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Descriptive epidemiology of spot urine sodium-to-potassium ratio clarified close relationship with blood pressure level: The Nagahama study

SHORT TITLE

Spot urine sodium/potassium ratio

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CONFLICTS OF INTEREST

The authors have no conflict of interest to disclose.

ABSTRACT

Objectives: We undertook descriptive epidemiology of spot urine sodium-to-potassium ratio (Na/K) in a population sample to clarify the close relationship between Na/K and blood pressure level independently of potential confounding factors.

Methods: Study participants consisted of 9,144 apparently healthy citizens (aged 54±13 years). All clinical parameters were obtained at baseline.

Results: Na/K was significantly higher in hypertensive subjects irrespective of antihypertensive medication status (normotension, 3.12±1.82; untreated hypertension 3.50±1.96; treated hypertension, 3.72±2.53). As urinary Na (β=0.092, P<0.001) and K (β=−0.050, P<0.001) levels were inversely associated with blood pressure (BP), Na/K (β=0.118, P<0.001) was more closely associated with BP than Na or K alone, as well as daily salt intake estimated from urinary Na (β=0.088, P<0.001). Several factors were significantly associated with Na/K, namely age, sex, obesity, blood pressure, renal function, salt restriction status, serum phosphate and urinary creatinine level, and fasting period and season at urine sample collection. However, the association between Na/K and BP was independent of these factors (adjusted β=0.112, P<0.001). No direct association was observed between Na/K and large arterial remodeling assessed by pulse wave analysis (P=0.496) or retinal arteriolar morphological change (P=0.431). Further, a genome-wide association study failed to identify any particular genotype influencing urinary Na and K levels.

Conclusions: Although we clarified several factors that might affect spot urine Na/K, these relationships were not substantial enough to confound the association between urinary Na/K and BP. A simple measure of Na/K might be more representative of salt loading obtained from spot urine samples than Na excretion alone.

KEYWORDS

Urinary Na/K, salt loading, descriptive epidemiology, blood pressure

INTRODUCTION

Excessive salt intake is a risk factor for hypertension [1] and might directly increase risk of stroke [2, 3], while direct association with coronary heart disease remains inconclusive [3]. Conversely, a modest reduction in salt intake decreases blood pressure (BP) levels in both hypertensive patients and normotensives individuals [4], and the degree of BP decline (approximately 6 mmHg systolic BP [SBP] per 100 mmol reduction in 24-h urinary sodium) [4] substantially influences reduction in risk of cardiovascular disease [5]. Accurate assessment of daily salt load is therefore important when evaluating the risk of hypertension and cerebrovascular disease at both the individual and whole population levels.

The most reliable method for estimating daily salt intake in both clinical and epidemiological settings is measurement of 24-h urinary sodium excretion. A number of studies have confirmed an association between elevated salt intake as assessed by the 24-h urine method and increased BP levels [6]. However, 24-h urine collection is inconvenient for participants and cannot be easily adapted for large-scale cohort studies. Another simple method of estimating daily salt intake is an equation developed by Kawasaki et al. [7], which estimates 24-h urinary sodium (Na) and potassium (K) excretion based on second morning voiding urine. Tanaka et al. [8] developed another equation, which can also estimate 24-h urinary Na and K excretion from a spot urine sample. Although these equations are easy to use and have been adopted in several epidemiological studies [9], the accuracy of estimation

is limited due to the large dispersion between the actual and estimated 24-h Na and K excretion levels [7, 8].

Sodium-to-potassium ratio (Na/K) of a urine sample is another index of salt loading. Given that potassium excretion level is inversely associated with BP [9] and potassium supplementation decreases BP levels [10, 11], the combined effect of lower potassium and higher sodium levels (i.e. higher Na/K) on BP appears to be greater than either alone [11, 12]. The superiority of Na/K was also observed in association with target organ damage, namely left ventricular hypertrophy [13] and cardiovascular disease (CVD) [14]. However, these studies used 24-h urine samples, and availability of Na/K data for spot urine samples is limited, despite a strong correlation being observed between spot urine Na/K and BP levels [15]. Recently, Iwahori et al. [16] reported that mean value of repeated measurement of spot urine Na/K was closely correlated to 24-h urine sample Na/K. These previous data emphasize the importance of elucidating the association of spot urine Na/K with BP levels and target organ damage, as well as clinical and environmental factors that might affect Na/K.

Here, we conducted a cross-sectional study by analyzing a dataset of the Nagahama Prospective Cohort for Comprehensive Human Bioscience (the Nagahama Study), which is a large-scale population-based cohort study in Japan, to further clarify factors that affects spot urine Na/K level, as well as the significance of spot urine Na/K with respect to BP level. We also conducted a genome-wide association study (GWAS) to identify any particular genotype

influencing urinary Na and K levels.

METHODS

Study subjects

Subjects consisted of 9,144 apparently healthy middle-aged to elderly citizens who participated in the Nagahama Prospective Cohort for Comprehensive Human Bioscience (the Nagahama Study). This cohort was recruited from 2008 to 2010 from the general population of Nagahama City, a largely rural city of 125,000 inhabitants located in central Japan. Community residents aged 30 to 74 years, living independently in the community and with no physical impairment or dysfunction, were recruited for the Nagahama cohort. Of 9,804 potential participants, those meeting any of the following conditions were excluded: women whose urinary Na and K levels were not measured due to menstruation (n=447) or pregnancy (n=41), individuals with unsuccessful measurement of clinical parameters required for this study (n=79), individuals undergoing cancer therapy (n=58) or insulin therapy (n=24), or individuals with extreme deviation of renal function (estimated glomerular filtration rate [eGFR] = $194 \times \text{creatinine}^{-1.094} \times \text{age}^{-0.287} \times 0.739$ [if female], <30 mL/min/1.73 m^2) (n=11). All study procedures were approved by the ethics committee of Kyoto University Graduate School of Medicine and by the Nagahama Municipal Review Board. Written informed

consent was obtained from all participants.

Spot urine sample

Spot urine samples were collected at baseline measurements (09:00-17:00), and urinary Na, K, and creatinine (Cre) levels were measured on the day of sampling. Time since last meal was inquired about for each subject.

Clinical parameters

Basic clinical parameters, including plasma markers, were measured at baseline. Smoking habit, alcohol consumption, and salt restriction status were assessed using a structured self-administered questionnaire. Daily salt intake was estimated from urinary Na and Cre values using the following formula [8]:

$$\text{Daily salt intake} = 21.98 \times \{\text{Na (mEq/l)} \times \text{predicted 24 h Cre excretion} / [\text{Cre (mg/dl)} \times 10]\}^{0.392} \times 0.0585$$

where 24 h Cre excretion was predicted using the following formula:

$$\text{24 h Cre excretion} = \text{body weight (kg)} \times 14.89 + \text{body height (cm)} \times 16.14 - \text{age} \times 2.04 - 2444.45.$$

Brachial and central BP was measured simultaneously after 5 min rest in the sitting position. Measurements were taken twice, and the mean value was used in analysis.

Brachial-to-ankle pulse wave velocity (baPWV) was measured as an index of large arterial stiffness, while central retinal arteriolar equivalent (CRAE) measured from a fundus photograph was used as an index of small arteriole change. Methods used for BP, baPWV, and CRAE measurement are detailed in the Supplementary Methods.

Genetic analysis

DNA was extracted from peripheral blood samples by a conventional phenol-chloroform method. Genome-wide single nucleotide polymorphism (SNP) analysis was performed with a subset of the Nagahama cohort sample (n=3,095) using a series of BeadChip DNA arrays (Illumina, San Diego, CA, USA). Replication analysis was performed with the remaining Nagahama samples with DNA available for genotyping, and the genotype of the candidate SNP was successfully analyzed (n=5,716). Genotypes were analyzed with a TaqMan probe assay using commercially available primer and probe sets purchased from Life Technologies Corporation (Carlsbad, CA, USA). Detailed methods of genotyping and association analysis are described in the Supplementary Methods.

Statistical analysis

Differences in numeric variables among subgroups were assessed by analysis of variance, while differences in frequency were assessed by a chi-squared test. Factors independently

associated with Na/K, BP, baPWV, and CRAE were identified by multiple logistic regression analysis. All statistical analyses were performed using JMP 9.0.2 software (SAS Institute, Cary, NC, USA), with a *P*-value less than 0.05 considered to indicate statistical significance.

RESULTS

Clinical characteristics of study subjects are shown in Table 1. Distribution of spot urine Na and K levels are shown in Supplementary Figure 1 and their scatter plots in Supplementary Figure 2. Although Na/K showed wide distribution (Supplementary Figure 3), 99.5% of subjects had ratios between 0.06 and 11.8. Spot urine Na/K was inversely associated with urinary Cre level (Supplementary Figure 4) due to the differences between urinary Na and K levels in relation to Cre levels (Supplementary Figure 5). Mean estimated daily salt intake was 9.8±2.1 g, and distribution of salt intake is shown in Supplementary Figure 6.

Factors associated with spot urine Na/K

Associations of clinical and lifestyle factors with spot urine Na/K are summarized in Table 2. Male sex, obesity, and current smoking, but not drinking habit, were positively associated with Na/K after adjustment for basic covariates. Further, subjects with careful salt intake had substantially lower Na/K. Older subjects had somewhat lower Na/K, but no clear

age-dependency was observed (Supplementary Figure 7). No significant differences in Na/K were found between subjects with and without metabolic disorders, namely type 2 diabetes and dyslipidemia.

Fasting condition was strongly and inversely associated with spot urine Na/K (Figure 1A), with substantially higher values during the non-fasting phase. Poorer renal function (Figure 1B) and higher serum phosphate (Figure 1C), but not calcium (Figure 1D), levels were also inversely associated with Na/K. Differences between Na and K in relation to environmental and clinical factors (Supplementary Figure 8) might explain the inverse association of Na/K with these factors.

Time at urine sample collection also influenced Na/K. However, while samples collected in the afternoon had significantly higher Na/K values than those collected in the morning ($P<0.001$) (Supplementary Figure 9), this difference nearly lost significance ($P=0.031$) after adjustment for covariates such as fasting time. Seasonal variations in spot urine Na/K were also noted, with lower values observed in the summer season. Mean monthly Na/K and temperature showed inverse relationships (Figure 2A).

Given the results of these correlation analyses, factors independently associated with urinary Na/K were identified by multivariate analysis (Supplementary Table 1). Factors showing a strong positive association with Na/K were body mass index (BMI) ($\beta=0.051$), SBP ($\beta=0.126$), eGFR ($\beta=0.106$), and antihypertensive medication status ($\beta=0.048$), while age

(β=-0.104), salt restriction status (β=-0.057), serum phosphate levels (β=-0.152), urinary Cre levels (β=-0.312), season (β=−0.076) and fasting duration at urine sample collection (β=−0.177) showed an inverse association with urinary Na/K (P<0.001).

Association of spot urinary Na/K with BP

The association of spot urine Na/K with BP is shown in Figure 3. Na/K was significantly higher in hypertensive subjects, irrespective of antihypertensive medication status (Figure 3A), and linearly increased with BP (Figure 3B). Frequency analysis also revealed a linear positive association between urinary Na/K and prevalence of hypertension (Supplementary Figure 10).

Creatinine-adjusted urinary Na levels also increased linearly with BP, while urinary K levels showed an inverse association with BP (Supplementary Figures 11 and 12), even in a multivariate analysis adjusted for major covariates, namely age, sex, BMI, and antihypertensive medication status (Na/Cre: β=0.092, P<0.001, K/Cre: β=−0.050, P<0.001). Standardized coefficient of urinary Na/K to SBP (Table 3, Model 1) was therefore larger than that of Na or K alone, as was the coefficient of daily salt intake estimated from urinary Na (Model 2). When both Na/K and estimated daily salt intake were included in a same regression model, only Na/K was identified as a determinant for SBP (Na/K, β=0.105, P<0.001, variation inflation factor [VIF]=1.65; estimated daily salt intake, β=0.021, P=0.070, VIF=1.72). No substantial change was observed in the β of Na/K after full adjustment for

covariates (Model 3) that were significantly associated with urinary Na/K. In contrast, no direct association was detected between urinary Na/K and large arterial remodeling as assessed by PWV (Model 4) or small arteriolar morphological changes as assessed by CRAE (Model 5). Although central BP has been suggested to be more closely associated with cardiovascular outcomes than brachial BP, association of urinary Na/K with central BP was not superior to that with brachial BP (Table 3, Model 6).

GWAS of urinary Na and K levels

We also conducted a GWAS to identify particular genotype influencing urinary Na and K levels (Supplementary Figures 13 to 16) using a subset of the Nagahama sample (Supplementary Table 4). Although *P*-values of several SNPs located in chromosome 1 reached nearly genome-wide significance, a replication analysis using the remaining Nagahama samples failed to confirm any possible association (Supplementary Table 5).

DISCUSSION

In this cross-sectional study of a large general population, we clarified that spot urine Na/K was more closely associated with BP than estimated daily salt intake calculated from urinary Na. Although many clinical and environmental factors substantially affected spot urine Na/K,

these factors did not confound the association between urinary Na/K and BP.

Epidemiological significance of spot urinary Na/K

Several epidemiological studies for hypertension [9] used estimated daily salt intake calculated from spot urine Na level as an index of salt loading. However, as demonstrated in the present study, uncorrected Na/K exhibited a stronger correlation with BP than estimated salt intake. One reason for this improved association might be the limited accuracy of salt intake estimation; indeed, for both equations, when used in epidemiological studies [7, 8], the estimated daily salt intake showed wide dispersion compared to the actual value. Another reason might be the use of K as a factor in the calculation. Suggestions that urinary K is inversely associated with BP [9] may explain why Na/K rather than the salt intake estimated from Na excretion level exhibited a stronger correlation with BP. We also demonstrated the inverse association between urinary K and SBP in this study.

Spot urine Na/K was influenced by several clinical and environmental factors, namely fasting condition, sampling time and season, renal function, and anthropometric factors. Therefore, studies using spot urine Na/K should consider these confounding factors. Further investigations are required to clarify which urine sample is most appropriate for Na/K measurements, namely first morning urine, second morning voiding urine or other.

Factors associated with spot urinary Na/K

Several factors considerably influenced urinary Na/K, with urinary Cre exhibiting the strongest correlation. The reverse J-shape association between urinary Na and Cre, presumably representing lower urine volume in cases with elevated Cre levels, might have caused the inverse association between urinary Na/K and Cre. A second significant factor was fasting status with considerably lower urinary Na/K in the fasting phase. Large time-dependent declines in urinary Na levels versus K levels might be the cause of the fasting-time dependency of Na/K. The inverse association between Na/K and eGFR might also be due to the large decrease in urinary Na levels relative to renal functional decline. In contrast, serum phosphate concentration was positively associated with urinary K but not Na level. As a large proportion of urinary phosphate is reabsorbed at the proximal tubule by sodium-phosphate co-transporters [17], concomitant reabsorption of Na and consequently larger K efflux might be a plausible reason for this relationship between higher serum phosphate levels and greater K excretion. The lack of any association between serum calcium and urinary K level supports this consideration. These covariates were, however, unlikely to have any marked influence on the relationship between Na/K and BP. As shown in the results of our regression analysis for BP, the relatively small effect of these potential confounding factors on BP might be a reason for the independent association between Na/K and BP.

GWAS of urinary Na and K levels failed to identify any particular genotype

influencing urinary Na and K levels. Environmental factors may strongly influence urinary Na and K excretion rather than the individual genetic variations. However, larger-scale GWAS might identify susceptible genotypes, as the sample size of our GWAS was limited compared to recent genetic association analyses of common diseases and clinical traits.

Association of spot urine Na/K with BP and arterial properties

Spot urine Na/K was linearly increased with BP level. Although Na/K of controlled hypertensive subjects were similar to those of healthy individuals, hypertensive subjects exhibited significantly higher Na/K irrespective of medication status. These results emphasize the importance of salt restriction not only for the prevention of hypertension but also in therapeutic intervention for hypertensive patients.

No direct association was observed between Na/K and large arterial stiffness assessed by baPWV. Although our study was based on a spot urine sample and might be a potential limitation in estimation of salt loading, the present results did not support the previously postulated direct adverse effects of excessive salt loading on target organ damage [2, 3]. Retinal vessels are the only visible arterioles and venules whose caliber can be easily measured by fundus photography, and retinal vessel signs such as the narrowing of CRAE have been associated with cardiovascular risk factors [18, 19], systemic inflammation [20], incidence of stroke [21, 22], and stroke death [23]. However, as shown in our previous report

[24] and the present study, BP was the strongest determinant for CRAE, and urinary Na/K also did not appear to have direct adverse effects on small arterioles. Of note, to our knowledge, the present study is the first to investigate the association between urinary Na/K and morphological changes in retinal arterioles.

Study limitations

Several limitations to the present study warrant discussion. First, we used a spot urine sample, with no 24-h urine data available. Similarities and differences between spot urine Na/K and that of 24-h collected urine samples should be clarified. Second, as the present study was conducted in a cross-sectional setting, further longitudinal investigation is warranted to clarify the prognostic significance of spot urine Na/K on incidence of hypertension and cardiovascular diseases.

Summary

In conclusion, we clarified several covariates that might affect spot urine Na/K, though the association of Na/K with BP level was relatively unaffected by these clinical and environmental factors. A simple measure of spot urine Na/K might be more representative of salt loading obtainable from spot urine than estimated daily salt intake. Further longitudinal investigations are required to clarify the prognostic significance of spot urine Na/K.

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FIGURE LEGENDS

Figure 1. Factors associated with spot urine Na/K.

Values are mean. Numbers of subjects in each subgroup are shown in columns. Estimated glomerular filtration rate (eGFR) was calculated using the following formula: $194 \times Cre^{-1.094} \times age^{-0.287} \times 0.739$ [if female].

Figure 2. Seasonal variation in spot urine Na/K.

A) Monthly mean urinary Na/K and temperature. Mean temperature during sampling period (2008 to 2010) was obtained from a database of the Japan Meteorological Agency. Months with mean temperature greater than 20 °C and less than 10 °C were regarded as summer and winter seasons, respectively. B) Seasonal mean urinary Na/K. Numbers of subjects in each subgroup are shown in columns.

Figure 3. Association between BP and spot urine Na/K.

Values are mean. A) Hypertension was defined as any or all of brachial SBP ≥140 mmHg, DBP ≥90 mmHg, or taking antihypertensive drugs. B) BP was graded according to the guideline for the management of hypertension by the Japanese Society of Hypertension; optimal, SBP <120 mmHg and DBP <80 mmHg; normal, SBP 120-129 mmHg and/or DBP 80-84 mmHg; high normal, SBP 130-139 mmHg and/or DBP 85-89 mmHg; grade I

hypertension, SBP 140-159 mmHg and/or DBP 90-99 mmHg; grade II hypertension, SBP 160-179 mmHg and/or DBP 100-109 mmHg; grade III hypertension, SBP ≥180 mmHg and/or DBP ≥110 mmHg.

Table 1. Clinical characteristics of study subjects (n=9,144)

Age (years)		54±13
Sex (male/female)		3,139/6,005
BMI (kg/m^2)		22.3±3.3
Salt restriction (%)		51.9
Blood pressure	*Brachial SBP (mmHg)*	124±18
	Central SBP (mmHg)	114±18
	DBP (mmHg)	76±11
	PP amplification (mmHg)	10±6
	Antihypertensive medication (%)	17.6
Glucose metabolism	*Glucose (mg/dl)*	90±14
	HbA1c (%)	5.5±0.5
	Antihyperglycemic treatment (%)	2.7
Plasma lipids	*Total cholesterol (mg/dl)*	207±35
	HDL cholesterol (mg/dl)	65±17
	Triglyceride (mg/dl)	99±67
	Antihyperlipidemic treatment (%)	12.4
Electrolyte	*Phosphate (mg/dl)*	3.51±0.46
	Calcium (mg/dl)	9.05±0.35
Renal function	*Serum Cre (mg/dl)*	0.70±0.16
	eGFR (ml/min/1.73m^2)	79±16
Urinary markers	*Cre (mg/dl)*	90±65
	Na (mEq/l)	128±62
	Na/Cre	1.84±1.00
	K (mEq/l)	49±31
	K/Cre	0.62±0.26
	Na/K	3.23±1.94
Estimated daily salt intake (g)		9.8±2.1
baPWV (cm/sec)		1271±226
CRAE (μm) §		126±12

BMI, body mass index; PP, pulse pressure; Cre, creatinine; baPWV, brachial-to-ankle pulse wave velocity; CRAE, central retinal arteriolar equivalent.

Estimated glomerular filtration rate (eGFR) was calculated using the following formula: $194 \times \text{Cre}^{-1.094} \times \text{age}^{-0.287} \times 0.739$ [if female]. Daily salt intake (g) was estimated from spot urine Na and Cre values using the following formula: $21.98 \times \{\text{Na (mEq/l)} \times \text{predicted 24 h Cre excretion} / [\text{Cre (mg/dl)} \times 10]\}^{0.392} \times 0.0585$, where 24 h Cre excretion was predicted using the following formula: body weight (kg) × 14.89 + body height (cm) × 16.14 − age × 2.04 − 2444.45 [8].

§Values available for 7,700 subjects.

Table 2. Association of clinical and lifestyle factors with spot urine Na/K

		Spot urine Na/K	*P* (ANOVA)	*P* (adjusted)
Sex	*Male (3,139)*	3.38±2.02	<0.001	
	Female (6,005)	3.15±1.88		
Obesity	*Obese (219)*	3.69±2.02	<0.001	
	Overweight (1,515)	3.37±2.93		
	Normal weight (7,410)	3.18±1.91		
Drinking	*Habitual (2,141)*	3.55±2.18	<0.001	0.657
	Occasional (936)	3.32±2.15		0.841
	Never or ever (6,066)	3.13±1.79		Reference
Smoking	*Current (1,338)*	3.37±2.00	<0.001	<0.001
	Past (1,882)	3.25±1.91		0.889
	None (5,924)	3.17±1.91		Reference
Salt restriction	*Yes (4,745)*	3.11±1.82	<0.001	<0.001
	No (4,399)	3.36±2.04		
Type 2 diabetes	*Diabetes (382)*	3.36±2.10	0.170	0.911
	Control (8,762)	3.22±1.93		
Dyslipidemia	*Dyslipidemia (4,264)*	3.28±1.96	0.011	0.355
	Control (4,880)	3.18±1.92		

Values are mean ± standard deviation. Numbers of subjects in each subgroup are shown in parentheses.

Obesity was defined as follows: obese, body mass index ≥30 kg/m^2; overweight, ≥25 kg/m^2; normal weight, <25 kg/m^2.

Type 2 diabetes was defined as any or all of HbA1c ≥6.5%, fasting plasma glucose ≥126 mg/dl, occasional plasma glucose ≥200 mg/dl, or taking antihyperglycemic drugs.

Dyslipidemia was defined as any or all of LDL cholesterol ≥140 mg/dl, HDL cholesterol <40 mg/dl, triglyceride ≥150 mg/dl, or taking antihyperlipidemic drugs. Group differences were assessed by analysis of variance. Adjusted factors in the multivariate analysis were age, sex, body mass index, SBP, and antihypertensive medication.

Table 3. Multivariate analysis for BP and arterial parameters

	Dependent variables					
	Brachial SBP			baPWV	CRAE	Central SBP
	Model 1[§]	Model 2[§]	Model 3[#]	Model 4[¶]	Model 5[¶]	Model 6[§]
Spot urine Na/K	0.118 (*P*<0.001)		0.112 (*P*<0.001)	0.005 (*P*=0.496)	-0.009 (*P*=0.431)	0.109 (*P*<0.001)
Estimated daily salt intake (g)		0.088 (*P*<0.001)				

baPWV, brachial-to-ankle pulse wave velocity; CRAE, central retinal arteriolar equivalent (values are available for 7,700 subjects); Cre, urinary creatinine.

Values are standardized regression coefficient and *P*-value.

Daily salt intake (g) was estimated from spot urine Na and Cre values using the following formula: $21.98 \times \{Na\ (mEq/l) \times predicted\ 24\ h\ Cre\ excretion / [Cre\ (mg/dl) \times 10]\}^{0.392} \times 0.0585$, where 24 h Cre excretion was predicted using the following formula: body weight (kg) × 14.89 + body height (cm) × 16.14 − age × 2.04 − 2444.45 [8].

[#]Fully adjusted model including age, sex, BMI, salt restriction, current smoking habit, serum phosphate and urinary creatinine levels, seasonal variation, antihypertensive medication status, eGFR, and fasting duration at urine sampling as covariates. Full results of the regression analyses are shown in Supplementary Table 2.

[¶]Adjusted for age, sex, BMI, current smoking habit, SBP, antihypertensive medication status, type 2 diabetes, and dyslipidemia. Full results of the regression analysis are shown in Supplementary Table 3.

[§]Adjusted for age, sex, BMI, and antihypertensive medication status.

FIGURE 1

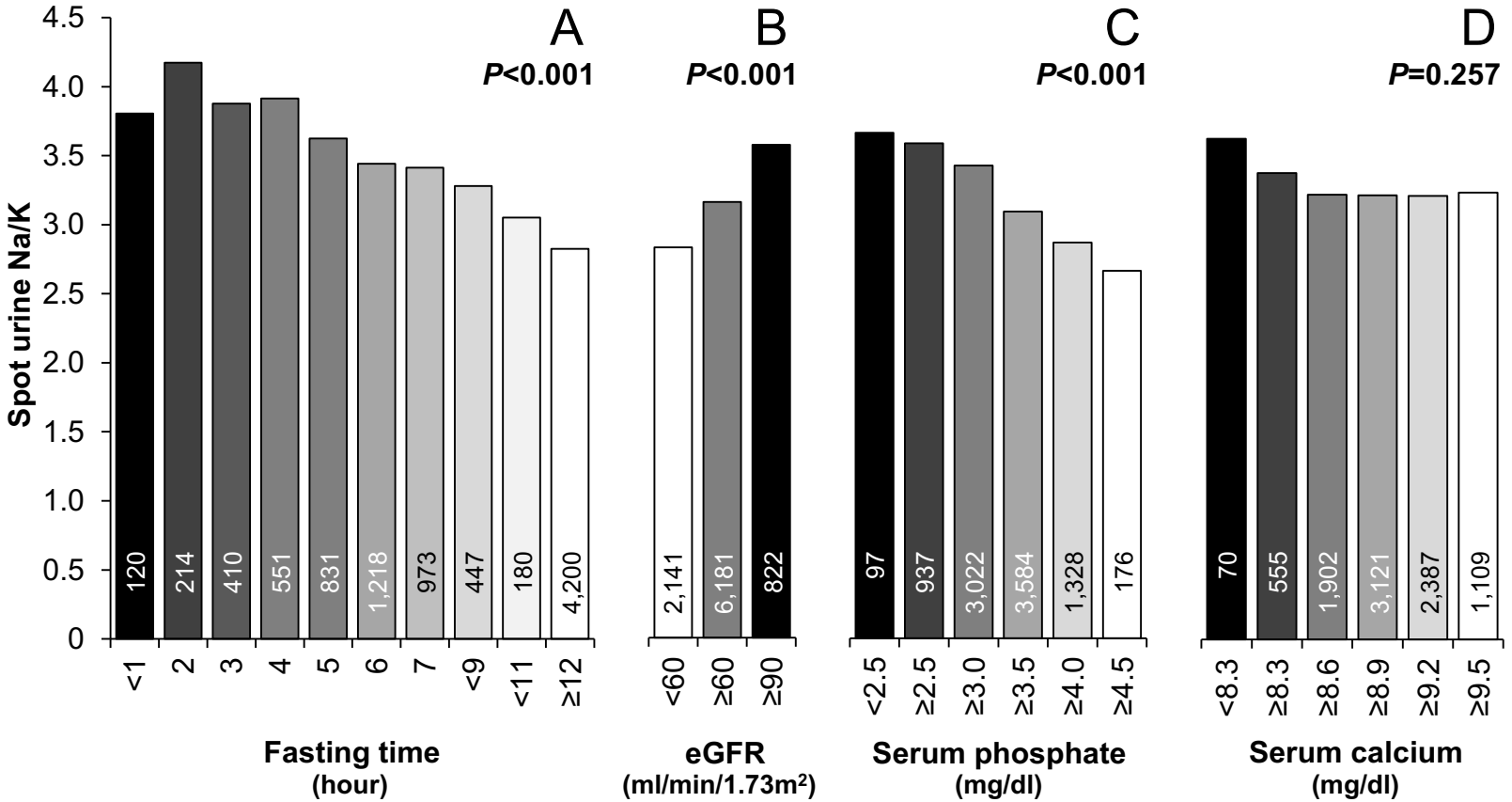


FIGURE 2

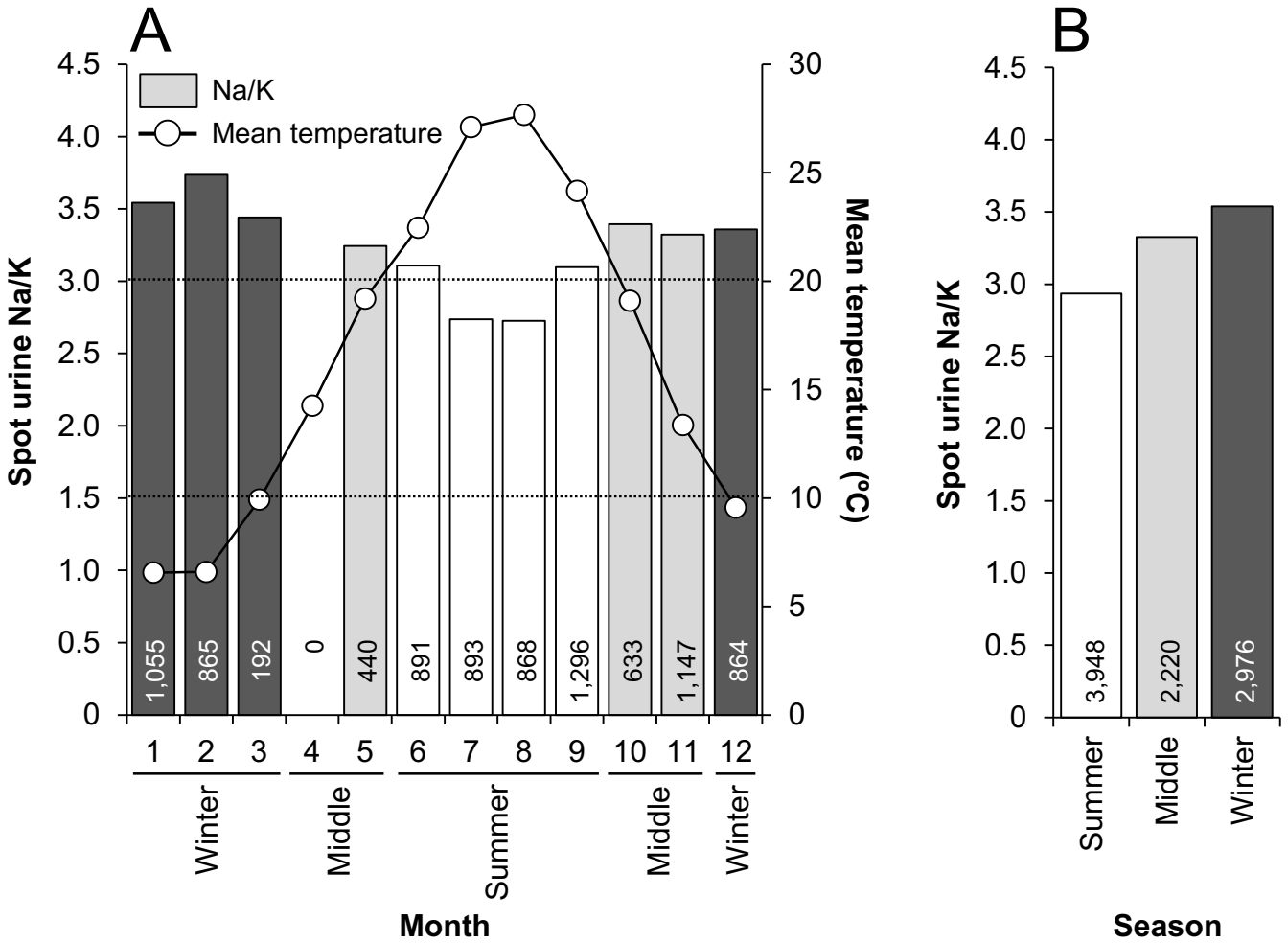
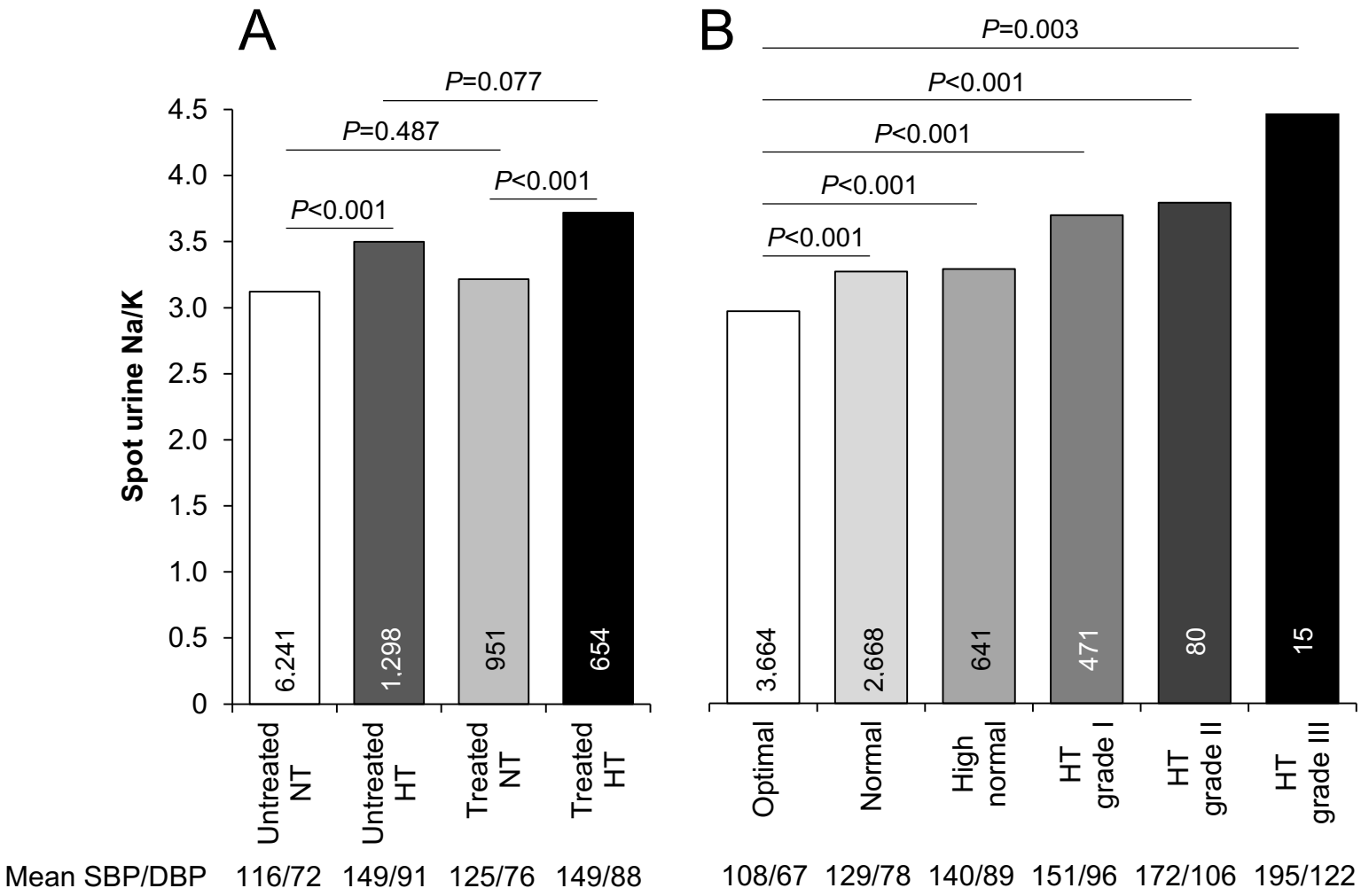


FIGURE 3



SUPPLEMENTARY MATERIALS

Descriptive epidemiology of spot urine sodium-to-potassium ratio clarified close relationship with blood pressure level: The Nagahama study

Yasuharu Tabara, Yoshimitsu Takahashi, Kyoko Kumagai, Kazuya Setoh, Takahisa Kawaguchi, Meiko Takahashi, Yuki Muraoka, Akitaka Tsujikawa, Norimoto Gotoh, Chikashi Terao, Ryo Yamada, Shinji Kosugi, Akihiro Sekine, Nagahisa Yoshimura, Takeo Nakayama, and Fumihiko Matsuda, on behalf of the Nagahama study group

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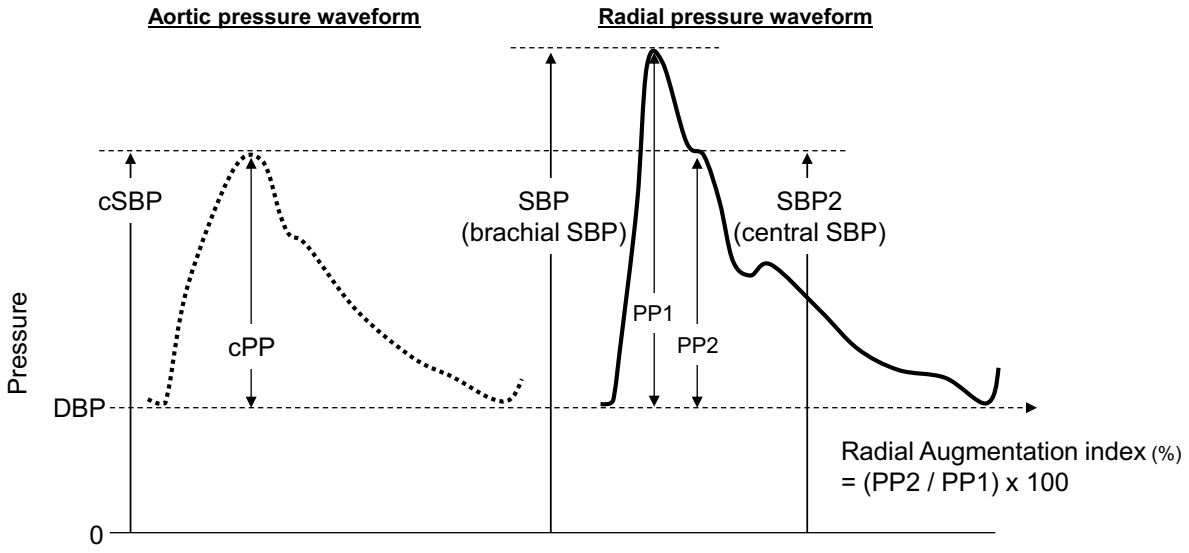
SUPPLEMENTARY METHODS

Blood pressure (BP) measurements

Radial arterial waveform and brachial BP were simultaneously measured (HEM-9000AI; Omron Healthcare, Kyoto, Japan) after 5 min rest in the sitting position. Briefly, brachial BP was measured at the right upper arm using a cuff-oscillometric device, and the radial arterial waveform was obtained from the left wrist using a multi-element tonometric sensor. The augmentation index (AIx) was calculated from the radial arterial waveform as the ratio of the late systolic peak to the first systolic peak. When AIx was less than 100%, absolute pressure of the late systolic peak (SBP2) of the radial arterial waveform, which was obtained by calibrating the first systolic peak with brachial SBP, was considered the central SBP. When AIx was more than 100%, brachial SBP was considered equal to central SBP. Central BP (SBP2) measured using the HEM-9000AI was therefore always lower than or equal to brachial SBP. Pulse pressure (PP) amplification was calculated by subtracting central PP from brachial PP and expressed in absolute values (mmHg). The validity of SBP2 in estimating central SBP has been demonstrated by invasive simultaneous measurement of the ascending aorta and radial artery pressure [1, 2]. Measurements were taken twice, and the mean value was used in analysis.



Measurement of brachial-to-ankle pulse wave velocity (PWV)

Cuffs were applied to both brachia and ankles, and all BPs were simultaneously measured using a cuff-oscillometric device (Vasera-1500, Fukuda Denshi, Tokyo, Japan). The pulse volume waveforms were also recorded simultaneously using a plethysmographic sensor connected to the cuffs. The baPWV was calculated from the time interval between the wave fronts of the brachial and ankle waveforms, and the path length from the brachia to ankle (0.597 × height + 14.4014) [3, 4].

Retinal vessel caliber measurements

A fundus photograph of the right eye was used for retinal caliber measurements. Measurements were taken in each of the 6 largest arterioles and venules within a 0.5- to 1-disc diameter from the optic disc margin. Central retinal arteriolar equivalent (CRAE) were calculated using the formulas first developed by Hubbard et al. (1999) [5] and later modified by Kundtson (2003) [6], and then adjusted for ocular magnification by axial length, corneal curvature, and ocular refractive value using the Littmann method [7]. Corneal curvature and ocular refraction were measured using an automatic refractometer (Autorefractor ARK-530; Nidek, Tokyo, Japan). Axial lengths, from anterior pole to posterior pole of eyeball, were measured using a partial coherence interferometry device (IOLMaster; Carl Zeiss, Jena, Germany). Analysis was performed by three trained graders with no clinical information about the subject.

Genome-wide association study (GWAS)

Genome-wide single nucleotide polymorphism (SNP) analysis was performed with a subset of the Nagahama cohort sample using DNA extracted from peripheral blood samples by the phenol-chloroform method. Briefly, a series of BeadChip DNA arrays (Illumina, San Diego, CA, USA) were used for analysis, namely HumanHap610 quad (1,828 samples),

HumanOmni2.5-4 (1,616 samples), HumanOmni2.5-8 (378 samples), HumanOmni2.5s (192 samples), HumanExome (192 samples), and HumanCoreExome (112 samples) (Illumina), and several samples were repeatedly genotyped using different arrays. Of a total of 3,712 genotyped samples, 445 met the following criteria and were excluded from analysis: call rate of analyzed SNPs <95% (n=137), high degrees of kinship (Pi-hat >0.35, PLINK ver. 1.07 [8]) (n=301), and ancestry outliers as identified by principal component analysis with HapMap Phase 2 release 28 JPT dataset as reference (EIGENSTRAT ver.2.0 [9]) (n=7). Of the 3,267 remaining samples, the available urinary sodium and potassium values (n=3,095) were used in the association analysis.

After applying a standard quality control for the genome-wide genotype data (call rate <99%, minor allele frequency <0.1%, or extreme deviation from Hardy-Weinberg equilibrium [$p<1.0 \times 10^{-6}$]), genotype imputation was performed by a standard two-step procedure using MACH ver. 1.0.16 software [9] with 1000 Genomes Phase I Integrated Release Version 3 for ASN (CHB+CHS+JPT) as a reference. As imputed SNPs with a minor allele frequency of <0.01 or R^2 <0.3 were excluded, 7,280,945 SNPs were ultimately used in the association analysis.

Rank-based inverse normal transformation was applied to the urinary sodium (Na) and potassium (K) levels. GWAS of the normalized Na and K levels was performed by linear regression analysis under an additive genetic model adjusted for age, sex, and BMI. GWAS of urinary creatinine-adjusted Na and K levels was also performed by the same method.

Replication genotype

Replication analysis was performed with a subset of the remaining Nagahama samples for which clinical data and genomic data were available (n=5,732). Genotypes were analyzed with a TaqMan probe assay using commercially available primer and probe sets purchased from Life Technologies Corporation (Carlsbad, CA, USA).

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Supplementary Table 1. Multivariate analysis for spot urine Na/K

		β	*P*
Age (years)		-0.104	<0.001
Sex (female)		-0.035	0.002
BMI (kg/m^2)		0.051	<0.001
Salt restriction status		-0.057	<0.001
Current smoking habit		0.027	0.009
Seasonal variation[#]	*Middle*	0.002	0.866
	Summer	-0.076	<0.001
Brachial SBP (mmHg)		0.126	<0.001
Antihypertensive medication status		0.048	<0.001
Fasting time (h)		-0.177	<0.001
eGFR (ml/min/1.73 m^2)		0.106	<0.001
Serum phosphate (mg/dl)		-0.152	<0.001
Urinary creatinine (mg/dl)		-0.312	<0.001

BMI, body mass index; eGFR, estimated glomerular filtration rate

eGFR was calculated using the following formula: $194 \times \text{creatinine}^{-1.094} \times \text{age}^{-0.287} \times 0.739$ (if female).

[#]Calculated using winter season as a reference

Supplementary Table 2. Multivariate analysis for brachial SBP

		β	*P*
Age (years)		0.344	<0.001
Sex (female)		-0.153	<0.001
BMI (kg/m^2)		0.170	<0.001
Salt restriction status		0.024	0.009
Current smoking habit		0.006	0.549
Seasonal variation[#]	*Middle*	0.006	0.566
	Summer	-0.046	<0.001
Antihypertensive medication status		0.104	<0.001
Fasting time (h)		0.031	0.001
eGFR (ml/min/1.73 m^2)		-0.016	0.128
Serum phosphate (mg/dl)		-0.046	<0.001
Urinary creatinine (mg/dl)		-0.016	0.117
Urinary Na/K		0.112	<0.001

BMI, body mass index; eGFR, estimated glomerular filtration rate

eGFR was calculated using the following formula: $194 \times \text{creatinine}^{-1.094} \times \text{age}^{-0.287} \times 0.739$ (if female).

[#]Calculated using winter season as a reference

Supplementary Table 3. Multivariate analysis for arterial parameters

	baPWV		CRAE	
	β	*P*	*β*	*P*
Age (years)	0.420	<0.001	-0.078	<0.001
Sex (female)	-0.008	0.259	-0.028	0.020
BMI (kg/m^2)	-0.037	<0.001	0.007	0.562
Current smoking habit	-0.005	0.478	0.073	<0.001
SBP (mmHg)	0.415	<0.001	-0.269	<0.001
Antihypertensive medication status	0.105	<0.001	-0.046	<0.001
Type 2 diabetes	0.058	<0.001	0.018	0.107
Hyperlipidemia	0.034	<0.001	0.016	0.162
Urinary Na/K	0.005	0.496	-0.009	0.431

baPWV, brachial-to-ankle pulse wave velocity; CRAE, central retinal arteriolar equivalent (values are available for 7,700 subjects); BMI, body mass index

Type 2 diabetes was defined as any or all of HbA1c ≥6.5%, fasting plasma glucose ≥126 mg/dl, occasional plasma glucose ≥200 mg/dl, or taking antihyperglycemic drugs. Dyslipidemia was defined as any or all of LDL cholesterol ≥140 mg/dl, HDL cholesterol <40 mg/dl, triglyceride ≥150 mg/dl, or taking antihyperlipidemic drugs.

Supplementary Table 4. Clinical characteristics of study subjects of GWAS

		GWAS (n=3,095)	**Replication (n=5,716)**
Age (years)		53±14	55±13
Sex (male/female)		1,089/2,006	1,924/3,792
BMI (kg/m^2)		22.3±3.2	22.4±3.3
Urinary markers	*Na (mEq/l)*	127±62	128±63
	Na/Cre	1.85±1.02	1.83±0.98
	K (mEq/l)	49±31	49±31
	K/Cre	0.63±0.27	0.62±0.26
	Na/K	3.22±1.97	3.22±1.92

BMI, body mass index; Cre, urinary creatinine; GWAS, genome-wide association study

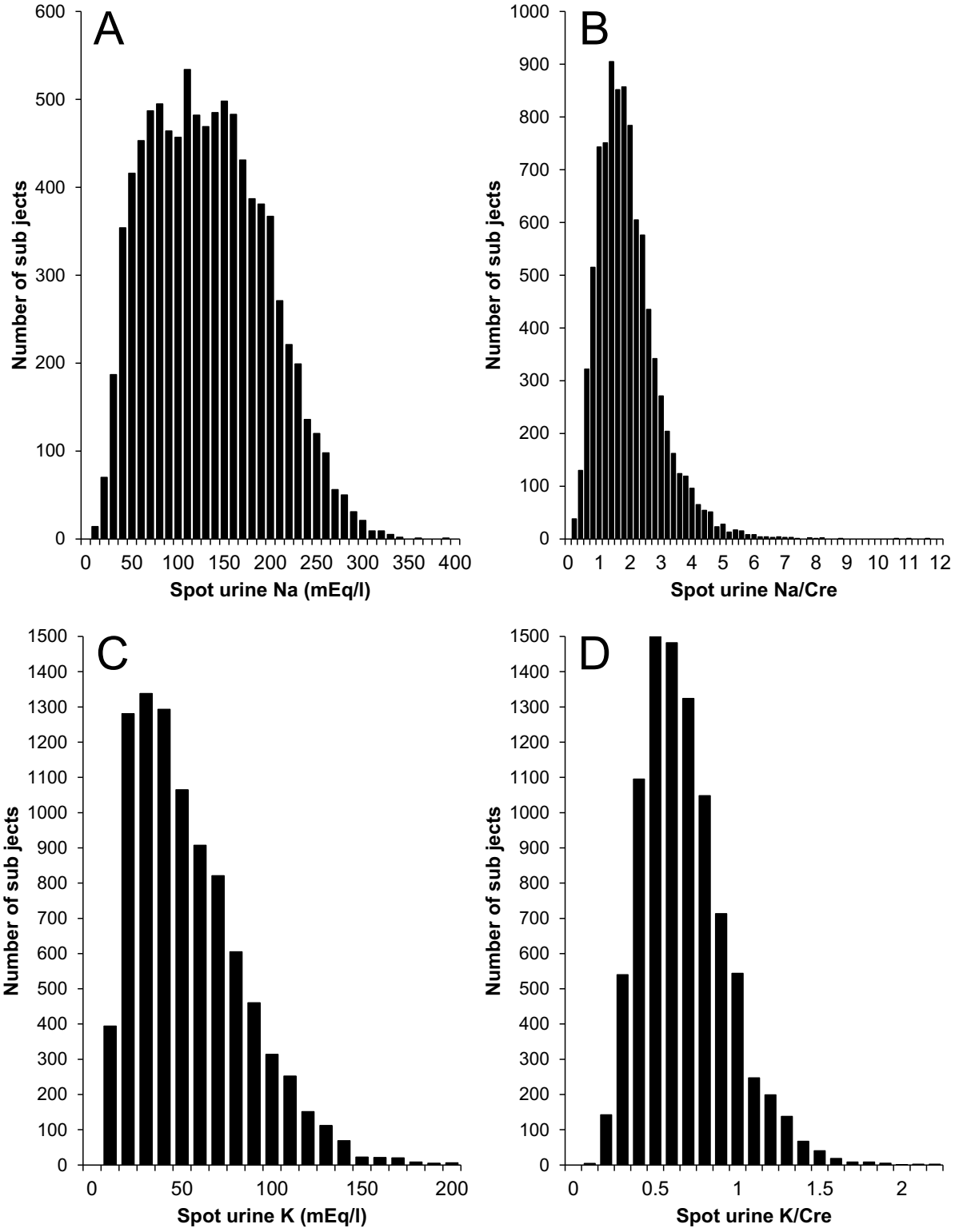
Values are mean ± standard deviation.

Supplementary Table 5. A top-hit SNP in GWAS of urinary sodium level

Chr	rs Number	GWAS				Replication		
		Coded allele (freq)	*n (HWE p)*	*β (s.e.)*	*P*	*n (HWE p)*	*β (s.e.)*	*P*
1	rs12092050	A (0.168)	3,095 (0.003)	0.191 (0.034)	$1.35^{*}10^{-8}$	5,716 (0.185)	0.012 (0.024)	0.618

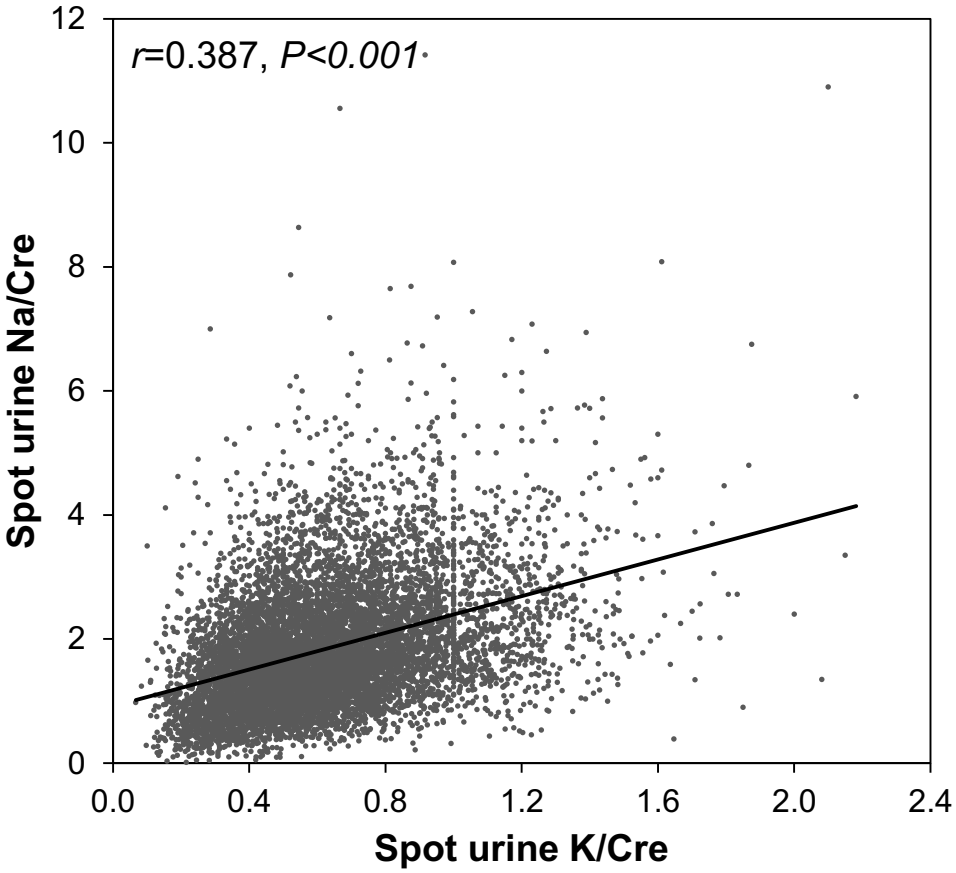
GWAS, genome-wide association study; Chr, chromosome; HWE, Hardy-Weinberg equilibrium; s.e., standard error

Urinary sodium value was transformed by rank-based inverse normal transformation. *β* and *P*-values in the additive regression models adjusted for age, sex, and BMI are shown.

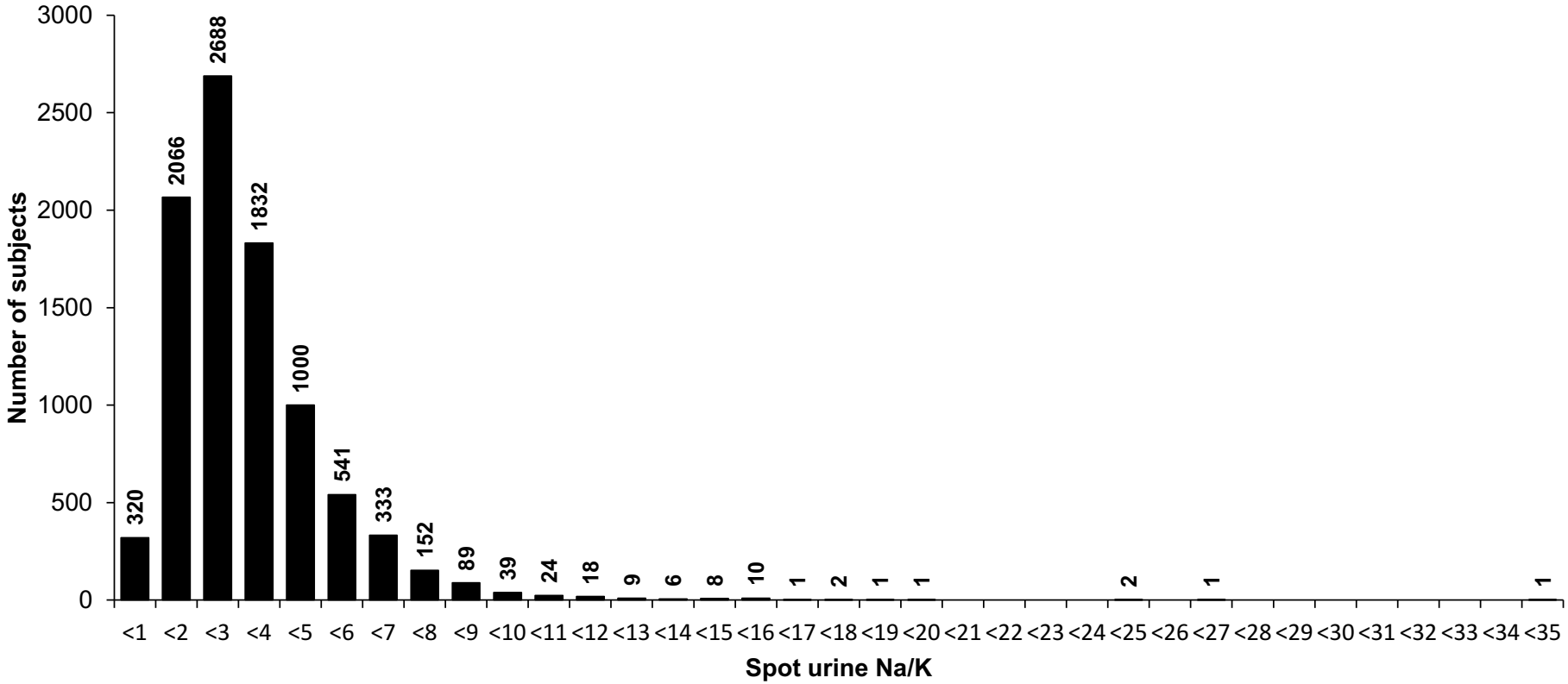


Supplementary Figure 1. Histograms of spot urine Na and K

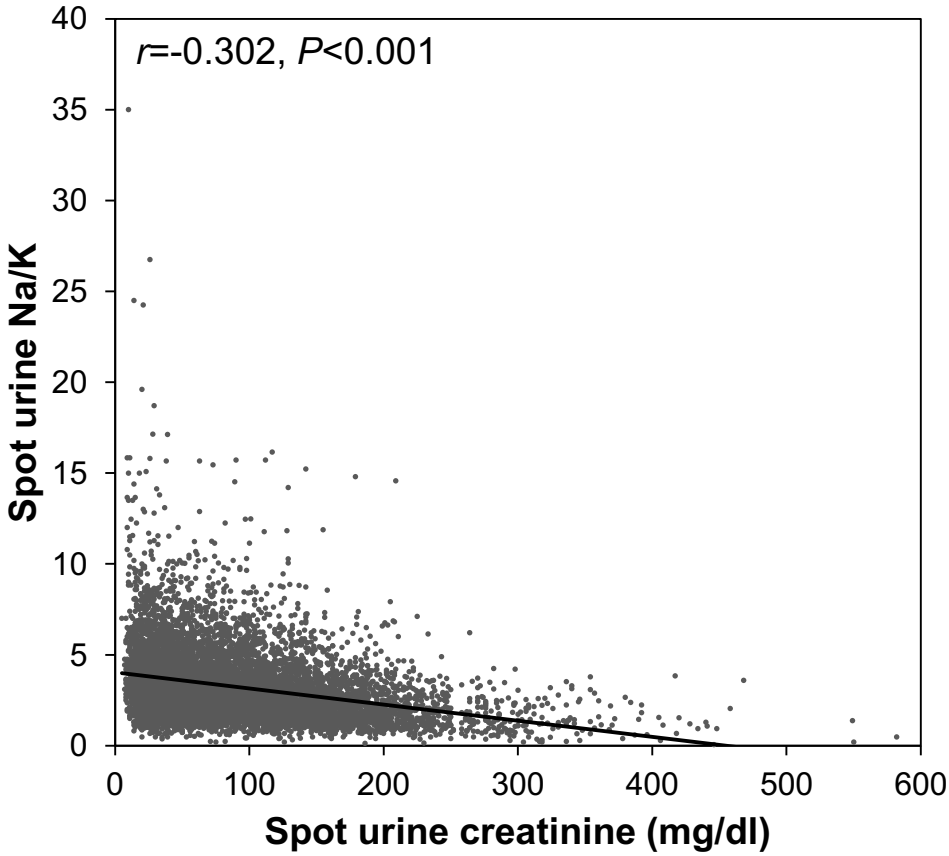
A) Sodium, **B**) sodium/creatinine, **C**) potassium, **D**) potassium/creatinine



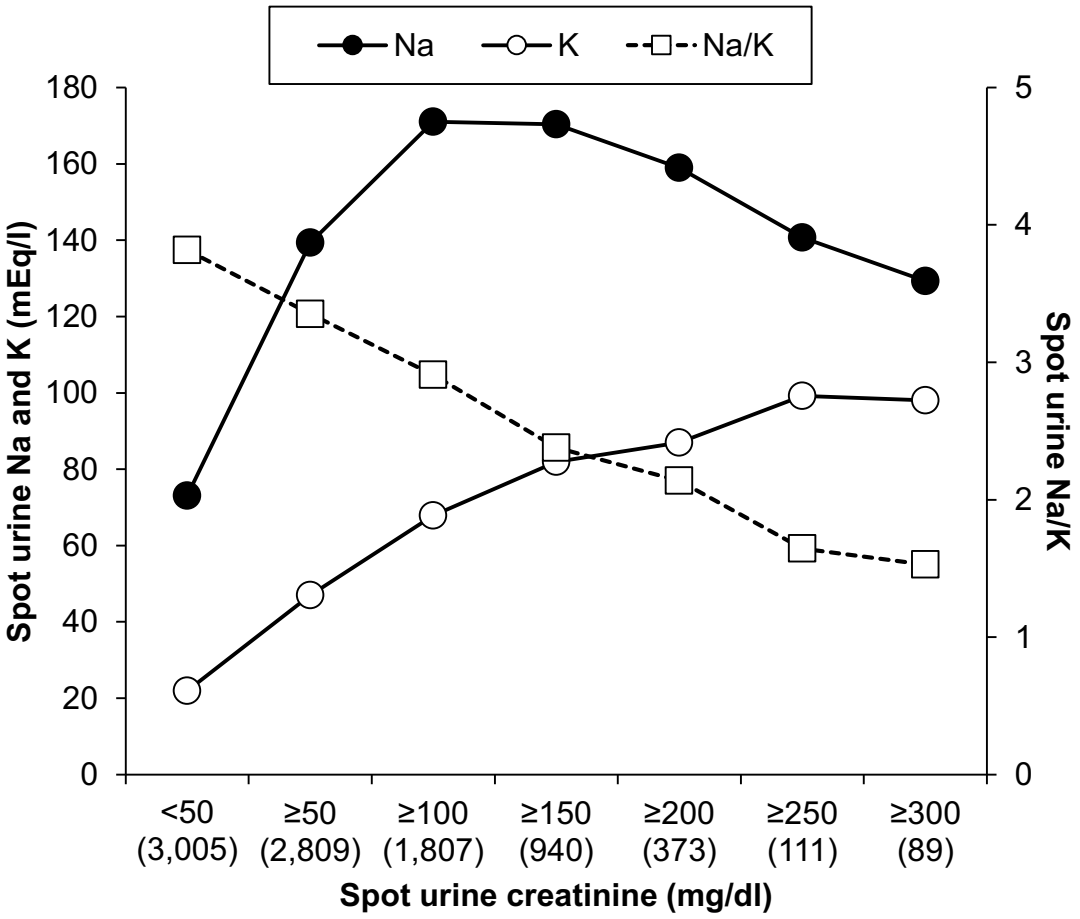
Supplementary Figure 2. Scatter plot of spot urine Na and K.
Values are correlation coefficient and *P*-value. Cre, urinary creatinine



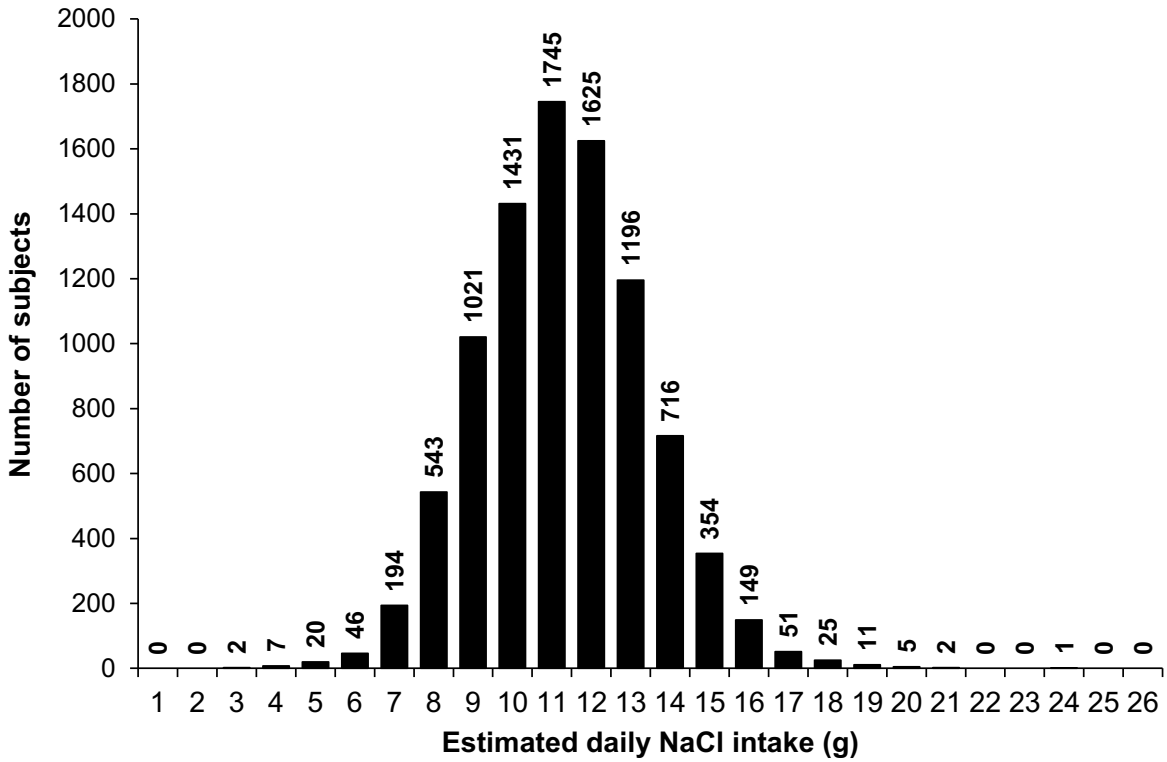
Supplementary Figure 3. Histogram of spot urine Na/K
Number of subjects in each group is indicated above each bar



Supplementary Figure 4. Scatter plot of spot urine creatinine and Na/K
Values are correlation coefficient and *P*-value



Supplementary Figure 5. Associations of spot urine creatinine with Na, K, and Na/K
Number of subjects in each creatinine subgroup in parentheses



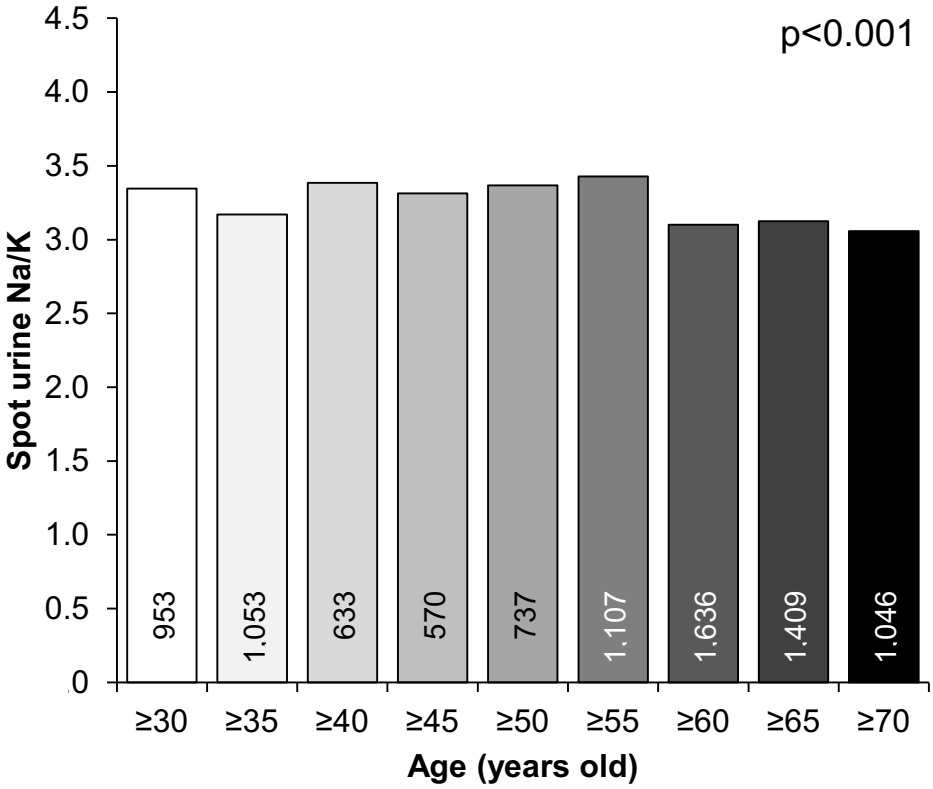
Supplementary Figure 6. Histogram of estimated daily salt intake

Number of subjects in each subgroup is indicated above each bar. Daily salt intake (g) was estimated from spot urinary sodium and creatinine values using the following formula (Tanaka et al., ***J Hum Hypertens***. 2002;**16**:97-103):

$21.98 \times \{Na\ (mEq/l) \times predicted\ 24\ h\ Cre\ excretion\ /\ [Cre\ (mg/dl) \times 10]\}^{0.392} \times 0.0585$,

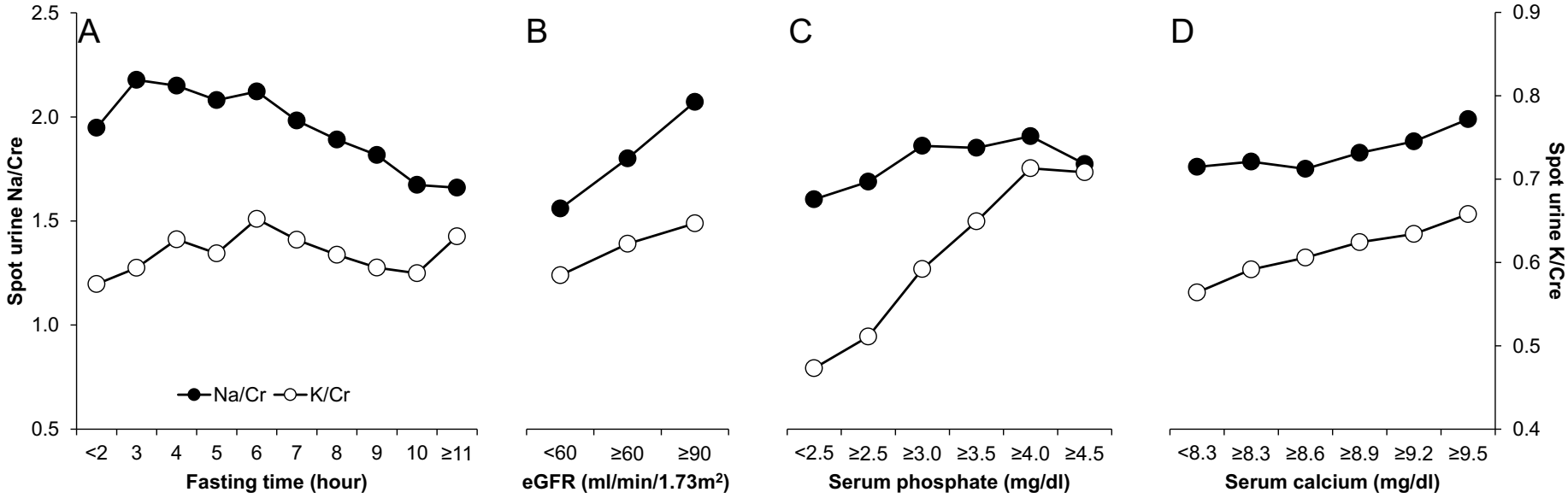
where 24 h Cre excretion was predicted using the following formula:

body weight (kg) × 14.89 + body height (cm) × 16.14 − age × 2.04 − 2444.45.



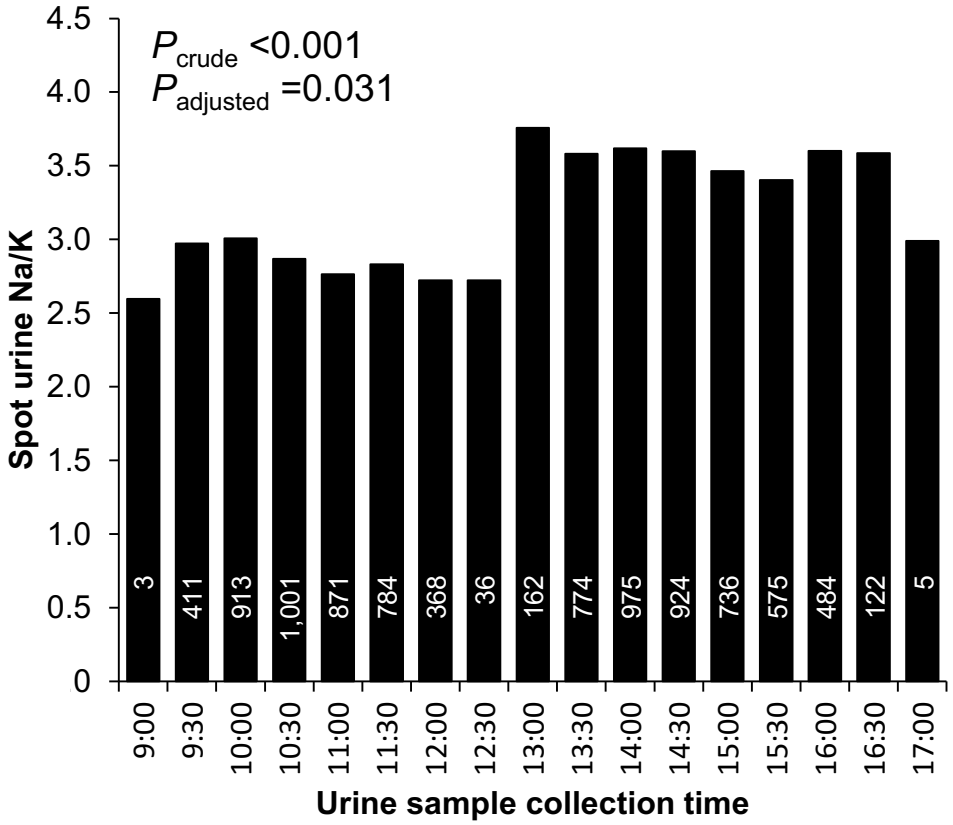
Supplementary Figure 7. Age related changes in spot urine Na/K

Each column indicates mean value. Numbers of subjects in each subgroup are shown in columns. Statistical significance was assessed by analysis of variance.



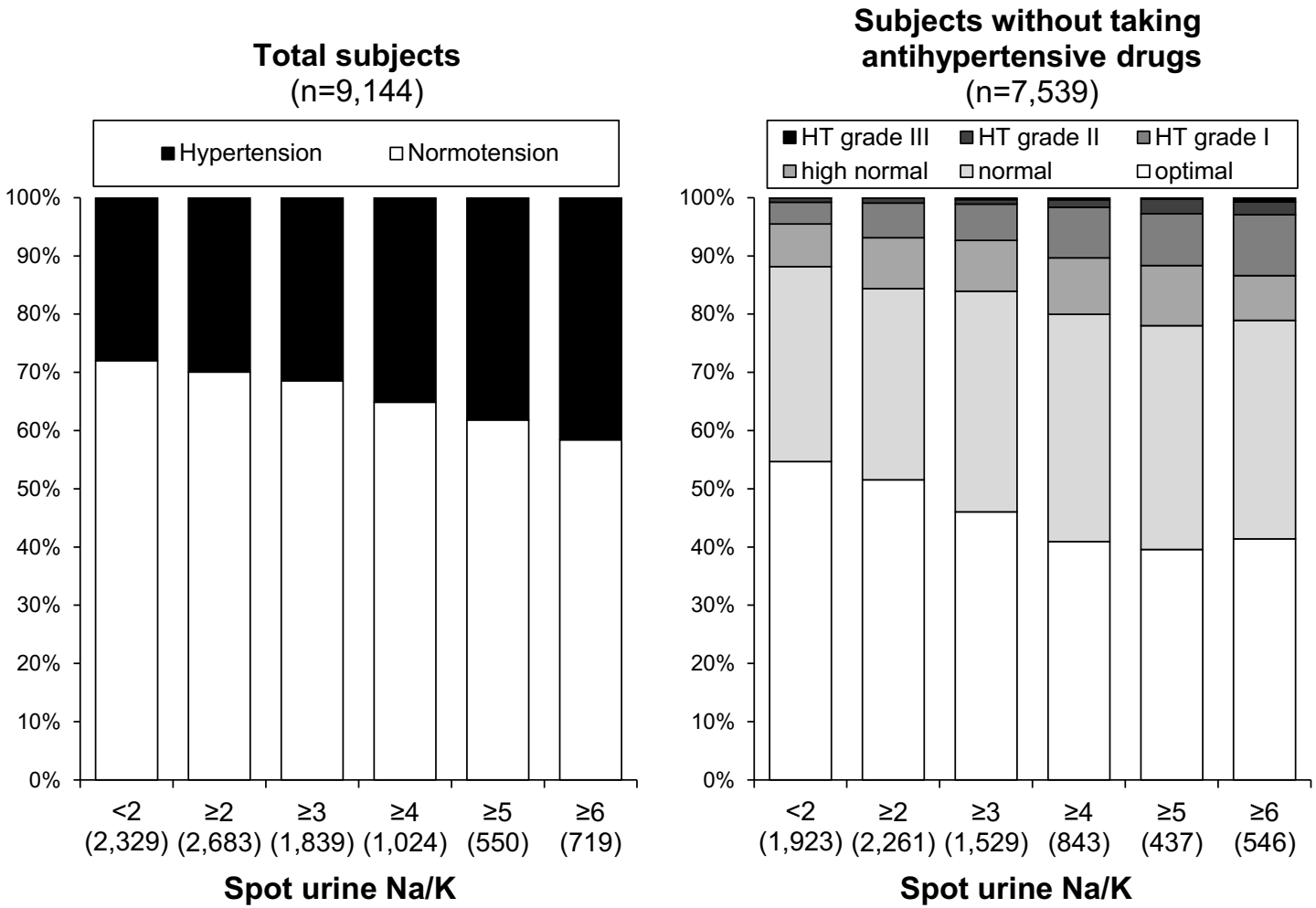
Supplementary Figure 8. Association of environmental and clinical factors with spot urine Na and K levels

Association of urinary Na and K with A) time since last meal, B) eGFR, C) serum phosphate, and D) serum calcium. Urinary Na and K levels were adjusted by urinary Cre.



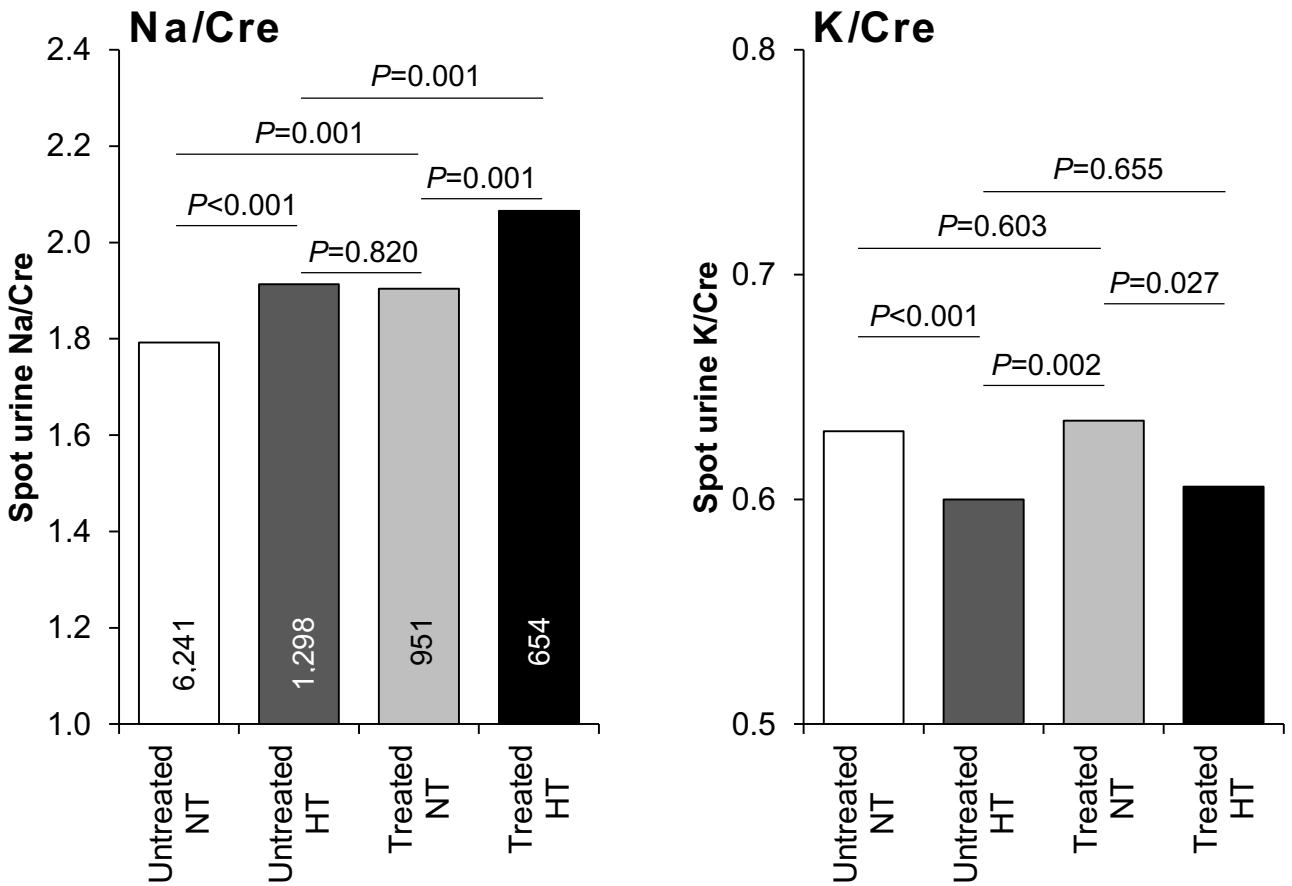
Supplementary Figure 9. Association of urine sample collection time with Na/K

Each column indicates mean value. Number of subjects in each subgroup is indicated in columns. Timestamp of pulse wave velocity measurement was used as a substitute for urine sample collection. Differences in mean values were assessed by analysis of variance. Covariate adjusted analysis was performed using a linear regression model with adjustment for the following factors: age, sex, body mass index, salt restriction, current smoking, seasonal variation, brachial SBP, antihypertensive medication, fasting time, estimated glomerular filtration rate, serum phosphate, and urinary creatinine.

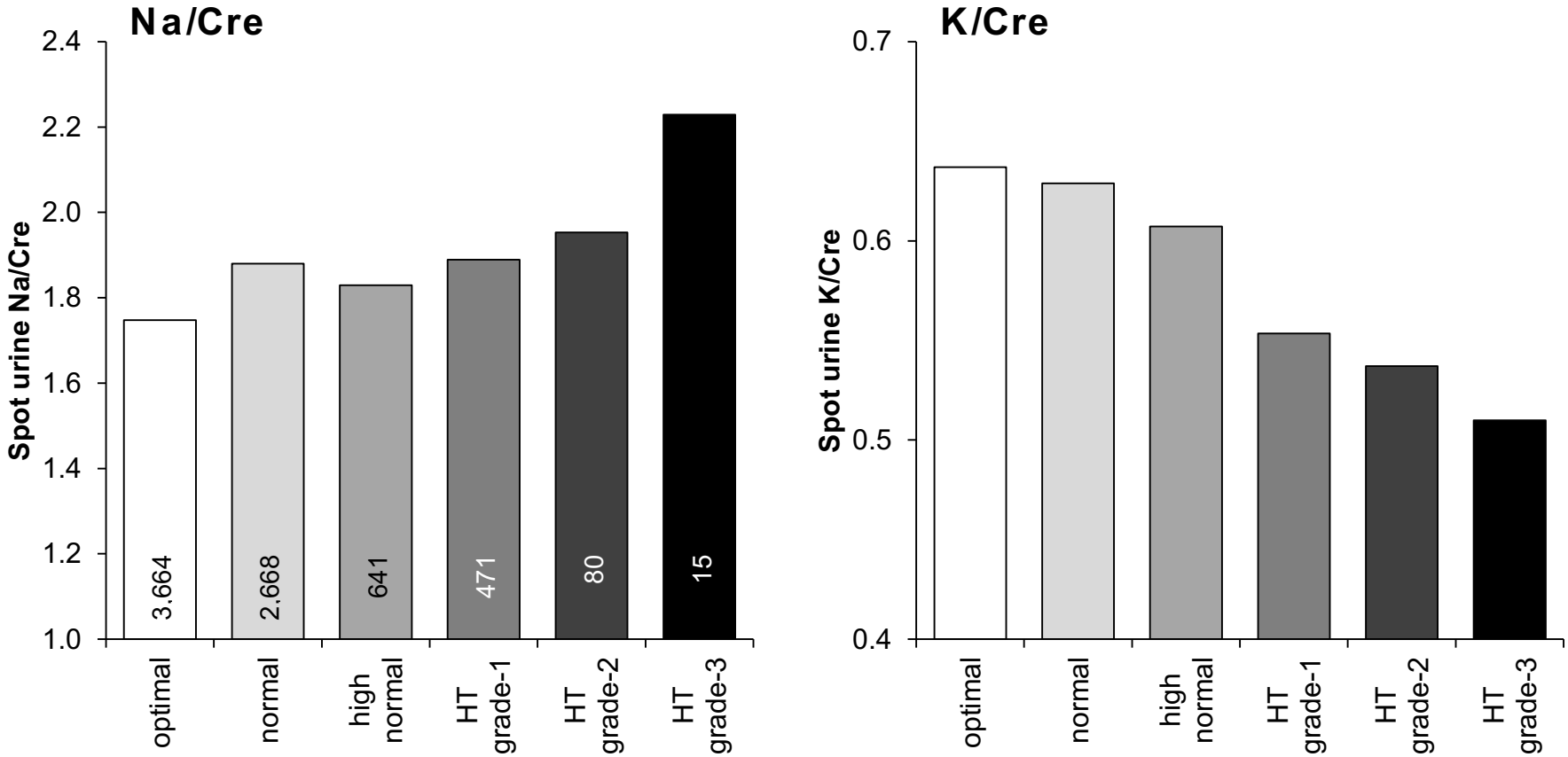


Supplementary Figure 10. Differences in frequency of hypertension by spot urine Na/K

Number of subjects in each subgroup is shown in the parentheses. Left panel: hypertension was defined as any or all of SBP ≥140 mmHg, DBP ≥90 mmHg, or taking antihypertensive drugs. Right panel: grade of hypertension was defined according to the guideline for the management of hypertension by the Japanese Society of Hypertension.

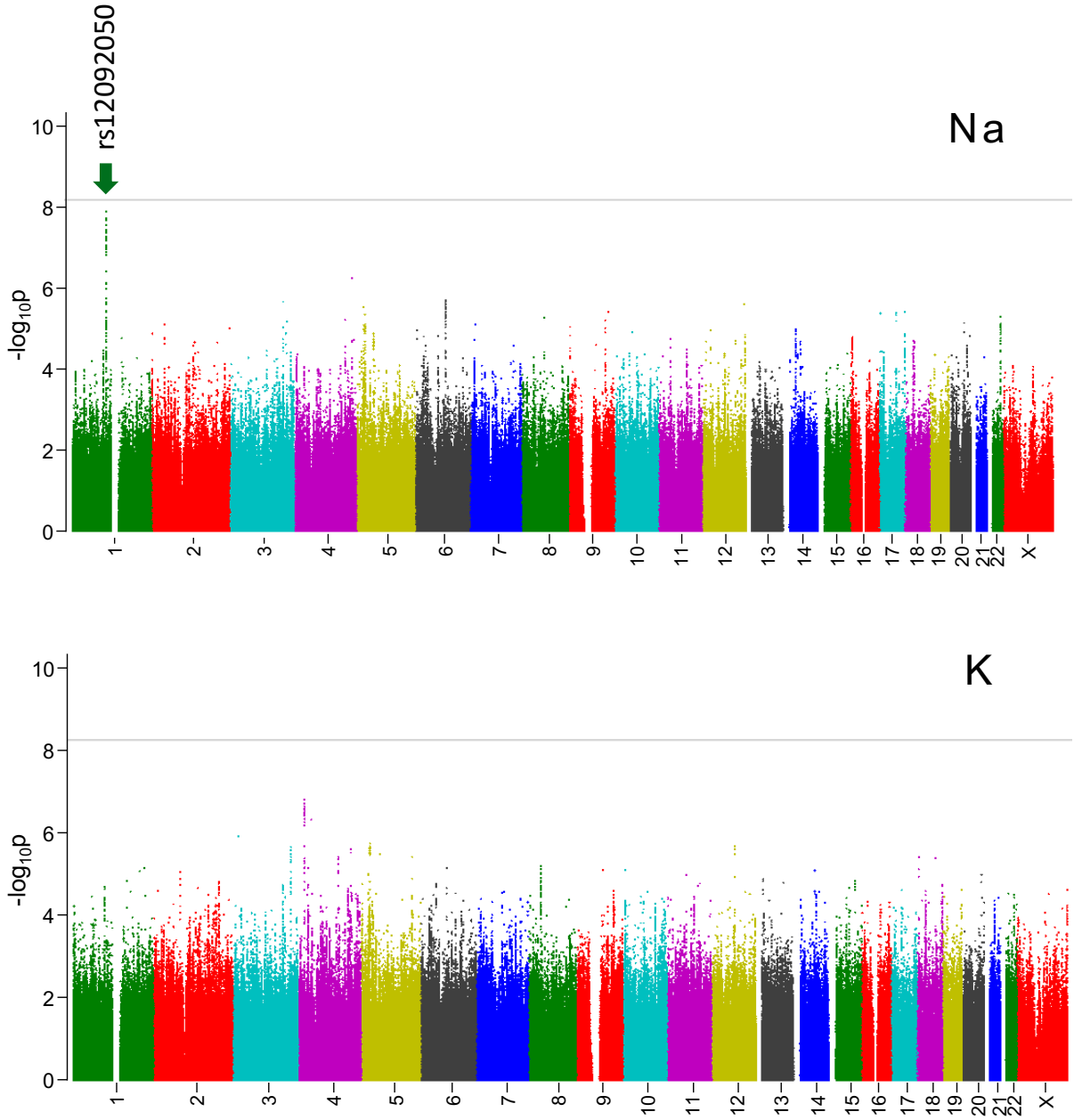


Supplementary Figure 11. Association of BP with creatinine-adjusted spot urine Na and K levels.
Number of subjects in each subgroup is shown in the parentheses. Hypertension was defined as any or all of brachial SBP ≥140 mmHg, DBP ≥90 mmHg, or taking antihypertensive drugs.



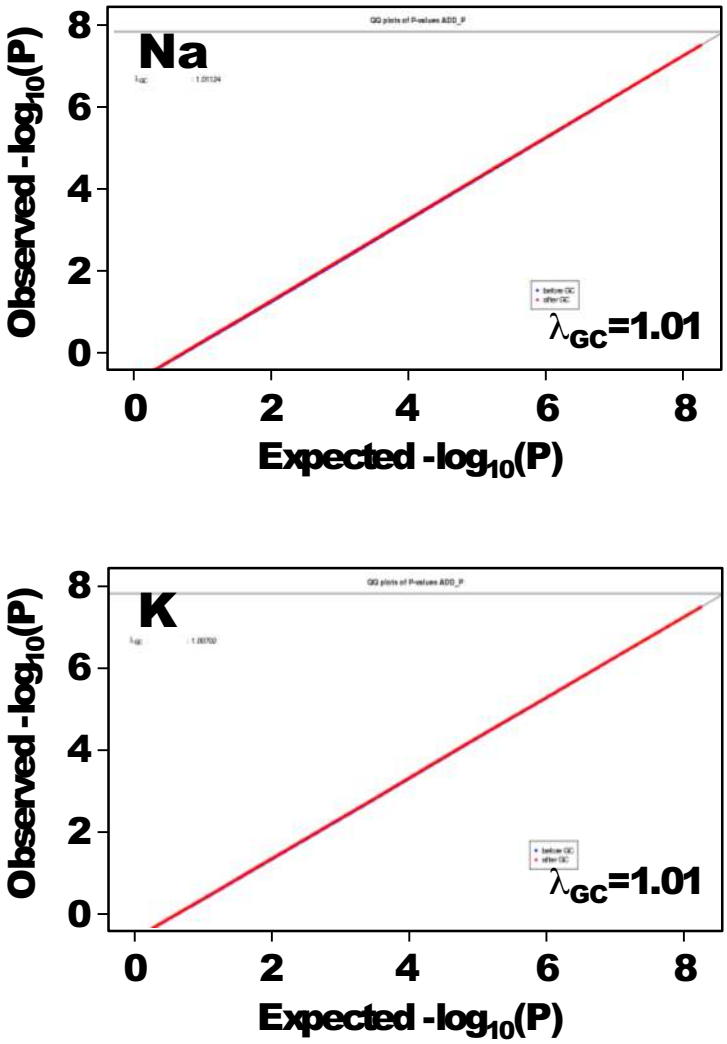
Supplementary Figure 12. Association of BP with creatinine-adjusted spot urine Na and K levels.

Number of subjects in each subgroup is shown in the parentheses. BP was graded according to the guideline for the management of hypertension by the Japanese Society of Hypertension; optimal, SBP <120 mmHg and DBP <80 mmHg; normal, SBP 120-129 mmHg and/or DBP 80-84 mmHg; high normal, SBP 130-139 mmHg and/or DBP 85-89 mmHg; grade I hypertension, SBP 140-159 mmHg and/or DBP 90-99 mmHg; grade II hypertension, SBP 160-179 mmHg and/or DBP 100-109 mmHg; grade III hypertension, SBP ≥180 mmHg and/or DBP ≥110 mmHg.



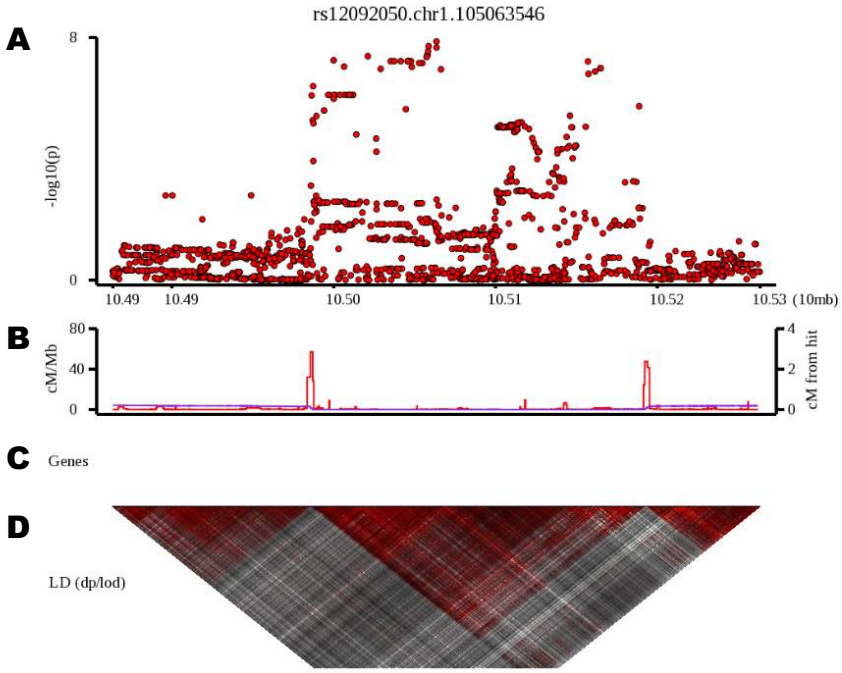
Supplementary Figure 13. Manhattan plots of association analysis for spot urine Na and K levels

Rank-based inverse normal transformation was applied to urinary Na and K values to normalize their distributions. Adjusted factors in the association analysis were age, sex, and body mass index. Association analysis was performed using PLINK v1.07 software.

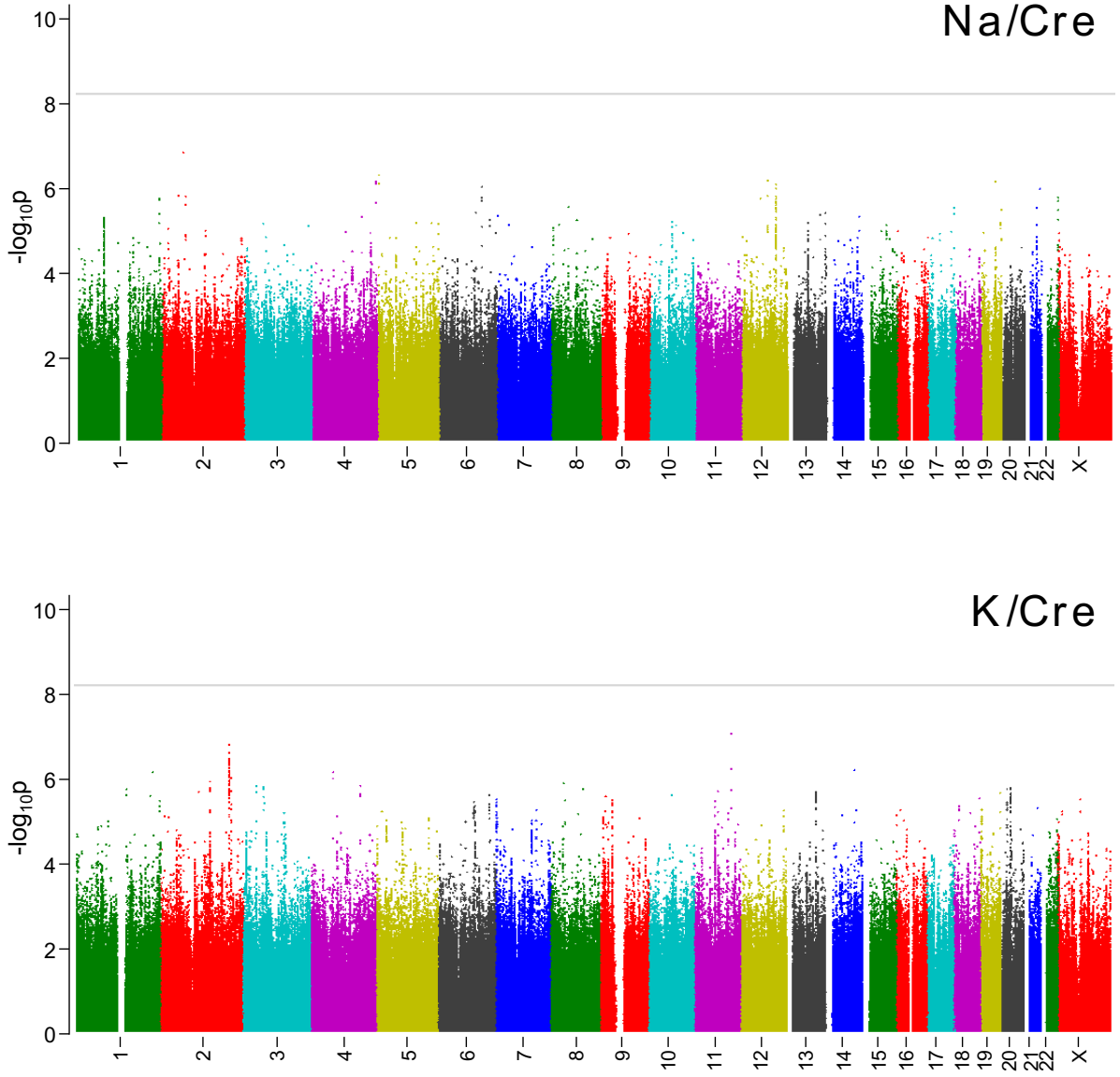


Supplementary Figure 14. Quantile-Quantile plots of the association analysis for spot urine Na and K

The Quantile-Quantile plot (QQ-plot) for the *P*-values of all SNPs that passed quality control criteria and for the *P*-values corrected for genomic control factor are indicated by blue and red dots, respectively. Lambda values before genomic control correction (λ_{GC}) are shown in each graph.



Supplementary Figure 15. Regional association plots of loci around the SNP rs12092050
A) *P*-values ($-\log_{10}$[p]) of genotyped SNPs around 200 kb to either side of the most strongly associated signal are shown in red dots. Genomic positions are based on NCBI reference sequence build 36 and dbSNP build 126. B) Red line indicates the recombination rate (cM/Mb) estimated from a HapMap JPT (release 28) dataset. Purple line indicates cumulative genetic distance (in cM) from the strongest signal. C) Blue lines (intron) and boxes (exon) indicate the locations of known genes (no gene in this region). D) Linkage disequilibrium (LD) map calculated using the Hapmap JPT dataset.



Supplementary Figure 16. Manhattan plots of association analysis for creatinine-adjusted spot urine Na and K levels

Adjusted factors in the association analysis were age, sex, and body mass index. Association analysis was performed using PLINK v1.07 software.