improvement (NRI) and integrated discrimination improvement (IDI).

Methods

Study population

We used a dataset derived from the kidney biopsy records at our institutions, made up of patients with IgAN who received a pathological diagnosis between January 1982 and December 2010. The cohort consisted of 846 subjects with primary IgAN, but not Henoch–Schönlein purpura or systemic lupus erythematosus, who were treated at one of 6 participating institutions (Kyushu University Hospital, Hamanomachi Hospital, Munakata Medical Association Hospital, Japan Seamen's Relief Association Moji Hospital, Karatsu Red Cross Hospital, and Hakujyuji Hospital). Subjects whose biopsy specimen contained <10 glomeruli ($n = 85$), subjects for whom data on one or more clinical parameters were unavailable ($n = 54$), and subjects with diabetes mellitus ($n = 13$) were excluded. Finally, 694 individuals with primary IgAN were enrolled in the present study. The patients were followed until December 31, 2012. The present study was performed with the approval of the Clinical Research Ethics Committee of the Kyushu University Institutional Review Board (approval number 469-04).

Clinical parameters

In this analysis, we reviewed medical records at the time of kidney biopsy to define potential confounders, including age, sex, blood pressure, cholesterol levels, triglycerides, serum creatinine, and 24-hour urinary protein excretion or urinary protein–creatinine ratio. Hypertension was defined as blood pressure ≥140/90 mmHg and/or current use of antihypertensive agents. Dyslipidemia was defined as serum total cholesterol ≥220 mg/dL and/or serum triglycerides ≥150 mg/dL. Total cholesterol concentrations and triglycerides were determined enzymatically. Hypoalbuminemia was defined as serum albumin <4.0 g/dL. Serum creatinine was measured by Jaffe's method until April 1988, and by the enzymatic method from May 1988 at Kyushu University. At the other participating institutions, serum creatinine was measured by Jaffe's method until December 2000, and by the enzymatic method from January 2001. We converted serum creatinine values measured by Jaffe's method to values for the enzymatic method by subtracting 0.207 mg/dL [19]. eGFR was calculated using the Schwartz formula in patients under the age of 18 and the following formula in patients over the age of 18: eGFR (mL/min per 1.73 m^2) = 194 × $Cr^{-1.094}$ × $Age^{-0.287}$ (if female, ×0.739) [20–22]. Persistent proteinuria was defined as a protein excretion ≥1.0 g/24 h at the time of biopsy [23]. Anemia was defined as hemoglobin level <13.0 g/dL in men and <12.0 g/dL in women. The use of an angiotensin-converting enzyme inhibitors (ACEIs), angiotensin receptor blockers (ARBs) or steroid for at least 6 months was defined as treatment.

Pathologic parameters

We evaluated pathologic lesions according to the Oxford classification [24, 25]. The mesangial hypercellularity score (M) was defined as M0 if the score was ≤0.5 and M1 if the score was >0.5. Endocapillary hypercellularity (E), segmental glomerulosclerosis (S) and extracapillary proliferation (Ex) were, respectively, expressed as E0, S0 and Ex0 if absent and E1, S1 and Ex1 if present. Tuft adhesions were classified as S1 lesions. Tubular atrophy/interstitial fibrosis (T) was semiquantitatively classified according to

the ratio of the cortical area involved with the tubular atrophy or interstitial fibrosis: T0, if 0–25 %; T1, if 26–50 %; and T2, if >50 %, respectively. Arterial lesions are scored by comparing thickness of intima to that of media (0, if no intimal thickening; 1, if intima thickened and < thickness of media; and 2, if intima thickened and > thickness of media), separately in the segment of vessel: interlobular, arcuate and larger arteries, respectively. Arteriolar hyalinosis was semiquantitatively classified as the proportion of arterioles affected (0, 1–25 %, 26–50 % and >50 %) [25].

Renal outcome

The primary endpoint was incidence of ESRD. ESRD was defined as the initiation of renal replacement therapy, including hemodialysis, peritoneal dialysis and kidney transplantation. The kidney outcomes were surveyed by reviewing the medical records or by telephone consultation with the clinics and hospitals where the patients visited or with the patients themselves.

Statistical analysis

The patients were divided into quintile groups (<0.40, 0.40–0.50, 0.51–0.66, 0.67–0.80, and >0.80 mg/dL), and the linear trends in the mean values and frequencies of covariates across quintiles of bilirubin levels were determined using a linear regression or logistic regression model. Age-adjusted trend was tested by the Mantel-extension test. The incidence rates of ESRD according to the quintiles of serum bilirubin levels were calculated with the person-year method. The age- and sex-adjusted and multivariate-adjusted hazard ratios (HRs) with 95 % CIs of each risk factor for the development of ESRD were calculated using a Cox proportional hazards model. Patients were censored at the date of their death, or at the end of follow-up for those still alive. The heterogeneity in the relationship between subgroups was tested by adding a multiplicative interaction term to the relevant Cox model. Predictive performance for ESRD with or without serum bilirubin was evaluated by calculating c-statistics, and the improvement in predictive accuracy was evaluated by calculating the NRI and IDI. The predicted probabilities of incident ESRD were classified into three categories of <3.5 %, 3.5–35 %, and ≥35 %, because the median values of the predicted probabilities among the subjects were 3.6 % in individuals without incident ESRD and 36.7 % in those with incident ESRD. Continuous NRI was calculated by taking the predicted probabilities as a continuous variable. The difference in the AUC between models was estimated using DeLong's method [26]. Statistical analyses were conducted using the SAS software package version 9.2 (SAS Institute, Cary, NC, USA) and R version 3.0.2 (http://www.r-project.org). A two-tailed value of $P < 0.05$ was considered statistically significant.

Results

The clinical characteristics of the patients according to the quintile of serum bilirubin concentration are provided in Table 1; subjects with higher bilirubin levels had a greater mean age and a higher proportion of men. The mean values of serum total cholesterol, serum triglycerides, urinary protein excretion, eGFR and hypertension frequency, and the incidence of Oxford pathological lesions with M1, S1, T2 and Ex1 were significantly lower in the population with higher bilirubin levels. The median length of follow-up was not significantly associated with the serum bilirubin quintile. The patients with higher bilirubin levels tended to have a lower prevalence of pathological lesions of types E1, but the difference was not statistically significant. During the median 4.9-year follow-up period, 77 patients (11.0 %) developed ESRD. The cumulative incidence rates of ESRD decreased linearly with higher bilirubin levels (P for trend <0.01), as shown in Fig. 1. As shown in Table 2, the age and sex-adjusted HRs for the development of ESRD increased linearly (P for trend <0.001). After adjusting for other clinical confounding factors, such as urinary protein excretion and eGFR, and the pathological parameters of the Oxford classification (M, S and T1, T2), the HRs for ESRD increased linearly as the serum bilirubin levels decreased (P for trend =0.01). Every 0.1 mg/dL decrease in bilirubin levels was associated with a 1.18-fold (95 % confidence intervals [CIs] 1.04–1.33) increased risk of ESRD after adjusting for the above-mentioned confounding factors.

To assess the consistency of the association between serum bilirubin and the development of ESRD, we also conducted subgroup analysis stratified by potential confounders and treatment assignment (Fig. 2). In the subgroup analysis, no significant interactions were detected between serum bilirubin and baseline characteristics and treatment assignment—namely, age, sex, hypoalbuminemia, anemia, hypertension, dyslipidemia, persistent proteinuria (≥1.0 g/24 h), reduced eGFR (<60 mL/min/1.73 m^2), the pathological parameters of the Oxford classification (M, S and T1, T2), extracapillary proliferation, and use of ACEIs, ARBs or steroids (all P for interaction >0.20). We also compared c-statistics between models with and without bilirubin to evaluate the possible impact of bilirubin on the risk assessment for ESRD, but there was no evidence of a significant difference in the c-statistics (0.86–0.87; $P = 0.2$). As shown in Table 3, adding serum bilirubin to the relevant model significantly improved the accuracy of the risk assessment for future ESRD: the

Table 1 Baseline characteristics according to quintile of serum bilirubin

Characteristics	Serum bilirubin levels (mg/dL)					*P* for trend
	<0.40 (n = 105)	0.40–0.50 (n = 198)	0.51–0.66 (n = 110)	0.67–0.80 (n = 154)	>0.80 (n = 127)	
Age (years)	38.1 ± 16.2	38.3 ± 16.1	35.7 ± 14.5	35.8 ± 14.7	33.2 ± 15.1	0.003
Gender (male)	35.2	46.0	50.0	48.1	57.5	0.002
Follow-up (years)	4.5 (2.1–8.4)	5.2 (2.4–9.1)	3.8 (1.2–8.0)	5.3 (1.6–12.0)	6.7 (2.0–9.9)	0.328
Mean arterial pressure, mmHg	93 ± 15	92 ± 14	92 ± 14	93 ± 14	90 ± 12	0.174
Hypertension[a]	34.6	24.8	25.7	26.3	17.5	0.009
Serum albumin (g/dL)	3.8 ± 0.59	3.9 ± 0.52	4.0 ± 0.50	4.1 ± 0.47	4.1 ± 0.47	<0.001
Serum hemoglobin (g/dL)	12.5 ± 1.93	13.9 ± 1.83	13.5 ± 1.76	13.8 ± 1.75	14.0 ± 1.66	<0.001
Serum total cholesterol (mg/dL)	207.8 ± 47.7	208.6 ± 49.3	201.0 ± 45.6	206.4 ± 55.5	189.5 ± 44.5	0.004
Serum triglycerides (mg/dL)	144.5 ± 86.7	135.3 ± 92.1	128.5 ± 83.8	115.8 ± 67.7	110.8 ± 59.4	<0.001
Urinary protein excretion (g/24 h)	1.8 ± 2.0	1.4 ± 1.6	1.1 ± 1.5	1.0 ± 1.1	0.9 ± 0.9	<0.001
eGFR (mL/min/1.73 m^2)	74.2 ± 42.8	74.5 ± 30.8	79.9 ± 28.0	84.3 ± 36.9	85.4 ± 27.9	<0.001
Pathologic parameters (oxford classification)						
Mesangial hypercellularity score						
M0 (≤0.5 of glomeruli)	80.9	87.9	88.2	90.3	89.0	0.008
M1 (>0.5 of glomeruli)	19.1	12.1	11.8	9.7	11.0	
Endocapillary hypercellularity						
E0 (absence)	58.1	64.1	61.8	69.5	63.0	0.295
E1 (presence)	41.9	35.9	38.2	30.5	37.0	
Segmental glomerulosclerosis						
S0 (absence)	20.9	26.3	23.6	34.4	30.7	0.0295
S1 (presence)	79.1	73.7	76.4	65.6	69.3	
Tubular atrophy/interstitial fibrosis						
T0 (≤25 %)	62.9	75.9	79.9	86.4	85.8	<0.001[b]
T1 (26–50 %)	21.9	15.1	14.6	11.0	11.0	
T2 (>50 %)	15.2	9.0	5.5	2.6	3.2	
Extracapillary proliferation						
Ex0 (absence)	28.6	35.9	38.2	55.8	48.0	<0.001
Ex1 (presence)	71.4	64.1	61.8	44.2	52.0	
Use of ACEI/ARB	47.1	50.8	58.9	44.1	44.8	0.325
Use of steroid	37.3	38.0	34.6	28.1	31.2	0.071

Values are given as the mean ± standard deviation or percentages. Duration of follow-up is shown as the median (interquartile range)

eGFR estimated glomerular filtration rate, *ACEI* angiotensin-converting enzyme inhibitor, *ARB* angiotensin receptor blocker

[a] Hypertension was defined as blood pressure ≥140/90 mmHg and/or current treatment with anti-hypertensive agents

[b] Chi-square test for linear association

categorical NRI was 0.097 (P = 0.03). Continuous NRI and IDI were also significantly increased after including serum bilirubin in the model, to 0.281 (P = 0.02) and 0.019 (P = 0.01), respectively.

The association between serum bilirubin levels and degree of vascular atherosclerosis is presented in Fig. 3. A significant trend was observed between the serum bilirubin quintile and the crude degree of arteriolar hyalinosis (P for trend =0.007) and interlobular artery wall thickness (P for trend =0.006). Since age was generally associated with the frequency of vascular lesions in renal biopsy specimen, we estimated the age-adjusted trend. Significant age-adjusted trend was only found between the serum bilirubin quintile and arteriolar hyalinosis (Mantel-extension P for trend =0.035).

Discussion

The present study clearly demonstrated that a lower bilirubin level was associated with a higher risk of developing ESRD in patients with IgAN. This relationship was found to remain significant even after adjustment for all other confounders, including age, sex, serum albumin,

urinary protein excretion, eGFR and the pathological parameters of the Oxford classification (M, S and T1, T2). Importantly, the serum bilirubin level provided additional predictive information for future ESRD beyond the conventional risk factors in patients with IgAN. These results suggest that serum bilirubin will be a useful marker for the risk assessment of progression in patients with IgAN.

Only one previous study investigated the relationships between serum bilirubin level and IgAN. Chin et al. [27] demonstrated that a higher bilirubin level was correlated inversely with incidence of ESRD in a cohort comprising 1,458 adult patients with IgAN, which result was consistent with the present study. However, there has been no evidence of an association between serum bilirubin levels and histological findings in patients with IgAN. Notably, in the present study, the serum bilirubin level was inversely correlated with histopathological severity according to the Oxford classification. Further, serum bilirubin has consistently been found to be an independent risk factor for future ESRD in patients with IgAN even after adjusting for all potential confounding factors, including the pathological parameters of the Oxford classification (M, S and T1, T2). To our knowledge, the present study is the first investigation to demonstrate a meaningful relationship between serum bilirubin and histological findings of renal biopsy in patients with IgAN.

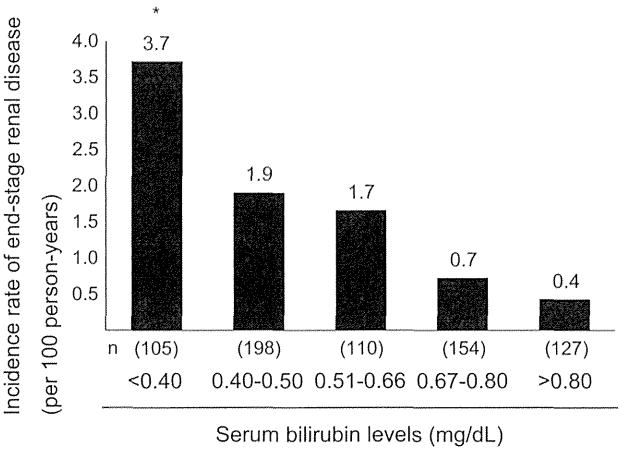

Fig. 1 The cumulative incidence rate of end-stage renal disease (ESRD) by serum bilirubin quintile. The differences and trends in the serum bilirubin concentration quintiles were tested by means of a Cox proportional hazards model. *P for trend <0.01

This study demonstrated that addition of the serum bilirubin level increased the NRI, and improved overall sensitivity measured by IDI. This result indicates that the serum bilirubin level could provide additional clinical information about the risk of future ESRD, beyond that provided by conventional risk factors. The NRI could provide clinical information by showing a quantifiable improvement by the addition of new biomarkers to the previous model. The IDI indicated the increased sensitivity by the addition of new biomarkers without sacrificing specificity. Hence, the NRI and the IDI would be more sensitive than c-statistics for identifying improvements in predictive value [28]. Recently, a scientific statement on clinical research from the American Heart Association has recommended that the evaluation of a novel risk marker of disease should be included if it provides more prognostic information than that provided by the standard risk markers [29]. We believe that our findings provide strong evidence that serum bilirubin would be a useful novel clinical marker, providing information beyond that from the established risk factors in the risk assessment of progression in IgAN.

The mechanism by which the inverse relationship between the serum bilirubin level and development of ESRD in patients with IgAN might be mediated is of great interest. The anti-oxidative or anti-inflammatory property of bilirubin might be related to the protective effect not only against atherosclerosis but also against progression of

Table 2 Age and sex-adjusted or multivariate-adjusted hazard ratios for the development of ESRD

Variable	N of ESRD/N of patients	Age- and sex-adjusted			Multivariate-adjusted[a]		
		HR (95 % CI)	P value	P for trend	HR (95 % CI)	P value	P for trend
Serum bilirubin levels (mg/dL)							
>0.80	4/127	1.00 (reference)			1.00 (reference)		
0.67–0.80	9/154	1.63 (0.50–5.31)	0.421		2.15 (0.63–7.37)	0.225	
0.51–0.66	11/110	3.81 (1.21–12.0)	0.022	<0.001	2.76 (0.84–9.12)	0.096	0.01
0.40–0.50	28/198	4.50 (1.57–12.9)	0.005		2.28 (0.74–7.00)	0.151	
<0.40	25/105	9.81 (3.39–28.4)	<0.001		5.05 (1.57–16.3)	0.007	
Every 0.1 mg/dL decrease in bilirubin	77/694	1.37 (1.22–1.53)	<0.001		1.18 (1.04–1.33)	0.001	

HR hazard ratio, *CI* confidence interval, *ESRD* end-stage renal disease

[a] Multivariate-adjusted model is adjusted for age, sex and clinical parameters (serum albumin, urinary protein excretion, estimated glomerular filtration rate) and pathological parameters (mesangial hypercellularity score, segmental glomerulosclerosis, tubular atrophy/interstitial fibrosis)

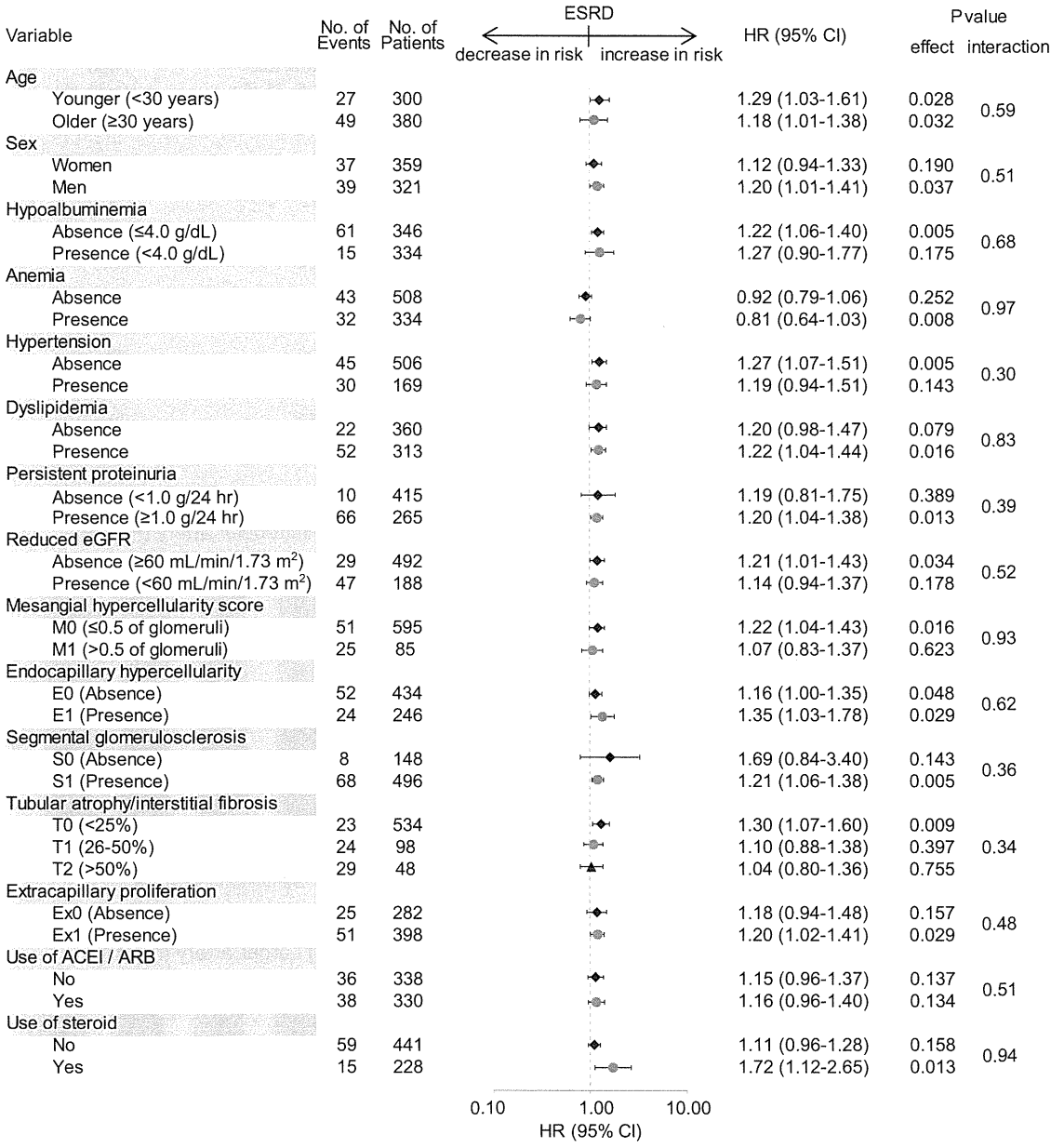

Fig. 2 Multivariate-adjusted hazard ratios and 95 % confidence intervals for the development of end-stage renal disease (ESRD) for every 0.1-mg/dL decrease in serum bilirubin levels according to subgroups of baseline characteristics and treatment assignment. The results were adjusted using the final selected model, which included age, sex, serum albumin, urinary protein excretion, estimated glomerular filtration rate, mesangial hypercellularity score, segmental glomerulosclerosis, and tubular atrophy/interstitial fibrosis. Variables relevant to the subgroups were excluded from each model. *HR* hazard ratio, *CI* confidence interval, *ACEI* angiotensin-converting enzyme inhibitor, *ARB* angiotensin receptor blocker

IgAN. A role for atherosclerosis in the progression of IgAN is supported by the association between renal prognosis of IgAN and metabolic factors involved in atherosclerosis, such as obesity [15], smoking status [30], hypertriglyceridemia [16], and hyperuricemia [16]. A previous in vitro study demonstrated that bilirubin might have anti-atherosclerotic effects due to a direct inhibition of certain isoforms of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, which is a key source of oxidative stress in atherosclerotic disease [31]. Another possible explanation for the renal protection is that bilirubin might mediate a potential link between progression of glomerulus sclerosis and non-traditional atherosclerotic risk factors such as insulin resistance and endothelial dysfunction. It has been reported that increased serum insulin and homeostasis model assessment of insulin resistance (HOMA-IR)

Table 3 Reclassification table of predicted probabilities of incident ESRD before and after reclassification with serum bilirubin in patients with and without the development of ESRD

Model 1[a]	Model 1 + serum bilirubin			
	Low (<3.5 %)	Moderate (3.5–35 %)	High (≥35 %)	Total
Patients who developed ESRD				
Low (<3.5 %)	6	3	0	9
Moderate (3.5–35 %)	1	23	5	29
High (≥35 %)	0	1	38	39
Total	7	27	43	77
Patients who did not develop ESRD				
Low (<3.5 %)	266	47	0	313
Moderate (3.5–35 %)	65	206	10	281
High (≥35 %)	0	4	19	23
Total	331	257	29	617

ESRD end-stage renal disease

[a] Model 1 is adjusted for age, sex, serum albumin, urinary protein excretion, estimated glomerular filtration rate, mesangial hypercellularity score, segmental glomerulosclerosis, and tubular atrophy/interstitial fibrosis

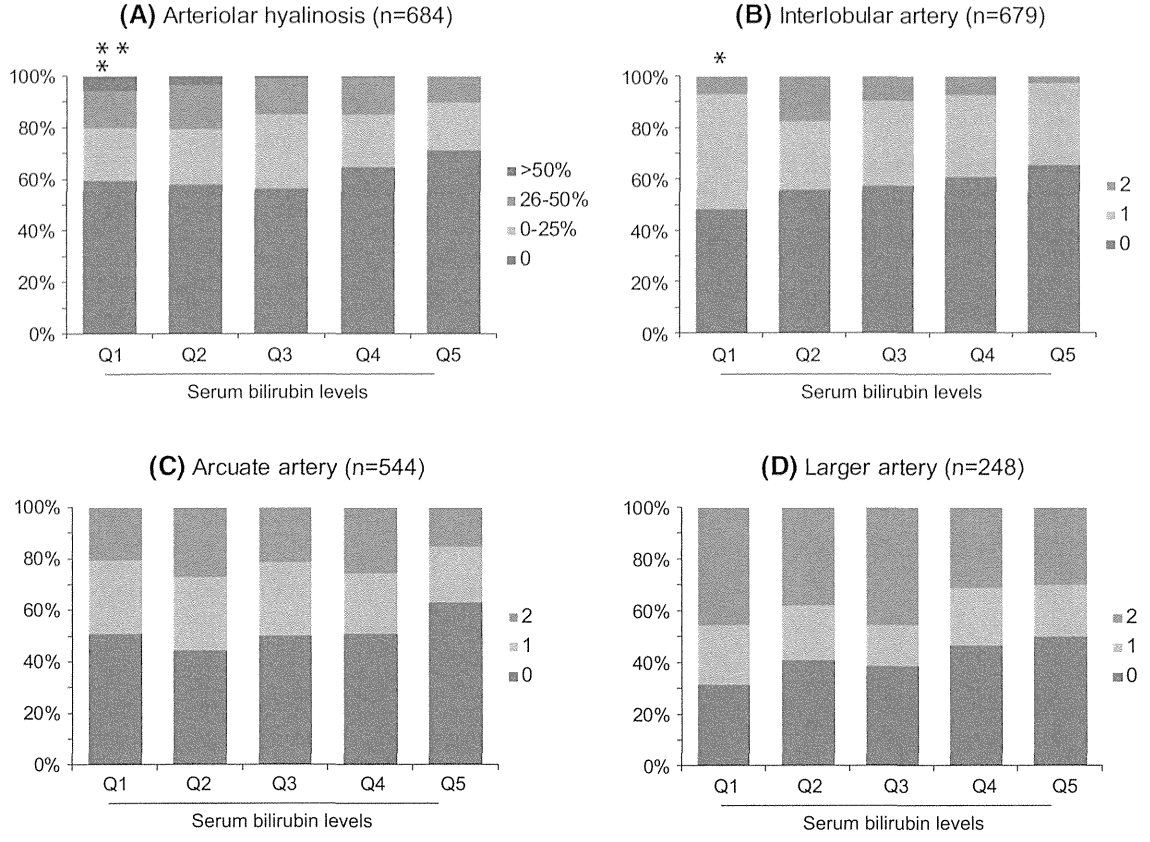

Fig. 3 Distribution of histopathological grade of vascular atherosclerosis by bilirubin quintile groups (Q1; <0.40 mg/dL, Q2; 0.40–0.50 mg/dL, Q3; 0.51–0.69 mg/dL, Q4; 0.70–0.79 mg/dL, Q5; ≤0.80 mg/dL). Arteriolar hyalinosis was categorized into three groups (0 %, 1–25 %, 26–50 % and >50 %), and arterial lesions were divided into three category (0, if no intimal thickening; 1, if intima thickened and < thickness of media; and 2, if intima thickened and > thickness of media). Age-adjusted trend was calculated by the Mantel-extension test. *P for trend <0.05. **Age-adjusted P for trend <0.05

levels were associated with the progression of IgAN [17]. Furthermore, it has been reported that serum bilirubin levels were inversely correlated with HOMA-IR and insulin levels in a general population of children and adolescents [32]. Endothelial dysfunction is also thought to accelerate atherosclerosis in patients with kidney disease [33]. Recent evidence suggests that the anti-viral drug atazanavir would ameliorate oxidative stress and improve endothelial function in patients with type 2 diabetes mellitus, most likely due to its bilirubin-increasing effect [34].

In our survey, the degree of arteriolar hyalinosis showed the only significant trend for the serum bilirubin levels independently of age. Arteriolar hyalinosis has been assumed to derive from plasma protein leakage coupled with vascular matrix proteins such as collagen type I and IV and fibronectin, which is probably induced by endothelial cell injury in various process, e.g., aging, hypertension and hyperglycemic state [35]. These findings provide strong evidence for the possible link among serum bilirubin, oxidative stress-mediated tissue injury, and non-traditional atherosclerotic risk factors in the progression of IgAN.

Several limitations of the present study should be noted. First, a single measurement of serum bilirubin might fail to capture the intra-individual variability of bilirubin levels over time in individual subjects, and would be considered a potential source of misclassification of study subjects into different bilirubin level categories. Such misclassification would weaken the association found in this study, biasing the results toward the null hypothesis. Second, another potential source of error is that we had no information on the fractionation of bilirubin. The antioxidant properties of bilirubin may differ depending on whether it is conjugated or unconjugated [36]. Third, although we adjusted for potential confounders that are likely to affect the association between serum bilirubin levels and kidney outcomes of IgAN, the effect of unmeasured residual confounders such as insulin resistance, endothelial dysfunction, smoking status and genetic activity of the hemeoxygenase-1 (HO-1) protein and gene, which is the rate-limiting enzyme in the production of bilirubin, cannot be eliminated in our study.

Conclusion

In conclusion, we demonstrated that lower bilirubin levels were significantly associated with higher risk of future ESRD in patients with IgAN. Moreover, we showed that serum bilirubin could provide additional predictive information beyond the established risk factors. We believe that low serum bilirubin might be a therapeutic target for the prevention of ESRD, but the evidence of a causal relationship between low serum bilirubin and the risk of ESRD is currently very limited. Therefore, further interventions will be needed to elucidate whether bilirubin can be a potential therapeutic target to reduce the burden of ESRD in patients with IgAN.

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Conflict of interest Honoraria: Kazuhiko Tsuruya (Kyowa Hakko Kirin Co., Chugai Pharmaceutical Co.), Research funding: Kazuhiko Tsuruya (Kyowa Hakko Kirin Co., Chugai Pharmaceutical Co., Fuso Pharmaceutical Industries, MSD K.K., Takeda Pharmaceutical Co.), Endowed department: Kazuhiko Tsuruya (Baxter). The authors declare that they have no relevant financial interests.

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Anti-albuminuric effect of the aldosterone blocker eplerenone in non-diabetic hypertensive patients with albuminuria: a double-blind, randomised, placebo-controlled trial

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Summary

Background Renin-angiotensin system inhibitors have renoprotective effects in patients with chronic kidney disease, but most patients treated with these drugs have residual urinary albumin excretion. Some small clinical studies show that mineralocorticoid receptor blockade reduces albuminuria. Our study aimed to examine the beneficial effects of addition of a selective aldosterone antagonist, eplerenone, to renin-angiotensin system inhibitors in hypertensive patients with non-diabetic chronic kidney disease.

Methods In this double-blind, randomised, placebo-controlled trial, we enrolled hypertensive patients, aged 20–79 years, with albuminuria (urinary albumin-to-creatinine ratio [UACR] in the first morning void urine of 30–599 mg/g), an estimated glomerular filtration rate of 50 mL/min per 1·73 m² or more, and who had received an angiotensin-converting enzyme inhibitor, an angiotensin receptor blocker, or both, for at least 8 weeks. Participants were from 59 clinics and hospitals in Japan. Eligible patients were randomly assigned (1:1), stratified by baseline characteristics, to either low-dose eplerenone (50 mg/day) or placebo, with continuation of standard antihypertensive treatment to attain therapeutic goals (<130/80 mm Hg) for 52 weeks. We assessed efficacy in all patients who received allocated treatment, provided a baseline and post-treatment urine sample, and remained in follow-up. We assessed safety in all patients who received allocated treatment. The primary efficacy measure was percent change in UACR in the first morning void urine at week 52 from baseline. The trial is registered at the clinical trials registry of University Hospital Medical Information Network (UMIN), trial identification number UMIN000001803.

Findings Between April 1, 2009, and March 31, 2012, we randomly allocated 170 patients to the eplerenone group and 166 patients to the placebo group. In the primary efficacy analysis, mean percent change in UACR from baseline was −17·3% (95% CI −33·65 to −0·94) for 158 patients in the eplerenone group compared with 10·3% (−6·75 to 22·3) for 146 patients in the placebo group (absolute difference −27·6% [−51·15 to −3·96]; p=0·0222). In the safety analyses, 53 (31%) of 169 patients in the eplerenone group had adverse events (five serious), as did 49 (30%) of 163 in the placebo group (seven serious). Although mean serum potassium concentration was higher in the eplerenone group than the placebo group, severe hyperkalaemia (>5·5 mmol/L) was not recorded in either group.

Interpretation Addition of low-dose eplerenone to renin-angiotensin system inhibitors might have renoprotective effects through reduction of albuminuria in hypertensive patients with non-diabetic chronic kidney disease, without serious safety concerns.

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See Online for appendix

Introduction

Hypertension and albuminuria are known risk factors for cardiovascular and renal events. The renin-angiotensin system (RAS) inhibitors, including angiotensin receptor blockers and angiotensin-converting enzyme inhibitors, have renoprotective and blood pressure-lowering effects; thus, guidelines recommend RAS inhibitors as first-line antihypertensive agents for patients with chronic kidney disease. In patients with non-diabetic nephropathy, inhibition of RAS activity reduces urinary protein excretion and slows the decline of the glomerular filtration rate.[1] Renoprotective effects might be at least partly independent of blood pressure-lowering effects, as the effects of RAS inhibitors are greater than those of other antihypertensive drugs at similar levels of blood pressure control.[2,3] However, hypertensive patients with chronic kidney disease treated with a RAS inhibitor often have substantial residual urinary albumin excretion. Because albuminuria is a crucial predictor of poor renal outcomes,[2,4,5] more extensive treatment in these patients is necessary.

Accumulating evidence, especially from animal studies, has shown that aldosterone plays a crucial part in renal injury and that blockade of the mineralocorticoid receptor, an aldosterone receptor, is renoprotective.[6,7] Activation of the mineralocorticoid receptor in the kidney may be independent of plasma aldosterone concentrations, as shown in a rat model of chronic kidney disease where a mineralocorticoid receptor antagonist suppressed the progression of renal injury in both high[6] and low[7] plasma aldosterone levels. Thus, a mineralocorticoid receptor

antagonist might have beneficial effects on renal injury in patients with chronic kidney disease who are already treated with RAS inhibitors. Indeed, several small-scale clinical studies have suggested a renoprotective effect of such antagonists. The selective mineralocorticoid receptor antagonist eplerenone was superior to a calcium antagonist in elderly patients with hypertension and microalbuminuria,[8] and to an angiotensin-converting enzyme inhibitor in hypertensive patients with albuminuria.[9] In addition, renal protection is seen with the addition of a mineralocorticoid receptor antagonist to an angiotensin-converting enzyme inhibitor in patients with diabetic nephropathy.[10–13] However, high doses of eplerenone (up to 200 mg), or of a more potent but less selective mineralocorticoid receptor antagonist spironolactone, were used in most studies to date, and resulted in an increased risk of severe hyperkalaemia in patients treated with RAS inhibitors. Notably, reduced doses of eplerenone (25–50 mg/day), that do not induce severe hyperkalaemia, suppressed urinary albumin excretion in elderly patients treated with angiotensin receptor blockers or angiotensin-converting enzyme inhibitors.[14] However, this study enrolled few patients and treatment with mineralocorticoid receptor antagonists was only for a short period.

Our study aimed to investigate the effects of long-term, low-dose eplerenone (50 mg/day) added to standard therapy with an angiotensin-converting enzyme inhibitor, angiotensin receptor blocker, or both, on the urinary albumin-to-creatinine ratio (UACR) in hypertensive patients with non-diabetic chronic kidney disease.

Methods

Study design and participants

The Eplerenone Combination versus Conventional Agents to Lower Blood Pressure on Urinary Anti-albuminuric Treatment Effect (EVALUATE) study was a prospective, multicentre, double-blind, placebo-controlled, randomised trial that assessed the effects of the mineralocorticoid receptor antagonist eplerenone on albuminuria in hypertensive patients with non-diabetic chronic kidney disease.[15] The study was investigator-driven and managed by the EVALUATE steering committee.

Patients from 59 clinics and hospitals in Japan participated and were enrolled in this study. Eligible patients were aged 20–79 years, were hypertensive with systolic blood pressures of 130–179 mm Hg or diastolic blood pressures of 80–99 mm Hg, had pre-treatment UACR in the first morning void urine (a mean of three measurements in three consecutive visits) of 30–599 mg/g, had an estimated glomerular filtration rate (eGFR) of 50 mL/min per 1·73 m^2 or more, and had received an angiotensin-converting enzyme inhibitor, an angiotensin-receptor blocker, or both, for at least 8 weeks. Major exclusion criteria included hypertensive emergencies that required intravenous administration of antihypertensive agents; serum potassium concentrations of 5·0 mmol/L or more; diabetes (fasting blood glucose concentration ≥126 mg/dL or treatment with anti-diabetic drugs); severe liver damage (Child-Pugh score: class C); severe heart failure (New York Heart Association class ≥III); severe arrhythmia (frequent ventricular or atrial extrasystole, prolonged ventricular tachycardia, atrial tachyarrhythmia with severe tachycardia, atrial fibrillation or flutter with severe tachycardia, sick sinus syndrome with severe bradycardia, or atrioventricular block with severe bradycardia); angina; myocardial infarction or cerebrovascular disease within 6 months before registration; pregnancy, possibility of pregnancy, or a desire to become pregnant; a history of severe adverse effects from mineralocorticoid receptor antagonists, angiotensin-converting enzyme inhibitors, or angiotensin-receptor blockers; administration of a mineralocorticoid receptor antagonist less than 8 weeks before registration; taking contraindicated drugs (including adreno-corticosteroidal drugs, immunosuppressants, potassium-sparing diuretics, potassium supplementation, itraconazole, ritonavir, and nelfinavir); and treatment for more than 2 weeks with non-steroidal anti-inflammatory drugs at registration. The study protocol was approved by the institutional review board of the University of Tokyo Clinical Research Center (reference number P2008028-11X) as the central review board and by review boards of other participating hospitals. The study was done in full accordance with the principles of the Declaration of Helsinki. Written informed consent was obtained after the participants received verbal and written explanations about the study from the attending physicians. We followed the Consolidated Statement of Reporting Trials (CONSORT) guidelines.[16]

Randomisation and masking

After confirmation of eligibility, we randomly allocated patients in a 1:1 ratio to either the eplerenone (50 mg/day) group or placebo group with a centralised computer-generated allocation procedure stratified by the following patient characteristics: UACR (<300 mg/g *vs* ≥300 mg/g), systolic blood pressure (<140 mm Hg *vs* ≥140 mm Hg), and previous treatment with the RAS inhibitors (angiotensin-converting enzyme inhibitor or angiotensin receptor blocker, *vs* dual treatment with angiotensin-converting enzyme inhibitor plus angiotensin receptor blocker). We adopted the web-based allocation system used by UMIN, a data centre run as a public institution in Japan. The allocation table, which was created by stratified block randomisation (block size 4) by the trial statistician, was registered with UMIN. Block size was concealed to all investigators until code breaking.

DBcaps capsules (Capsugel Japan, Sagamihara, Japan) were used to mask the test drugs (eplerenone and placebo). Encapsulated study drugs were prepared and packaged centrally by the pharmacy of the University of Tokyo and distributed to the participating hospitals. The study investigators, patients, data collection and