**Table 2.** Clinical Characteristics of Included Employees

| | Baseline Sleep Duration | | | | |
|---|---|---|---|---|---|
| | ≤4 h | 5 h | 6 h | ≥7 h | $P_{trend}$ |
| No. of employees | 266 | 1,543 | 3,155 | 1,870 | |
| Mean sleep duration during observational period (h) | 4.5 ± 0.7 | 5.3 ± 0.6 | 6.0 ± 0.5 | 6.7 ± 0.6 | |
| Demographic and physical data | | | | | |
| Age (y) | 34 (29-41) | 34 (28-42) | 34 (29-42) | 34 (29-41) | 0.5 |
| Age category | | | | | |
| 20-29 y | 75 (28.2) | 473 (30.7) | 935 (29.6) | 514 (27.5) | |
| 30-39 y | 111 (41.7) | 598 (28.8) | 1,269 (40.2) | 811 (43.4) | |
| 40-49 y | 53 (19.9) | 304 (19.7) | 595 (18.9) | 313 (16.7) | |
| 50-59 y | 24 (9.0) | 151 (9.8) | 317 (10.0) | 195 (10.4) | |
| 60-65 y | 3 (1.1) | 17 (1.1) | 39 (1.2) | 37 (2.0) | |
| Men | 124 (46.6) | 752 (48.7) | 1,599 (50.7) | 970 (51.9) | 0.03 |
| Occupations | | | | | |
| Clerical workers | 60 (22.6) | 371 (24.0) | 917 (29.1) | 561 (30.0) | <0.001[a] |
| Academic researchers | 75 (28.2) | 489 (31.7) | 1,212 (38.4) | 865 (46.3) | <0.001[a] |
| Engineers and technical assistants | 22 (8.3) | 113 (7.3) | 216 (6.8) | 122 (6.5) | 0.2[a] |
| Health care workers | 104 (39.1) | 543 (35.2) | 788 (25.0) | 301 (16.1) | <0.001[a] |
| Other employees | 5 (1.9) | 27 (1.7) | 22 (0.7) | 21 (1.1) | 0.05[a] |
| BMI (kg/m$^2$) | 22.0 ± 3.4 | 22.1 ± 3.5 | 21.8 ± 3.2 | 21.6 ± 3.1 | <0.001 |
| MAP (mm Hg) | 85 ± 13 | 85 ± 12 | 85 ± 12 | 85 ± 12 | 0.2 |
| Lifestyle data | | | | | |
| Smoking status | | | | | |
| Nonsmokers | 216 (81.2) | 1,249 (80.9) | 2,540 (80.5) | 1,503 (80.4) | 0.6[b] |
| Past smokers | 19 (7.1) | 121 (7.8) | 282 (8.9) | 167 (8.9) | |
| Current smokers | | | | | |
| 1-10 cigarettes/d | 11 (4.1) | 62 (4.0) | 111 (3.5) | 75 (4.0) | |
| 11-20 cigarettes/d | 15 (5.6) | 87 (5.6) | 176 (5.6) | 99 (5.3) | |
| ≥21 cigarettes/d | 5 (1.9) | 24 (1.6) | 46 (1.5) | 26 (1.4) | |
| Alcohol consumption | | | | | |
| Rarely | 165 (62.0) | 836 (54.2) | 1,649 (52.3) | 975 (52.1) | 0.01[c] |
| 1-3 d/wk | 64 (24.1) | 443 (28.7) | 885 (28.1) | 488 (26.1) | |
| 4-6 d/wk | 20 (7.5) | 118 (7.6) | 268 (8.5) | 154 (8.2) | |
| 7 d/wk | 17 (6.4) | 146 (9.5) | 353 (11.2) | 253 (13.5) | |
| Excessive daytime somnolence | 30 (11.3) | 76 (4.9) | 100 (3.2) | 42 (2.2) | <0.001 |
| Laboratory data | | | | | |
| Urinary protein by dipstick test | | | | | <0.001 |
| Negative | 242 (91.0) | 1,463 (94.8) | 3,024 (95.8) | 1,806 (96.6) | |
| Trace | 24 (9.0) | 80 (5.2) | 131 (4.2) | 64 (3.4) | |
| Hematuria by dipstick test | | | | | |
| Negative | 230 (86.5) | 1,333 (86.4) | 2,671 (84.7) | 1,576 (84.3) | } 0.05[d] |
| Trace | 8 (3.0) | 27 (1.7) | 63 (2.0) | 36 (1.9) | |
| 1+ | 19 (7.1) | 129 (8.4) | 307 (9.7) | 187 (10.0) | |
| ≥2+ | 9 (3.4) | 54 (3.5) | 114 (3.6) | 71 (3.8) | |
| eGFR (mL/min/1.73 m$^2$) | 89 ± 15 | 90 ± 15 | 90 ± 15 | 91 ± 16 | 0.05 |
| eGFR category | | | | | |
| ≥120 mL/min/1.73 m$^2$ | 8 (3.0) | 55 (3.6) | 128 (4.1) | 98 (5.2) | |
| 105-119 mL/min/1.73 m$^2$ | 26 (9.8) | 175 (11.3) | 374 (11.9) | 239 (12.8) | |
| 90-104 mL/min/1.73 m$^2$ | 81 (30.5) | 493 (32.0) | 984 (31.2) | 558 (29.8) | |
| 75-89 mL/min/1.73 m$^2$ | 112 (42.1) | 584 (37.8) | 1,181 (37.4) | 713 (38.1) | |
| 60-74 mL/min/1.73 m$^2$ | 39 (14.7) | 236 (15.3) | 488 (15.5) | 262 (14.0) | |
| Hemoglobin $A_{1c}$ (%) | 5.0 ± 0.4 | 5.0 ± 0.4 | 4.9 ± 0.4 | 4.9 ± 0.4 | <0.001 |
| Total cholesterol (mg/dL) | 189 ± 33 | 190 ± 32 | 192 ± 32 | 192 ± 34 | 0.08 |
| Triglycerides (mg/dL) | 59 (43-92) | 62 (44-92) | 63 (44-95) | 65 (47-97) | <0.001 |
| Uric acid (mg/dL) | 5.0 ± 1.3 | 5.1 ± 1.4 | 5.1 ± 1.4 | 5.1 ± 1.4 | 0.03 |

*(Continued)*

**Table 2 (Cont'd).** Clinical Characteristics of Included Employees

| | Baseline Sleep Duration | | | | |
|---|---|---|---|---|---|
| | ≤4 h | 5 h | 6 h | ≥7 h | $P_{trend}$ |
| Treatments for comorbid conditions | | | | | |
| Hypertension | 5 (1.9) | 37 (2.4) | 61 (1.9) | 42 (2.2) | 0.9 |
| Diabetes | 0 (0.0) | 14 (0.9) | 15 (0.5) | 9 (0.5) | 0.4 |
| Dyslipidemia | 4 (1.5) | 27 (1.7) | 38 (1.2) | 17 (0.9) | 0.04 |
| Hyperuricemia | 2 (0.8) | 7 (0.5) | 22 (0.7) | 11 (0.6) | 0.8 |
| Heart diseases | 0 (0.0) | 4 (0.3) | 4 (0.1) | 5 (0.3) | 0.6 |
| Outcome and follow-up data | | | | | |
| Observational period (y) | 2.5 (1.2-4.0) | 2.4 (1.4-3.9) | 2.3 (1.3-3.9) | 2.5 (1.5-3.9) | 0.9 |
| No. of examinations | | | | | 0.4 |
| 1 | 81 (30.5) | 463 (30.0) | 1,016 (32.2) | 592 (31.7) | |
| 2 | 67 (25.2) | 380 (24.6) | 772 (24.5) | 448 (24.0) | |
| 3 | 44 (16.5) | 285 (18.5) | 566 (17.9) | 340 (18.2) | |
| 4 | 74 (27.8) | 414 (26.8) | 797 (25.3) | 483 (25.8) | |
| 5 | 0 (0.0) | 1 (0.1) | 4 (0.1) | 7 (0.4) | |
| Development of proteinuria | 33 (12.4) | 142 (9.2) | 240 (7.6) | 135 (7.2) | 0.002 |

*Note:* N = 6,834. Continuous variables are shown as mean ± standard deviation or median (25th-75th percentile); categorical variables given as number (percentage).

Abbreviations: BMI, body mass index; eGFR, estimated glomerular filtration rate; MAP, mean arterial pressure.

[a]Each occupation versus others (eg, clerical workers vs nonclerical workers).

[b]Nonsmokers versus others.

[c]Drinking rarely versus others.

[d]Negative or trace for hematuria by dipstick test versus 1+ or more.

## RESULTS

After excluding 2,703 employees whose outcome was unavailable due to a single examination during the entire observational period, the present study enrolled 6,834 employees without eGFR <60 mL/min/1.73 m$^2$, proteinuria, or treatment for self-reported kidney disease at baseline. Of these 6,834 individuals, sleep durations of those excluded and included were not significantly different (6.0 ± 0.9 vs 6.0 ± 0.9 hours; $P$ = 0.5; Table 1). Statistically significant differences were observed between the employees excluded and those included for age ($P$ < 0.001), occupation ($P$ < 0.001), MAP ($P$ < 0.001), smoking status ($P$ = 0.001), alcohol consumption ($P$ = 0.03), urinary protein and hematuria by dipstick test ($P$ < 0.001 and $P$ = 0.001), eGFR ($P$ = 0.009), hemoglobin $A_{1c}$ level ($P$ = 0.01), and total cholesterol level ($P$ < 0.001). However, the differences did not reach a clinically meaningful level, suggesting that the characteristics were clinically similar and that the 2 groups were at approximately the same risk of developing proteinuria.

Clinical characteristics of 266 (3.9%), 1,543 (22.6%), 3,155 (46.2%), and 1,870 (27.4%) employees with 4 or fewer, 5, 6, and 7 or more hours of sleep duration are listed in Table 2. Shorter sleep duration was associated significantly with female sex ($P_{trend}$ = 0.03), higher proportions of health care workers in the university hospitals ($P_{trend}$ < 0.001) and other occupations ($P_{trend}$ = 0.05), higher BMI ($P_{trend}$ < 0.001), higher proportion of those who consumed alcohol rarely ($P_{trend}$ = 0.01), higher prevalence of excessive daytime somnolence ($P_{trend}$ < 0.001), trace urinary protein ($P_{trend}$ < 0.001), negative/trace hematuria ($P_{trend}$ = 0.05), lower eGFR ($P_{trend}$ = 0.05), higher hemoglobin $A_{1c}$ level ($P_{trend}$ < 0.001), lower triglyceride level ($P_{trend}$ < 0.001), lower uric acid level ($P_{trend}$ = 0.03), and higher prevalence of current treatment for dyslipidemia ($P_{trend}$ = 0.04). During a median of 2.5 (25th-75th percentile, 1.4-3.9) years of the observational period, mean sleep durations during the observational period of employees with 4 or fewer, 5, 6, and 7 or more hours of sleep duration were 4.5 ± 0.7, 5.3 ± 0.6, 6.0 ± 0.5, and 6.7 ± 0.6 hours, respectively. Of 6,586 employees without baseline excessive daytime somnolence, 6,338 (96.2%) never reported it during the observational period, whereas 113 (45.6%) of 248 employees with baseline excessive daytime somnolence had at least one positive answer to the same question after the baseline visit and 61 employees (24.6%) reported it in ≥50% of visits. These results strongly suggested that baseline sleep duration and excessive daytime somnolence reflected their subsequent conditions after the baseline visit.

During the observational period, 33 (12.4%), 142 (9.2%), 240 (7.6%), and 135 (7.2%) employees with 4 or fewer, 5, 6, and 7 or more hours of sleep duration developed proteinuria, respectively ($P_{trend}$ = 0.002).

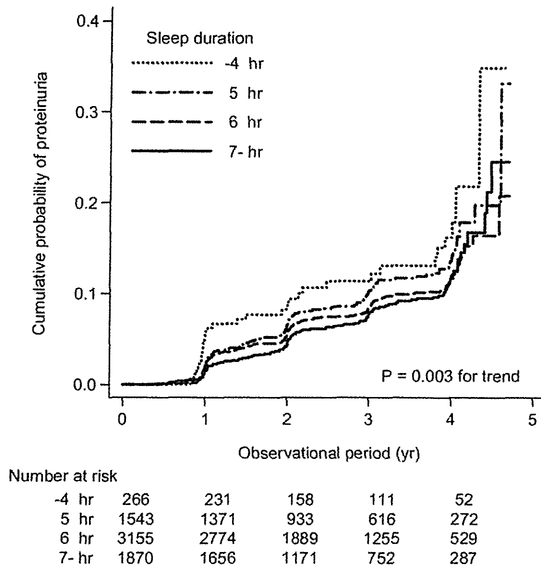


**Figure 1.** Estimated cumulative probability of the development of proteinuria in 6,834 included employees using the Kaplan-Meier method.

Cumulative probabilities of developing proteinuria at 1, 2, 3, and 4 years were 0.058, 0.088, 0.113, and 0.162 in employees with 4 or fewer hours of sleep duration; 0.020, 0.065, 0.102, and 0.139 in those with 5 hours; 0.020, 0.057, 0.084, and 0.116 in those with 6 hours; and 0.014, 0.047, 0.078, and 0.116 in those with 7 or more hours, showing that employees with shorter sleep duration were at higher risk of developing proteinuria ($P_{trend}$ = 0.003; Fig 1). Compared with 6,535 employees with negative baseline urinary protein results, 299 employees with trace baseline urinary protein were at significantly higher risk of developing proteinuria (mainly 1+: 499 [90.7%] of 550 employees who developed proteinuria; Table 3).

To identify predictors of proteinuria, the incidence rate ratio (IRR) for each covariate was calculated (Table 4). In univariate models, significant predictors of the development of proteinuria were younger age, female sex, health care workers in university hospitals, lower MAP, heavier smoking, shorter sleep duration, trace urinary protein, hematuria of 2+ or higher, higher eGFR, higher hemoglobin $A_{1c}$ level, lower uric acid level, and current treatment for heart disease. Even after adjustment for clinically relevant factors, shorter sleep duration of 5 or fewer hours was associated significantly with the development of proteinuria (compared with sleep duration ≥7 hours; IRRs of 1.28 [95% confidence interval (CI), 1.00-1.62] and 1.72 [95% CI, 1.16-2.53] for 5 and ≤4 hours, respectively). Also associated with proteinuria were younger age (compared with age 40-49 years, IRRs of 2.20 [95% CI, 1.61-3.00] and 1.48 [95% CI, 1.12-1.96] for ages 20-29 and 30-39 years, respectively), heavier smoking (compared with nonsmokers, IRR for current smokers with ≥21 cigarettes/d of 2.03 [95% CI, 1.16-3.56]), trace urinary protein (compared with negative result, IRR of 2.14 [95% CI, 1.56-2.92]), eGFR ≥120 mL/min/1.73 $m^2$ (vs 90-104 mL/min/1.73 $m^2$, IRR of 1.51 [95% CI, 1.05-2.16]), higher hemoglobin $A_{1c}$ level (IRR of 1.30 [95% CI, 1.06-1.60] per 1% increase), and current treatment for heart disease (IRR of 6.06 [95% CI, 2.48-14.8]). No significant effect modification between sleep duration and other covariates was observed in multivariate models.

As a sensitivity analysis, an association between sleep duration and the development of proteinuria was assessed in employees who did not work the night shift, which might have an influence on sleep duration. Compared with employees with at least one night shift per month, employees not working the night shift had significantly longer sleep durations (6.1 ± 0.9 vs 5.8 ± 0.8 hours; $P < 0.001$), although the difference in mean baseline sleep duration was only 17 minutes (Table 1). Clerical workers, engineers, and technical assistants more likely were included in employees not working the night shift (1,848 [96.8%] of 1,909 clerical workers and 441 [93.2%] of 473 engineers and technical assistants), whereas substantial proportions of academic researchers and health care workers in university hospitals worked night shifts (1,379 [52.2%] of 2,641 academic researchers and 1,289 [74.3%] of 1,736 health care workers), indicating that whether employees worked the night shift was dependent chiefly on their occupa-

**Table 3.** Urinary Protein at the Baseline Visit and End of the Observational Period

| Baseline Urinary Protein | No Proteinuria at End Point (n = 6,284; 92%) | | Proteinuria at End Point (n = 550; 8%) | | | Total |
|---|---|---|---|---|---|---|
| | Negative | Trace | 1+ | 2+ | 3+ | |
| Negative | 5,397 (82.6) | 634 (9.7) | 457 (7.0) | 45 (0.7) | 2 (0.0) | 6,535 (100.0) |
| Trace | 196 (65.6) | 57 (19.1) | 42 (14.0) | 4 (1.3) | 0 (0.0) | 299 (100.0) |
| Total | 5,593 (81.8) | 691 (10.1) | 499 (7.3) | 49 (0.7) | 2 (0.0) | 6,834 (100.0) |

*Note:* Proteinuria at end point is urinary protein by dipstick test at the time of the development of proteinuria for employees with the outcome and at the end of the observational period for employees without the outcome. Values given as number (percentage).

**Table 4.** Predictors of Proteinuria in Included Employees

| | Univariate Models | | Multivariate Model | |
|---|---|---|---|---|
| | **IRR (95% CI)** | ***P*** | **IRR (95% CI)** | ***P*** |
| Demographic and physical data | | | | |
| Age category | | | | |
| 20-29 y | 2.13 (1.63-2.77) | <0.001 | 2.20 (1.61-3.00) | <0.001 |
| 30-39 y | 1.36 (1.04-1.77) | 0.02 | 1.48 (1.12-1.96) | 0.006 |
| 40-49 y | 1.00 (reference) | | 1.00 (reference) | |
| 50-59 y | 1.17 (0.81-1.67) | 0.4 | 1.10 (0.76-1.60) | 0.6 |
| 60-65 y | 1.66 (0.76-3.59) | 0.2 | 1.47 (0.66-3.28) | 0.3 |
| Sex | | | | |
| Women | 1.00 (reference) | | 1.00 (reference) | |
| Men | 0.76 (0.64-0.89) | 0.001 | 0.79 (0.60-1.04) | 0.1 |
| Occupation | | | | |
| Clerical workers | 1.00 (reference) | | 1.00 (reference) | |
| Academic researchers | 0.96 (0.77-1.18) | 0.7 | 1.23 (0.96-1.58) | 0.1 |
| Engineers and technical assistants | 0.94 (0.65-1.36) | 0.7 | 1.03 (0.71-1.49) | 0.9 |
| Health care workers | 1.32 (1.06-1.65) | 0.01 | 1.10 (0.87-1.38) | 0.4 |
| Other employees | 1.22 (0.54-2.76) | 0.6 | 1.31 (0.56-3.06) | 0.5 |
| BMI (/1 kg/m$^2$) | 1.01 (0.98-1.03) | 0.6 | 1.03 (1.00-1.06) | 0.07 |
| MAP (/10 mm Hg) | 0.92 (0.86-0.99) | 0.02 | 1.00 (0.92-1.09) | 0.9 |
| Lifestyle data | | | | |
| Smoking status | | | | |
| Nonsmokers | 1.00 (reference) | | 1.00 (reference) | |
| Past smokers | 0.93 (0.69-1.26) | 0.7 | 1.10 (0.80-1.51) | 0.6 |
| Current smokers | | | | |
| 1-10 cigarettes/d | 1.24 (0.83-1.84) | 0.3 | 1.30 (0.87-1.94) | 0.2 |
| 11-20 cigarettes/d | 1.04 (0.72-1.49) | 0.8 | 1.17 (0.80-1.70) | 0.4 |
| ≥21 cigarettes/d | 1.71 (1.00-2.91) | 0.05 | 2.03 (1.16-3.56) | 0.01 |
| Alcohol consumption | | | | |
| Rarely | 1.00 (reference) | | 1.00 (reference) | |
| 1-3 d/wk | 0.89 (0.73-1.09) | 0.3 | 0.95 (0.77-1.16) | 0.6 |
| 4-6 d/wk | 0.76 (0.55-1.07) | 0.1 | 0.90 (0.64-1.26) | 0.5 |
| 7 d/wk | 0.79 (0.60-1.04) | 0.1 | 1.01 (0.74-1.37) | 0.9 |
| Sleep duration | | | | |
| ≥7 h | 1.00 (reference) | | 1.00 (reference) | |
| 6 h | 1.07 (0.86-1.32) | 0.5 | 1.07 (0.87-1.33) | 0.5 |
| 5 h | 1.28 (1.01-1.62) | 0.04 | 1.28 (1.00-1.62) | 0.05 |
| ≤4 h | 1.72 (1.17-2.51) | 0.005 | 1.72 (1.16-2.53) | 0.007 |
| Excessive daytime somnolence | 0.73 (0.44-1.22) | 0.2 | 0.67 (0.40-1.13) | 0.1 |
| Laboratory data | | | | |
| Urinary protein by dipstick test | | | | |
| Negative | 1.00 (reference) | | 1.00 (reference) | |
| Trace | 2.59 (1.92-3.51) | <0.001 | 2.14 (1.56-2.92) | <0.001 |
| Hematuria by dipstick test | | | | |
| Negative | 1.00 (reference) | | 1.00 (reference) | |
| Trace | 1.46 (0.82-2.59) | 0.2 | 1.26 (0.71-2.26) | 0.4 |
| 1+ | 1.07 (0.81-1.41) | 0.6 | 1.08 (0.81-1.43) | 0.6 |
| ≥2+ | 1.57 (1.08-2.28) | 0.02 | 1.25 (0.85-1.84) | 0.3 |
| eGFR | | | | |
| 120 mL/min/1.73 m$^2$ | 1.74 (1.22-2.47) | 0.002 | 1.51 (1.05-2.16) | 0.03 |
| 105-119 mL/min/1.73 m$^2$ | 1.33 (1.03-1.73) | 0.03 | 1.20 (0.92-1.56) | 0.2 |
| 90-104 mL/min/1.73 m$^2$ | 1.00 (reference) | | 1.00 (reference) | |
| 75-89 mL/min/1.73 m$^2$ | 0.86 (0.70-1.06) | 0.2 | 1.00 (0.80-1.24) | 0.9 |
| 60-74 mL/min/1.73 m$^2$ | 0.79 (0.60-1.04) | 0.09 | 1.03 (0.75-1.41) | 0.9 |
| Hemoglobin $A_{1c}$ (/1%) | 1.23 (1.04-1.46) | 0.02 | 1.30 (1.06-1.60) | 0.01 |
| Total cholesterol (/10 mg/dL) | 0.99 (0.96-1.01) | 0.3 | 1.00 (0.97-1.03) | 0.9 |
| Triglycerides (/1 log mg/dL) | 0.96 (0.68-1.36) | 0.8 | 1.47 (0.95-2.29) | 0.09 |
| Uric acid (/1 mg/dL) | 0.92 (0.86-0.98) | 0.006 | 0.93 (0.85-1.02) | 0.1 |
| Treatments for comorbid conditions | | | | |
| Hypertension | 0.95 (0.54-1.68) | 0.9 | 1.17 (0.62-2.20) | 0.6 |
| Diabetes | 2.18 (0.97-4.86) | 0.06 | 1.39 (0.52-3.71) | 0.5 |
| Dyslipidemia | 0.65 (0.27-1.56) | 0.3 | 0.60 (0.23-1.55) | 0.3 |
| Hyperuricemia | 0.58 (0.14-2.32) | 0.4 | 0.74 (0.18-3.04) | 0.7 |
| Heart diseases | 5.59 (2.32-13.5) | <0.001 | 6.06 (2.48-14.8) | <0.001 |

*Note:* N = 6,834.
Abbreviations: BMI, body mass index; CI, confidence interval; eGFR, estimated glomerular filtration rate; IRR, incidence rate ratio; MAP, mean arterial pressure.

tions ($P < 0.001$). Additionally, statistically significant differences were observed between night- and day-shift employees in age, sex, BMI, smoking status, alcohol consumption, urinary protein, hematuria, eGFR, hemoglobin $A_{1c}$ level, total cholesterol level, triglyceride level, uric acid level, and prevalence of hypertension. For 4,061 employees who did not work the night shift, shorter sleep duration was associated with older age ($P_{trend} = 0.008$), female sex ($P_{trend} = 0.01$), higher proportions of engineers and technical assistants ($P_{trend} < 0.001$), health care workers in university hospitals ($P_{trend} < 0.001$) and other occupations ($P_{trend} = 0.003$), higher prevalence of excessive daytime somnolence ($P_{trend} < 0.001$), lower eGFR ($P_{trend} = 0.002$), higher hemoglobin $A_{1c}$ level ($P_{trend} = 0.001$), lower triglyceride level ($P_{trend} = 0.04$), and higher prevalence of dyslipidemia ($P_{trend} = 0.01$; Table 5). A multivariate Poisson regression model identified shorter sleep duration as a significant predictor of the development of proteinuria (vs 7 hours; IRRs of 1.86 [95% CI, 1.07-3.24] and 1.47 [95% CI, 1.06-2.04] for ≤4 and 5 hours, respectively), along with younger age, heavier smoking, trace urinary protein, higher eGFR, higher hemoglobin $A_{1c}$ level, and current treatment for heart disease (Table 6), confirming the previous results.

## DISCUSSION

In this study, we identified short sleep duration as a modifiable lifestyle predictor of proteinuria, one of the vital risk factors for end-stage renal disease and CVD. In the cohort examined, employees with 5 or fewer hours of sleep duration were at significantly higher risk of developing proteinuria, even after adjusting for clinically relevant factors. These results provide novel insight into an association between sleep duration and proteinuria, which few studies have reported before. Key advantages of the present study were inclusion of a large number of younger employees (1,997 [29.2%] and 4,786 [70.0%] employees aged <30 and 40 years), age groups that rarely have been included in previous studies; ascertainment of mean sleep duration during the entire observational period; and sensitivity analysis performed after excluding employees who worked night shifts, a trait that might affect sleep duration.

Our study shows that sleep duration is associated with the development of proteinuria in a stepwise manner (Tables 4 and 6), whereas a large number of studies have shown an association between sleep duration and all-cause mortality[10,15,23,24] and cardiovascular events[12] and mortality[13] in a U-like fashion, chiefly with 7 hours of sleep duration at lowest risk. Compared with the previous studies, in this cohort, shorter mean sleep duration (6.0 ± 0.9 hours) with a small number of employees having 8 or fewer hours of sleep (n = 115 [1.7%]) hindered a statistically meaningful analysis to assess an association between longer sleep duration and development of proteinuria. Due to the large number of participants in the present study who had shorter sleep durations, many of these individuals might be at higher risk of future poor health. Because employment status and occupations greatly contribute to shorter sleep duration (Tables 2 and 5),[25,26] the present study suggests that sleep duration is one of the key factors to consider in improving occupational health.

The underlying mechanism of an association between short sleep duration and proteinuria remains to be investigated. One plausible factor is systemic inflammation,[27] which potentially leads to glomerular endothelial dysfunction and subsequent albuminuria.[28] Pertinent to this, a randomized controlled trial showed that serum C-reactive protein level significantly increased in healthy volunteers with sleep restriction (4.2 hours) for 10 days compared with those with 8 hours of sleep duration.[27] Sleep deprivation also induces an increase in peripheral white blood cell count[29] and serum interleukin 6 level.[30,31] These studies suggest that inflammation induced by short sleep duration may have contributed in part to proteinuria in the present study.

Excessive daytime somnolence, a cardinal symptom of sleep apnea,[32] was not identified as a predictor of proteinuria in the present study. Excessive daytime somnolence was associated with CVD in white and black Americans (National Health and Nutrition Examination Survey [NHANES] I epidemiologic follow-up study).[33] Because this previous study included a large number of obese participants (27.2% of participants with BMI ≥27.8 kg/m$^2$ in men and ≥27.3 kg/m$^2$ in women), who were highly vulnerable to sleep apnea, excessive daytime somnolence might partly reflect the concomitant sleep apnea. Several cross-sectional studies including many obese participants (mean BMI >30 kg/m$^2$) have shown that sleep apnea is associated with albuminuria.[34,35] On the contrary, the present study included a substantially smaller number of obese participants compared with previous studies, namely 1,058 (15.5%), 363 (5.3%), and 127 (1.9%) employees with BMI ≥25.0, ≥27.5, and ≥30.0 kg/m$^2$, respectively. An insignificant difference in BMI between 248 (3.6%) and 6,586 (96.4%) employees with and without excessive daytime somnolence (22.0 ± 3.2 and 21.8 ± 3.2 kg/m$^2$; $P = 0.2$) suggests that excessive daytime somnolence in the present study was unlikely to be due to sleep apnea. Rather, a significantly higher prevalence of excessive daytime somnolence in employees with shorter sleep durations (11.3%, 4.9%, 3.2%, and 2.2% in employees with ≤4, 5, 6, and ≥7 hours of sleep duration,

**Table 5.** Clinical Characteristics of Included Employees Who Did Not Work the Night Shift

| | Baseline Sleep Duration | | | | |
|---|---|---|---|---|---|
| | ≤4 h | 5 h | 6 h | ≥7 h | $P_{trend}$ |
| No. of employees | 130 | 749 | 1,864 | 1,318 | |
| Mean sleep duration during observational period (h) | 4.6 ± 0.7 | 5.3 ± 0.6 | 6.0 ± 0.6 | 6.8 ± 0.6 | |
| Demographic and physical data | | | | | |
| Age (y) | 37 (30-46) | 36 (30-46) | 35 (29-45) | 34 (29-43) | 0.008 |
| Age category | | | | | |
| 20-29 y | 32 (24.6) | 181 (24.2) | 496 (26.6) | 344 (26.1) | |
| 30-39 y | 44 (33.8) | 273 (36.4) | 683 (36.6) | 539 (40.9) | |
| 40-49 y | 34 (26.2) | 171 (22.8) | 397 (21.3) | 230 (17.5) | |
| 50-59 y | 17 (13.1) | 110 (14.7) | 250 (13.4) | 169 (12.8) | |
| 60-65 y | 3 (2.3) | 14 (1.9) | 38 (2.0) | 36 (2.7) | |
| Men | 48 (36.9) | 309 (41.3) | 769 (41.3) | 597 (45.3) | 0.01 |
| Occupation | | | | | |
| Clerical workers | 56 (43.1) | 345 (46.1) | 891 (47.8) | 556 (42.2) | 0.1[a] |
| Academic researchers | 23 (17.7) | 167 (22.3) | 546 (29.3) | 526 (39.9) | <0.001[a] |
| Engineers and technical assistants | 21 (16.2) | 101 (13.5) | 204 (10.9) | 115 (8.7) | <0.001[a] |
| Health care workers | 25 (19.2) | 113 (15.1) | 207 (11.1) | 102 (7.7) | <0.001[a] |
| Other employees | 5 (3.8) | 23 (3.1) | 16 (0.9) | 19 (1.4) | 0.003[a] |
| BMI (kg/m$^2$) | 21.6 ± 3.6 | 21.9 ± 3.5 | 21.5 ± 3.2 | 21.4 ± 3.1 | 0.07 |
| MAP (mm Hg) | 87 ± 14 | 86 ± 13 | 85 ± 12 | 85 ± 13 | 0.06 |
| Lifestyle data | | | | | |
| Smoking status | | | | | |
| Nonsmokers | 107 (82.3) | 624 (83.3) | 1516 (81.3) | 1067 (81.0) | 0.2[b] |
| Past smokers | 10 (7.7) | 48 (6.4) | 157 (8.4) | 112 (8.5) | |
| Current smokers | | | | | |
| 1-10 cigarettes/d | 6 (4.6) | 22 (2.9) | 58 (3.1) | 53 (4.0) | |
| 11-20 cigarettes/d | 4 (3.1) | 43 (5.7) | 100 (5.4) | 67 (5.1) | |
| ≥21 cigarettes/d | 3 (2.3) | 12 (1.6) | 33 (1.8) | 19 (1.4) | |
| Alcohol consumption | | | | | |
| Rarely | 77 (59.2) | 423 (56.5) | 1,029 (55.2) | 705 (53.5) | 0.1[c] |
| 1-3 d/wk | 33 (25.4) | 198 (26.4) | 491 (26.3) | 335 (25.4) | |
| 4-6 d/wk | 10 (7.7) | 50 (6.7) | 139 (7.5) | 105 (8.0) | |
| 7 d/wk | 10 (7.7) | 78 (10.4) | 205 (11.0) | 173 (13.1) | |
| Excessive daytime somnolence | 17 (13.1) | 41 (5.5) | 61 (3.3) | 32 (2.4) | <0.001 |
| Laboratory data | | | | | |
| Urinary protein by dipstick test | | | | | |
| Negative | 118 (90.8) | 725 (96.8) | 1,807 (96.9) | 1,273 (96.6) | 0.1 |
| Trace | 12 (9.2) | 24 (3.2) | 57 (3.1) | 45 (3.4) | |
| Hematuria by dipstick test | | | | | |
| Negative | 103 (79.2) | 629 (84.0) | 1,544 (82.8) | 1,089 (82.6) | |
| Trace | 6 (4.6) | 16 (2.1) | 38 (2.0) | 24 (1.8) | 0.5[d] |
| 1+ | 14 (10.8) | 80 (10.7) | 208 (11.2) | 148 (11.2) | |
| ≥2+ | 7 (5.4) | 24 (3.2) | 74 (4.0) | 57 (4.3) | |
| eGFR | 88 ± 14 | 88 ± 14 | 90 ± 15 | 91 ± 17 | 0.002 |
| ≥120 mL/min/1.73 m$^2$ | 3 (2.3) | 23 (3.1) | 75 (4.0) | 78 (5.9) | |
| 105-119 mL/min/1.73 m$^2$ | 11 (8.5) | 68 (9.1) | 225 (12.1) | 156 (11.8) | |
| 90-104 mL/min/1.73 m$^2$ | 42 (32.3) | 218 (29.1) | 569 (30.5) | 389 (29.5) | |
| 75-89 mL/min/1.73 m$^2$ | 50 (38.5) | 309 (41.3) | 698 (37.4) | 490 (37.2) | |
| 60-74 mL/min/1.73 m$^2$ | 24 (18.5) | 131 (17.5) | 297 (15.9) | 205 (15.6) | |
| Hemoglobin $A_{1c}$ (%) | 5.0 ± 0.4 | 5.0 ± 0.4 | 4.9 ± 0.4 | 4.9 ± 0.4 | 0.001 |
| Total cholesterol (mg/dL) | 193 ± 35 | 193 ± 34 | 193 ± 32 | 193 ± 34 | 0.9 |
| Triglycerides (mg/dL) | 61 (43-93) | 62 (44-90) | 60 (44-90) | 64 (46-93) | 0.04 |
| Uric acid (mg/dL) | 4.9 ± 1.4 | 4.9 ± 1.4 | 4.9 ± 1.3 | 5.0 ± 1.4 | 0.08 |

*(Continued)*

**Table 5 (Cont'd).** Clinical Characteristics of Included Employees Who Did Not Work the Night Shift

| | Baseline Sleep Duration | | | | |
|---|---|---|---|---|---|
| | ≤4 h | 5 h | 6 h | ≥7 h | $P_{trend}$ |
| Treatments for comorbid conditions | | | | | |
| Hypertension | 3 (2.3) | 22 (2.9) | 42 (2.3) | 33 (2.5) | 0.7 |
| Diabetes | 0 (0.0) | 7 (0.9) | 10 (0.5) | 8 (0.6) | 0.8 |
| Dyslipidemia | 2 (1.5) | 19 (2.5) | 23 (1.2) | 12 (0.9) | 0.01 |
| Hyperuricemia | 1 (0.8) | 6 (0.8) | 13 (0.7) | 7 (0.5) | 0.5 |
| Heart diseases | 0 (0.0) | 2 (0.3) | 3 (0.2) | 5 (0.4) | 0.4 |
| Outcome and follow-up data | | | | | |
| Observational period (y) | 2.2 (1.1-4.0) | 2.5 (1.5-3.9) | 2.3 (1.3-3.9) | 2.4 (1.5-3.9) | 0.7 |
| No. of examinations | | | | | 0.3 |
| 1 | 43 (33.1) | 212 (28.3) | 607 (32.6) | 419 (31.8) | |
| 2 | 39 (30.0) | 181 (24.2) | 462 (24.8) | 328 (24.9) | |
| 3 | 16 (12.3) | 135 (18.0) | 321 (17.2) | 221 (16.8) | |
| 4 | 32 (24.6) | 220 (29.4) | 473 (25.4) | 344 (26.1) | |
| 5 | 0 (0.0) | 1 (0.1) | 1 (0.1) | 6 (0.5) | |
| Development of proteinuria | 16 (12.3) | 68 (9.1) | 125 (6.7) | 89 (6.8) | 0.01 |

*Note:* N = 4,061. Continuous variables are shown as mean ± standard deviation or median (25th-75th percentile); categorical variables given as number (percentage).

Abbreviations: BMI, body mass index; eGFR, estimated glomerular filtration rate; MAP, mean arterial pressure.

[a]Each occupation versus others (eg, clerical workers vs nonclerical workers).

[b]Nonsmokers versus others.

[c]Drinking rarely versus others.

[d]Negative or trace for hematuria by dipstick test versus 1+ or more.

respectively; $P_{trend}$ < 0.001; Table 2) implies that excessive daytime somnolence merely reflected a shortage of sleep. Lower, although insignificant, IRRs of excessive daytime somnolence (0.67 [95% CI, 0.40-1.13; $P = 0.1$] in all included employees [Table 4] and 0.86 [95% CI, 0.47-1.60; $P = 0.6$] in employees who did not work the night shift [Table 6]) possibly might be due to employees with shorter sleep durations who took unmeasured daytime sleep as a nap.

In addition to shorter sleep duration, younger age was identified by this study as a significant predictor of proteinuria; furthermore, our analysis found a J-shaped association between age and development of proteinuria, although a small number of employees 50 years or older failed in a statistically significant association between older age and development of proteinuria (Tables 4 and 6). Most previous studies, including the PREVEND (Prevention of Renal and Vascular End-Stage Disease)[36] and KMIC (Korea Medical Insurance Corp) studies,[37] assessed predictors of proteinuria mainly in middle-aged or older persons. Little information was available about predictors of proteinuria in persons 40 years or younger, which was the main group enrolled in the present study (70.0%). Because the IRR for those aged 20-29 years decreased from 2.20 [95% CI, 1.61-3.00] to 1.69 [95% CI, 1.13-2.53] after excluding employees who worked the night shift in the present study and since several previous studies showed that rotating shift was a risk factor for CVD,[38-40] the occupational environment might contribute in part to the development of proteinuria in younger employees. Further study is essential to show an association between younger age and development of proteinuria.

The present study had several limitations. First, the present study was based on employees in a national university, including many academic researchers and health care workers in the university hospital, who are not representative of the general population. External validity should be verified in different cohorts. Second, inadequate follow-up of employees might lead to biased results. Considering the relative youth of the employees in the present study, loss to follow-up was ascribed not to retirement or leave due to severe illness, but probably a job change, which would be expected to be related to characteristics of their workplaces. Because sleep duration also was linked to occupation (Table 2),[25,26] results of the present study should be validated in cohorts comprising different occupations. Third, self-reported sleep duration might be biased, although several studies have shown that self-reported sleep duration is correlated moderately with those measured by polysomnography[41] and actigraphy.[42] Fourth, the outcome measure in the present study was time to first proteinuria (≥1+) after the baseline examination, not persistent proteinuria. This was because of a short observational period, namely, a median of 2.5 (25th-75th percentile, 1.4-3.9) years. Further observation would be essential to ascertain an association between sleep duration and persistent proteinuria. Fifth, inflammation might be a key factor in the link between

**Table 6.** Predictors of Proteinuria in Included Employees Who Did Not Work the Night Shift

| | Univariate Models | | Multivariate Model | |
|---|---|---|---|---|
| | IRR (95% CI) | *P* | IRR (95% CI) | *P* |
| Demographic and physical data | | | | |
| Age | | | | |
| 20-29 y | 1.63 (1.16-2.29) | 0.004 | 1.69 (1.13-2.53) | 0.01 |
| 30-39 y | 1.21 (0.87-1.69) | 0.3 | 1.28 (0.90-1.83) | 0.2 |
| 40-49 y | 1.00 (reference) | | | |
| 50-59 y | 1.06 (0.70-1.61) | 0.7 | 0.93 (0.60-1.45) | 0.8 |
| 60-65 y | 1.41 (0.61-3.29) | 0.4 | 1.26 (0.52-3.05) | 0.6 |
| Sex | | | | |
| Women | 1.00 (reference) | | | |
| Men | 0.95 (0.75-1.19) | 0.6 | 0.85 (0.59-1.24) | 0.4 |
| Occupation | | | | |
| Clerical workers | 1.00 (reference) | | | |
| Academic researchers | 0.96 (0.74-1.25) | 0.8 | 1.13 (0.83-1.55) | 0.4 |
| Engineers and technical assistants | 1.03 (0.71-1.48) | 0.9 | 1.10 (0.75-1.59) | 0.6 |
| Health care workers | 0.80 (0.53-1.23) | 0.3 | 0.76 (0.49-1.16) | 0.2 |
| Other employees | 1.42 (0.63-3.22) | 0.4 | 1.13 (0.47-2.74) | 0.8 |
| BMI (/1 kg/m$^2$) | 1.01 (0.98-1.05) | 0.5 | 1.00 (0.96-1.04) | 0.9 |
| MAP (/10 mm Hg) | 0.99 (0.91-1.09) | 0.9 | 1.02 (0.91-1.15) | 0.7 |
| Lifestyle data | | | | |
| Smoking status | | | | |
| Nonsmokers | 1.00 (reference) | | | |
| Past smokers | 1.01 (0.67-1.52) | 0.9 | 1.13 (0.73-1.75) | 0.6 |
| Current smokers | | | | |
| 1-10 cigarettes/d | 1.12 (0.61-2.06) | 0.7 | 1.14 (0.62-2.12) | 0.7 |
| 11-20 cigarettes/d | 1.26 (0.79-2.01) | 0.3 | 1.28 (0.78-2.10) | 0.3 |
| ≥21 cigarettes/d | 2.02 (1.07-3.79) | 0.03 | 2.06 (1.04-4.07) | 0.04 |
| Alcohol consumption | | | | |
| Rarely | 1.00 (reference) | | | |
| 1-3 d/wk | 0.86 (0.65-1.13) | 0.3 | 0.85 (0.64-1.13) | 0.3 |
| 4-6 d/wk | 0.86 (0.54-1.35) | 0.5 | 0.91 (0.57-1.45) | 0.7 |
| 7 d/wk | 0.85 (0.59-1.22) | 0.4 | 0.88 (0.59-1.33) | 0.5 |
| Sleep duration | | | | |
| ≥7 h | 1.00 (reference) | | | |
| 6 h | 1.01 (0.77-1.32) | 0.9 | 1.07 (0.81-1.41) | 0.6 |
| 5 h | 1.32 (0.96-1.81) | 0.08 | 1.47 (1.06-2.04) | 0.02 |
| ≤4 h | 1.84 (1.08-3.13) | 0.03 | 1.86 (1.07-3.24) | 0.03 |
| Excessive daytime somnolence | 0.96 (0.53-1.76) | 0.9 | 0.86 (0.47-1.60) | 0.6 |
| Laboratory data | | | | |
| Urinary protein by dipstick test | | | | |
| Negative | 1.00 (reference) | | | |
| Trace | 3.28 (2.16-4.97) | <0.001 | 2.78 (1.79-4.33) | <0.001 |
| Hematuria by dipstick test | | | | |
| Negative | 1.00 (reference) | | | |
| Trace | 1.84 (0.91-3.72) | 0.09 | 1.46 (0.70-3.03) | 0.3 |
| 1+ | 1.20 (0.85-1.69) | 0.3 | 1.30 (0.91-1.85) | 0.2 |
| ≥2+ | 1.62 (0.99-2.64) | 0.06 | 1.49 (0.90-2.48) | 0.1 |
| eGFR | | | | |
| ≥120 mL/min/1.73 m$^2$ | 1.95 (1.24-3.06) | 0.004 | 1.91 (1.20-3.06) | 0.007 |
| 105-119 mL/min/1.73 m$^2$ | 1.23 (0.85-1.79) | 0.3 | 1.14 (0.78-1.67) | 0.5 |
| 90-104 mL/min/1.73 m$^2$ | 1.00 (reference) | | | |
| 75-89 mL/min/1.73 m$^2$ | 0.90 (0.67-1.19) | 0.4 | 0.92 (0.69-1.24) | 0.6 |
| 60-74 mL/min/1.73 m$^2$ | 0.83 (0.57-1.19) | 0.3 | 0.88 (0.58-1.34) | 0.6 |
| Hemoglobin $A_{1c}$ (/1%) | 1.28 (1.04-1.58) | 0.02 | 1.29 (1.01-1.65) | 0.04 |
| Total cholesterol (/10 mg/dL) | 0.99 (0.96-1.03) | 0.7 | 1.00 (0.96-1.04) | 0.9 |
| Triglycerides (/1 $Log_{10}$ [mg/dL]) | 1.27 (0.79-2.02) | 0.3 | 1.47 (0.80-2.71) | 0.2 |
| Uric acid (/1 mg/dL) | 1.02 (0.93-1.10) | 0.7 | 1.05 (0.93-1.19) | 0.4 |
| Treatments for comorbid conditions | | | | |
| Hypertension | 1.03 (0.51-2.08) | 0.9 | 0.98 (0.44-2.20) | 0.9 |
| Diabetes | 2.78 (1.03-7.45) | 0.04 | 1.73 (0.51-5.84) | 0.4 |
| Dyslipidemia | 1.19 (0.49-2.87) | 0.7 | 1.08 (0.39-3.00) | 0.9 |
| Hyperuricemia | 1.06 (0.26-4.26) | 0.9 | 1.11 (0.26-4.77) | 0.9 |
| Heart diseases | 6.25 (2.33-16.8) | <0.001 | 7.07 (2.53-19.7) | <0.001 |

*Note:* N = 4,061.

Abbreviations: BMI, body mass index; CI, confidence interval; eGFR, estimated glomerular filtration rate; IRR, incidence rate ratio; MAP, mean arterial pressure.

short sleep duration and the development of proteinuria, but this association was not assessed in the present study.

In conclusion, the present study identified short sleep duration (≤5 hours) as a significant predictor of proteinuria, even adjusting for multiple clinically relevant metabolic and lifestyle factors. These results provide novel insight into the mechanism of the development of proteinuria, which is one of the critical predictors of end-stage renal disease and CVD.

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# Febuxostat suppressed renal ischemia–reperfusion injury via reduced oxidative stress

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ABSTRACT

Febuxostat is a novel selective inhibitor of xanthine oxidase (XO), approved for treating hyperuricemia. XO inhibits the generation of uric acid (UA) as well as the resulting generation of superoxide. During renal ischemia–reperfusion (I/R) injury, the burst of reactive oxygen species (ROS) can trigger the inflammation and the tubular cell injury. As XO is a critical source of ROS, inhibition of XO could be a therapeutic target for I/R injury. Therefore, we performed this study to test the therapeutic effect of febuxostat on renal I/R injury.

Sprague–Dawley rats, received vehicle or febuxostat, were subjected to right nephrectomy and left renal I/R injury. Febuxostat significantly suppressed XO activity, and thereby reduced oxidative stress, assessed by nitrotyrosine, thiobarbituric acid-reactive substances (TBARS) and urine 8-isoprostane. Furthermore, febuxostat reduced the induction of endoplasmic reticulum (ER) stress, assessed by GRP-78, ATF4, and CHOP. Vehicle-treated I/R injured rats exhibited elevated serum creatinine and UN, which were significantly suppressed in febuxostat-treated I/R-injured rats. Histological analysis revealed that fubuxostat-treated rats showed less tubular injury and interstitial fibrosis with reduction in ED1-positive macrophage infiltration, TUNEL positive apoptotic tubular cells, and interstitial smooth muscle α actin (SMαA) expression, compared to vehicle-treated rats. In conclusion; novel XO inhibitor, febuxostat, can protect kidney from renal I/R injury, and may contribute to preserve kidney function.

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## 1. Introduction

Renal ischemia–reperfusion (I/R) injury, frequently associated with shock or surgery, is a major cause of acute renal failure [1]. Reactive oxygen species (ROS) have been implicated as a major pathophysiological component in I/R injury in several tissues including kidney [2]. Several lines of insights have focused on xanthine oxidoreductase (XOR) inhibitor as a therapeutic tool for I/R injury. XOR inhibits the generation of uric acid (UA) as the final product of purine catabolism, as well as the resulting generation of superoxide. Under ischemic condition, adenosine triphosphate (ATP) is degraded to xanthine, and hypoxanthine, which are substrates for XOR. XOR functions as either xanthine dehydrogenase (XDH) form, which transfers electron to $NAD^+$, and generates NADH or xanthine oxidase (XO) form, which transfers electron to $O_2$ and generates oxidative stress. Because ischemia-induced cellular calcium overload convert XDH to XO [3], under reperfusion phase, enhanced XO can produce more ROS, such as superoxide, hydrogen peroxide, and hydroxyl radicals. These ROS can exaggerate cellular damages.

Recently, apoptosis is triggered by ROS-mediated activation of endoplasmic reticulum (ER) stress requiring involvement of CHOP pathway [4]. Disturbances such as hypoxia and oxidative stress may lead to ER dysfunction, which can induce ER stress in kidney [5]. Oxidative stress can cause aberrant unfolded and malfolded proteins, which in turn induces ER stress. Some unfolded protein responses enhance the protein-folding capacity by activating the transcription of target genes, such as glucose-regulated protein-78 (GRP-78) [6]. ER stress-induced apoptosis is mainly mediated C/EBP homologous protein-10 (CHOP). CHOP is a transcription factor, which induces several proapoptotic factors, and is downstream of activating transcription factor-4 (ATF4). Severe ER stress preferentially induces proapototic CHOP expression as compared to mild ER stress [5].

On the basis that XO produces ROS, XOR inhibitor might have a protective effect under renal I/R injury. Allopurinol, one of XOR inhibitor, is a classic "suicide inhibitor," as its binding to and

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reduction of the molybdenum (Mo) cofactor induces self-oxidation to form oxypurinol, an active inhibitory metabolite. Reduction of the Mo cofactor by allopurinol ultimately leads to electron transfer to the FAD, resulting in the ROS production [7]. In addition, oxypurinol binding and resultant inhibition also requires the Mo cofactor to be reduced [8]. In this point of view, both allopurinol and oxypurinol require enzyme turnover resulting in ROS formation before inhibition is attained. On the contrary, a new XOR inhibitor, febuxostat, is not affected by the above enzyme redox state and interaction with XO, and thereby produces less ROS. In this study, we examined whether treatment with febuxostat could protect the kidney from tubular ROS production under renal ischemia–reperfusion injury and, thereby, inhibit subsequent tubulointerstitial injury.

## 2. Materials and methods

### 2.1. Experimental design

Male Sprague–Dawley rats weighing 200 g were purchased from Japan SLC Inc. (Shizuoka, Japan) and were maintained under standard conditions until the experiments were done. All studies were performed in accordance with the principles of the Guideline on Animal Experimentation of Osaka University. The rats were randomly allocated into three groups: (1) vehicle-treatment group (Veh group); (2) febuxostat-treatment group (Feb group) and (3) sham-operated group (sham group). Vehicle and febuxostat group rats received orally 0.5 ml of 0.5% methylcellulose as a vehicle and 10 mg/kg/day of febuxostat in 0.5% methylcellulose 1 day and 60 min before I/R injury, respectively. On day 0, the rats were subjected to right renal nephrectomy and left renal I/R injury. Sham-operated rats were used as normal control. All rats were anesthetized with an intraperitoneal injection of sodium thiopentone (30 mg/kg). The animals were allowed to stabilize for 30 min before they were subjected to right nephrectomy and 45 min of left renal occlusion using artery clips to clamp the renal pedicles. Occlusion was confirmed visually by a change in the color of the kidneys to a paler shade. Reperfusion was initiated with the removal of the artery clips and was confirmed visually by noting a blush. The rats were sacrificed 4 h ($n = 5$ in each group), 24 h ($n = 8$ in each group), and 72 h ($n = 8$ in each group) after reperfusion.

### 2.2. Xanthine oxidoreductase/xanthine oxidase activity

XOR activity was determined with a fluorometric assay described by Beckman et al. Briefly, kidney tissue was homogenized and centrifuged at 12,000g for 15 min. The supernatant was used to the assay based on the conversion of pterin to a fluorescent product, isoxanthopterin (Excitation wave length: 355 nm, Emission wave length: 405 nm), and was performed with or without methylene blue to determine XOR (XO + XDH) activity and XO activity, respectively.

### 2.3. Antibodies

Specific polyclonal antibodies for anti-smooth muscle α actin (SMαA) antibody (EPOS System: clone 1A4; Dako, Glostrup, Denmark), and anti-rat ED1 antibody (1:100, clone ED1; MCA341R, AbD Serotec, Kidlington, Oxford, UK) for macrophage staining were used in this study.

### 2.4. Morphology and Immunohistochemical staining

Following fixation with 4% paraformaldehyde, the kidneys were processed to paraffin and histological sections (2 μm) of the kidneys were used for Periodic acid-Schiff (PAS), or for Immunohistochemical staining. Immunohistochemical staining was carried out by standard avidin–biotinylated peroxidase complex method (DakoCytomation LSAB2 System-HRP, Dako) with diaminobenzidine as the chromogen.

We have scored and calculated the number of infiltrated macrophages, and the percentage of SMαA staining positive areas by using a computer-aided manipulator (Win Roof; Mitani, Fukui, Japan). All of the slides were highlighted on digitized images using a computer-aided manipulator (Light microscopy; Nikon Eclipse 80i (Nikon, Tokyo, Japan), and pictures were taken with Nikon ACT-1 ver.2.63) Glomeruli and large vessels were excluded in the microscopic fields for image analysis. PAS-stained sections were scored by calculation of percentage of tubules in corticomedullary junction that displayed cell necrosis, loss of brush border, cast formation, and tubular dilation as follows: 0, none; 1, <10%; 2, 11–25%; 3, 26–45%; 4, 46–75%; and 5, >76%. At least 20 randomly selected areas per rat were assessed. The scores of ten fields per each kidney sections were averaged and used as the score of individual rat.

### 2.5. Terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling (TUNEL) staining

TUNEL staining was performed using the *in situ* Apoptosis Detection Kit (Takara Bio, Otsu, Japan), according to the manufacturer's instructions. Briefly, the sections were deparaffinized and treated with antigen retrieval in preheated 10 mmol/L sodium citrate (pH 7), using a steamer for 40 min. They were then incubated with 3% $H_2O_2$ for 10 min, which was followed by incubation with TdT enzyme solution for 90 min at 37 °C. The reaction was terminated by incubation in a stop/wash buffer for 30 min at 37 °C. The number of TUNEL-positive cell nuclei and the total numbers of cell nuclei stained with hematoxylin were counted in 10 random areas, and the percentages of the numbers of TUNEL-positive nuclei to the numbers of total cell nuclei were then calculated.

### 2.6. Real-time quantitative polymerase chain reaction (PCR)

Total RNA was extracted from the kidney cortex using an RNeasy mini kit (Qiagen, Hilden, Germany), and was reverse transcribed to cDNA. Gene expression was measured by real-time quantitative PCR using an Applied Biosystems Prism 7500 (Applied Biosystems, Foster City, CA, USA) with cDNA, SYBR Green PCR Core Reagents (Invitrogen) and a set of primers. Primers fwere as follows: Monocyte Chemotactic Protein-1 (MCP-1); 5′-atgcagttaatgc cccactc-3′ (forward), 5′-ttccttattggggtcagcac-3′ (back), IL-1β; 5′-caggaaggcagtgtcactca-3′ (forward), 5′-aaagaaggtgcttgggtcct-3′ (back), Transforming Growth Factor-β (TGF-β); 5′-ctactgcttcagctcc-acagaga-3′ (forward), 5′-accttgggttgcgacc-3′ (back), Type I collagen; 5′-aatggtgctcctggtattgc-3′ (forward), 5′- aatggtgctcctggtattgc -3′ (back), ATF4; 5′-gctatggatgggttggtcag-3′ (forward), 5′-agctcat ctggcatggtttc-3′ (back), CHOP; 5′-ttacagtcatggcagctgagtc-3′ (forward), 5′-gacctcctgcagatcctcatac-3′ (back), GRP-78; 5′-tgttccgct ctaccatgaaac-3′ (forward), 5′-aattcgagtagatccgccaac-3′ (back), 18s rRNA; 5′-gcaattattccccatgaacg-3′ (forward), 5′-ggcctcactaaaccatc-caa-3′ (back).18s rRNA transcript was used as an internal control.

### 2.7. Oxidative stress

Kidney cortex tissue was weighted and homogenized with 0.05 M potassium phosphate buffer containing 1 mM ethylenediaminetetraacetic acid (EDTA) and protease inhibitor cocktail (Roche Applied Science, Indianapolis, USA). Tissue suspension was centrifuged at 12,000×g for 15 min at 4 °C, and the supernatants were collected and used for assay. Nitrotyrosine levels were quantified by enzyme immunoassay using the NWLSS nitrotyro-

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sine ELISA kit (Northwest Life Science Specialties, LLC) according to the manufacturer's instructions. The measurement of thiobarbituric acid-reactive substances (TBARS) in the rat kidney was based on the formation of malondialdehyde by using a commercially available TBARS Assay kit (Cayman Chemical) according to the manufacturer's instructions.

Urinary concentrations of 8-isoprostane was determined using enzyme immunoassay kits from Japan institute for the control of aging (Shizuoka, Japan,). Results were adjusted by urine creatinine concentration, and averaged.

### 2.8. Statistical analysis

All values are expressed as mean ± SE. Comparisons between two parameters were analyzed by using the unpaired Student's *t*-test. Comparisons among the three groups were evaluated using the Tukey method by GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego, CA, USA), and $P < 0.05$ was considered to be statistically significant.

## 3. Results

### 3.1. Febuxostat suppressed renal XO and XDH activity

Renal tissue XO activity was not changed in vehicle-treated ischemia–reperfusion (I/R)-injured kidneys (1124 ± 187 μU/mg protein) compared with sham-operated kidney (1390 ± 397 μU/mg protein) 4 h after I/R injury. In contrast, XO activity was not detected in febuxostat-treated kidneys. Concomitant with the reduction of XO activity, febuxostat significantly reduced UA levels (0.3 ± 0.0 mg/dL) compared with vehicle treatment (1.4 ± 0.1 mg/dL) 4 h after I/R injury, but no difference was observed at 24 h between vehicle-treated and febuxostat-treated group (0.50 ± 0.14 and 0.58 ± 0.07 mg/dL, respectively).

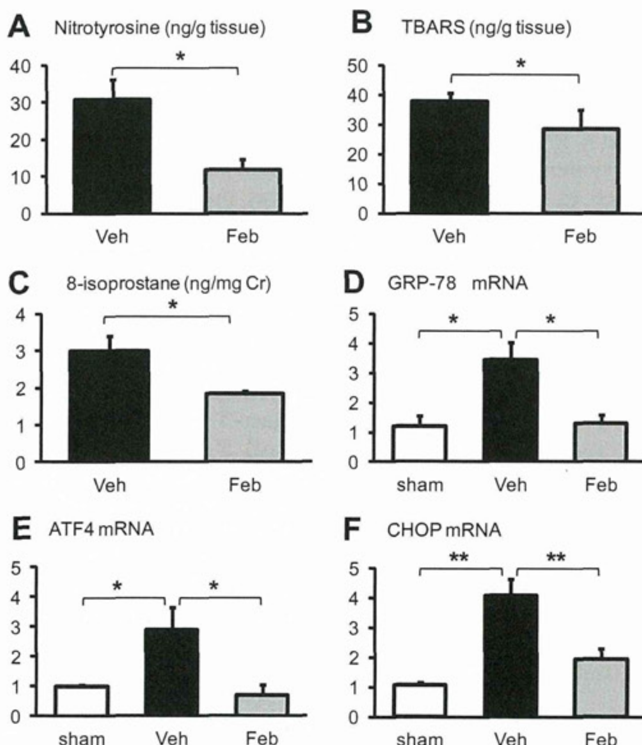


**Fig. 1.** Effect febuxostat on oxidative stress. ELISA demonstrated the renal concentration of nitro-tyrosine (A) and TBARS (B), and urinary excretion of 8-isoprostane (C) 4 h after I/R injury (*$P < 0.05$). Real-time PCR showed GRP-78 (D), ATF4 (E), and CHOP (F) mRNA expression 4 h after I/R injury. Result was expressed as relative expression against the expression in sham-operated rats (*$P < 0.05$, **$P < 0.01$).

### 3.2. Febuxostat inhibits oxidative stress and ER stress

As we observed the sufficient XO inhibition of febuxostat in I/R injured kidneys, we examined the resultant effect on oxidative stress. Nitrotyrosine concentration, a marker of nitro-oxidative stress, extracted from febuxostat-treated I/R-injured kidneys was lower (11.9 ± 2.6 pmol/g tissue) than that from vehicle-treated kidney (30.7 ± 5.4 pmol/g tissue) 4 h after disease induction ($P < 0.05$ vs. Veh group; Fig. 1A). Febuxostat treatment also significantly suppressed TBARS concentration, a marker of lipid peroxidation, (28.5 ± 3.7 nmol/g tissue) compared with vehicle treatment (37.6 ± 3.0 nmol/g tissue) 4 h after disease induction ($P < 0.05$ vs. Veh group; Fig. 1B). In addition, urinary excretion of 8-isoprostane was also significantly suppressed febuxostat-treated rats compared with vehicle-treated rats (1.83 ± 0.09 and 2.98 ± 0.44 ng/mg Cr, respectively, $P < 0.05$; Fig. 1C).

Several studies have indicated that oxidative stress induces ER stress [5]. Therefore, the expression of ER stress-related genes in kidney tissues was measured 4 h after I/R. RT-PCR demonstrated that marked elevation in GRP-78 (Fig. 1D), ATF4 (Fig. 1E), and CHOP (Fig. 1F) levels were observed in the vehicle-treated I/R injury model rats (3.43 ± 0.60-, 2.88 ± 0.77-, and 4.07 ± 0.55-fold, respectively, $P < 0.05$) compared with the sham group (1.19 ± 0.37-, 1.00 ± 0.05-, and 1.06 ± 0.09-fold, respectively). In contrast, I/R injury-induced ER stress was suppressed in the Feb treated group (1.29 ± 0.30-, 0.70 ± 0.33-, and 1.95 ± 0.34-fold, respectively, $P < 0.05$ vs. Veh group).

### 3.3. Effects on tubular damage and apoptosis in the I/R injury kidney

I/R-injured rats exhibited impaired renal function, assessed by serum UN and creatinine (95.9 ± 8.9 mg/dL and 1.59 ± 0.19 mg/dL, respectively, $P < 0.01$ vs. sham group), compared with sham-operated rats (17.2 ± 0.8 mg/dL and 0.36 ± 0.04 mg/dL, respectively). Febuxostat ameliorated the elevated serum UN and creatinine levels (38.2 ± 4.3 and 0.62 ± 0.06 mg/dL, respectively, $P < 0.01$ vs. Veh group) (Fig. 2A).

PAS staining of kidney sections from vehicle-treated rats 24 h after I/R injury showed marked disruption, including widespread degeneration of tubular architecture, tubular swelling, luminal congestion, loss of brush border, and increased interstitial infiltration (PAS score; 3.4 ± 0.1, Fig. 2B). Treatment with febuxostat ameliorated characteristic histological changes of I/R injury, including tubular damage and increased interstitial cells (PAS score; 1.8 ± 0.1, $P < 0.001$ vs. Veh group, Fig. 2C). To elucidate the protective mechanisms by which febuxostat administration ameliorated tubular injury, we did TUNEL staining to quantify the number of apoptotic cells. In the vehicle-treated I/R injury model rats, TUNEL-positive, apoptotic cells increased among the tubular epithelial cells at 24 h (TUNEL-positive cells per all nuclei, 9.02 ± 0.27%, Fig. 2D), while TUNEL-positive, apoptotic cells were significantly decreased by febuxostat treatment (1.23 ± 0.06%, $P < 0.001$ vs. Veh group, Fig. 2E).

### 3.4. Febuxostat ameliorates interstitial infiltration

As we observed the reduced interstitial infiltrated cells in febuxostat-treated kidney, we then examined the macrophage infiltration in the interstitium. The number of ED-1 positive macrophages was significantly increased in interstitial area of vehicle-treated I/R-injured kidneys at 24 h and 72 h (266.3 ± 17.1 and 503.6 ± 19.0 per low power field (LPF), respectively, $P < 0.001$

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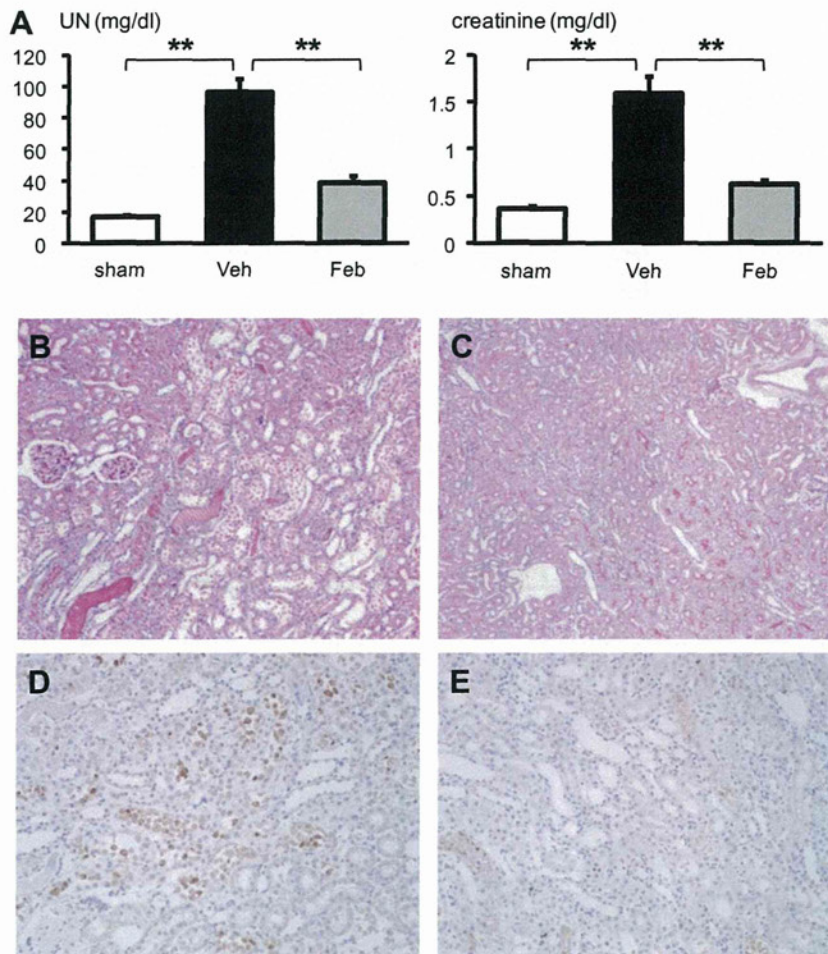


**Fig. 2.** Effects of febuxostat on renal injury. Effect of febuxostat on renal function was summarized. Serum UN and creatinine were examined 24 h after sham-operation (sham) or ischemia–reperfusion injury with heminephrectomy treated with vehicle (Veh) or febuxostat (Feb) (A) (**$P < 0.01$). Effect of febuxostat on tubular injury was assessed by staining with PAS (B, C) or TUNEL (D, E) from vehicle-treated (B, D) or febuxostat-treated (C, E) I/R injured rats. (magnification, 200×).

vs. sham group) compared with sham-operated kidneys (8.6 ± 0.6 field and 25.8 ± 2.9 per LPF, respectively), while febuxostat suppressed the infiltration of ED-1-positive macrophages (81.1 ± 7.6 and 228.9 ± 11.8 per LPF, respectively, $P < 0.001$ vs. Veh group) (Fig. 3A–C), which was consistent with the observation from PAS staining. As we observed the protective effect of febuxostat on macrophage infiltration, we examined the effect of febuxostat on MCP-1 expression in I/R-injured kidneys. Real-time RT-PCR revealed that MCP-1 mRNA expression was increased at 4 h and 24 h in I/R-injured kidney (8.85 ± 1.82-fold and 5.60 ± 1.42-fold, respectively, $P < 0.01$ vs. Sham group). Parallel with the significant reduction of macrophage infiltration, febuxostat suppressed the increment of MCP-1 expression (3.47 ± 0.74-fold and 3.24 ± 0.78-fold, respectively, $P < 0.05$ vs. Veh group) (Fig. 3D). Moreover, the IL-1β mRNA expression, a proinflammatory cytokine that related to macrophage infiltration, was also decreased in febuxostat treated rats (1.42 ± 0.40-fold, vs. Veh group 3.60 ± 0.65-fold, $P < 0.05$, Fig. 3E).

### *3.5. Effects on interstitial phenotypic changes in the I/R injury kidney*

To detect interstitial myofibroblasts, which are associated with interstitial damage and fibrosis, the expression of SMαA was examined immunohistochemically. The interstitial expression of SMαA increased 72 h after I/R injury in the vehicle-treated rats (7.08 ± 0.15%, $P < 0.001$ vs. sham group), while febuxostat treatment significantly suppressed interstitial expression of SMαA (3.63 ± 0.12%, $P < 0.001$ vs. Veh group) (Fig. 4A–C). Similarly, real-time RT-PCR analysis showed that febuxostat significantly decreased TGF-β mRNA expression at 24 h after reperfusion (0.91 ± 0.14-fold, vs. Veh group 1.35 ± 0.12-fold, $P < 0.05$ vs. Veh group, Fig. 4D), and decreased type I collagen mRNA expression at 72 h after reperfusion (1.56 ± 0.45-fold, vs. Veh group 4.12 ± 0.45-fold, $P < 0.05$ vs. Veh group, Fig. 4E).

## 4. Discussion

We demonstrated that febuxostat suppressed XO activity, reduced oxidative stress, and thereby ameliorated tubulointerstitial injury in a rat model of I/R injury. Untreated I/R-injured kidneys exhibited increased plasma creatinine, tubular apoptosis, interstitial macrophage infiltration and interstitial SMαA expression, while administration of febuxostat ameliorated these manifestations. Importantly, febuxostat reduced oxidative stress, assessed by nitrotyrosine, TBARS and urine 8-isoprostane, together with the reduction of XO activity. Nitrotyrosine is a tyrosine nitration product mediated especially under proinflammatory conditions by reactive nitrogen species. Peroxynitrite anion: $ONOO^-$ is one of the most powerful reactive oxygen species that is produced by the reaction of nitric oxide and superoxide radicals, and considered as a marker of reactive nitrogen species induced by iNOS accompanied with oxidative stress [9]. TBARS, a measure of lipid

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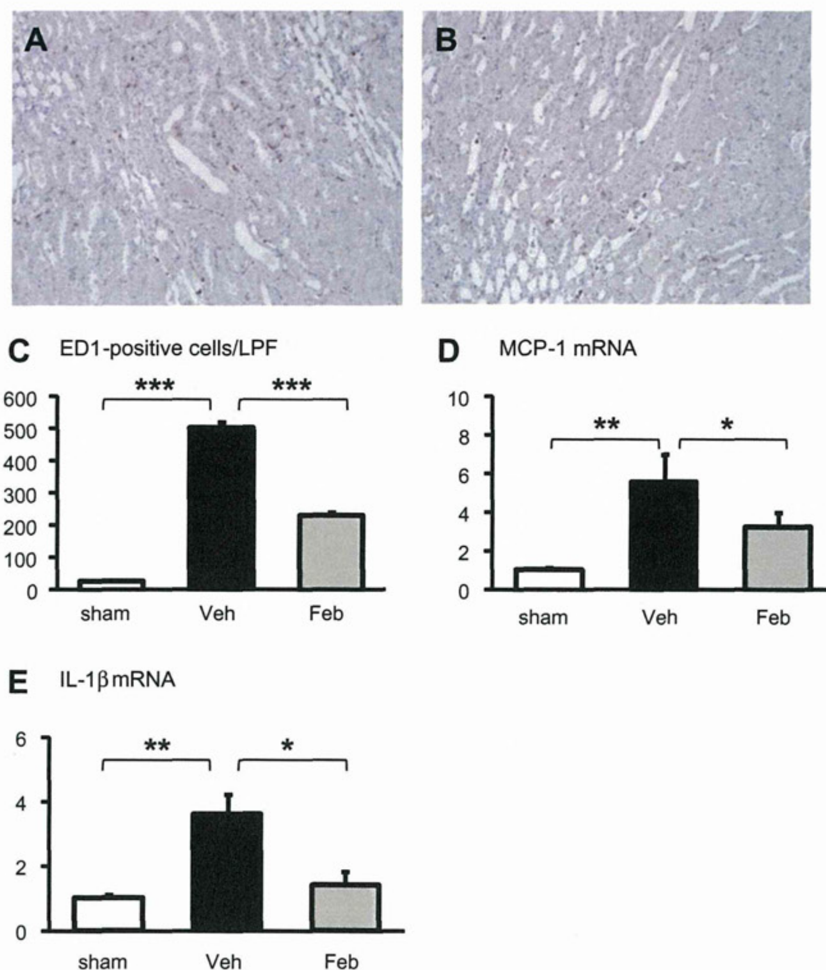


**Fig. 3.** Effects of febuxostat on macrophage infiltration. Representative immunohistochemical staining for ED-1-positive macrophages from vehicle-treated (A) or febuxostat-treated (B) I/R injured rats, and the number of ED-1 positive cells in interstitial space per 200× magnifier fields (C) 72 h after I/R injury. Real-time PCR showed the MCP-1 mRNA level at 24 h (D) and IL-1β level at 4 h (E) after I/R injury. Result was expressed as relative expression against the expression in sham-operated rats ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

peroxidation, was increased after I/R injury, but ameliorated in febuxostat-treated kidney. Racasan reported that infusion of XO increased urinary excretion of TBARS, which was completely normalized in the recovery period [10]. This observation was consistent with our results that inhibition of XO by febuxostat reduced renal TBARS. In addition, reduced 8-isoprostane revealed that febuxostat inhibited the production of the oxidative stress-mediated peroxidation of arachidonic acid. We identified less production of ROS in febuxostat-treated I/R kidneys which may originate from complete blockade of XO activity. Several lines of insights have focused on XO as a source for ROS production. XDH, which is unable to generate ROS, is converted to XO by cellular calcium overload [3]. XO can produce ROS, such as superoxide, hydrogen peroxide, and hydroxyl radicals. As we showed that febuxostat diminished the XO activity compared with the vehicle-treated I/R kidney, the reduction of XO activity might suppress renal content of nitrotyrosine and TBARS, and urinary excretion of 8-isoprostane in febuxostat-treated I/R kidneys.

We also showed that the macrophage infiltration on day 1 after increasing oxidative stress 4 h after disease induction. Previous report showed a positive interaction between ROS and macrophage infiltration. Oxidative stress promotes the expression of various inflammation-related molecules, including MCP-1 and IL-1β, which, in turn, promotes the inflammatory cell infiltration [11]. It has been reported that XO-induced oxidative stress stimulates MCP-1 and IL-1β expression [12,13]. It was also reported that hyperlipidemia caused XO activity in relation to MCP-1 expression in kidney, followed by macrophage infiltration and tubulointerstitial injury, but that inhibition of XO prevented interstitial macrophage infiltration, together with decreased MCP-1 expression [12]. These results points to an important role of XO in the early stage of I/R injury, mediating macrophage infiltration by a putatively oxidative stress-dependent up-regulation of MCP-1 and IL-1β.

Together with the reduction of macrophage infiltration, TUNEL-positive apoptotic tubular cells were also suppressed in febuxostat-treated I/R kidneys on day 1, which was also consistent with the reduction of XO activity. XO-derived ROS generation was reported to induce apoptosis in cultured hepatocytes [14]. One possible mechanism of XO inhibitor-induced beneficial effect is the preservation of mitochondrial function by protecting mitochondrial membrane integrity [15]. This is supported by our observation that fubuxostat-treatment decreased lipid peroxidation, assessed by TBARS concentration. In addition, febuxostat may provide beneficial effect by reducing intracellular uric acid production. In contrast to the role of plasma uric acid as a strong anti-oxidant [16], intracellular uric acid induces oxidative stress by the activation of NADPH oxidase [17], and promotes inflammation. Another possibility of suppressed apoptotic cells by febuxostat is mediated by the suppression of CHOP expression. CHOP has been identified

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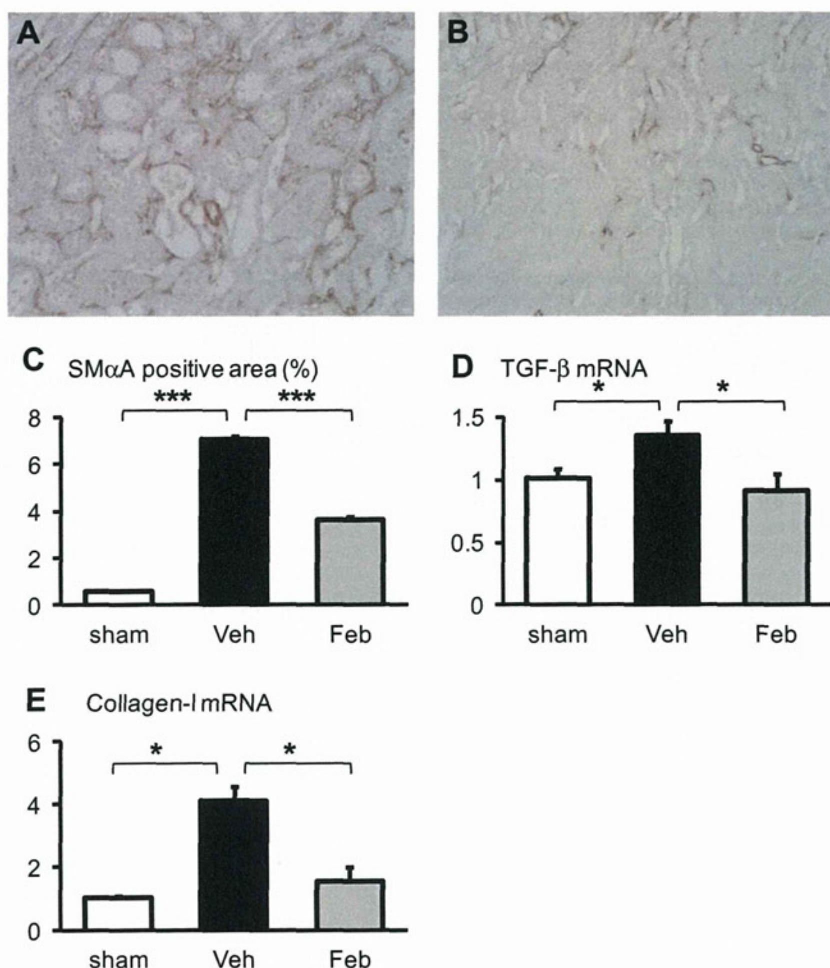


**Fig. 4.** Effects of febuxostat on phenotypic alteration. Representative immunohistochemical staining for SMαA from vehicle-treated (A) or febuxostat-treated (B) I/R injured rats, and the percentage of SMαA positive staining areas in interstitial space (C) 72 h after I/R injury. (magnification, 200×) Real-time PCR showed the TGF-β mRNA expression at 24 h (D) and type I collagen mRNA expression at 72 h (E) after I/R injury. Result was expressed as relative expression against the expression in sham-operated rats (* $P < 0.05$, *** $P < 0.001$).

as an ER-initiated proapoptotic signal that plays an important role in the pathogenesis of diabetes mellitus and neurodegenerative diseases [18]. In kidney, apoptosis is triggered by ROS-mediated activation of CHOP pathway [4]. We previously demonstrated that unfolded protein accumulation was observed in I/R-injured kidney tubules [19], and here showed that GRP-78, target gene of unfolded protein response, was upregulated in I/R kidney, but febuxostat reversed this induction. In addition, ATF4, and its downstream CHOP were increased in I/R kidney, while treatment of febuxostat ameliorated their increase. Thus, febuxostat suppressed apoptosis by inhibiting oxidative stress and ER stress.

The present study supports the current pathological concept that XO activity itself rather than hyperuricemia may play important roles in I/R injury. Several reports suggest the UA-independent therapeutic effect of XO inhibitor. Clinical study showed that benzbromaron lowered UA level, but had no effects on hemodynamic impairment in chronic heart failure patients [20]. XO inhibitor showed renoprotective effects in 5/6 nephrectomy rats without hyperuricemia [21]. Since XOR are expressed ubiquitously, targeting XO activity can be applied to a variety of tissue and disease conditions. CKD patients are shown to have high oxidative stress [22], and those patients are expected to have high protein conversion rate from XDH to XO. The use of XOR inhibitor in CKD patients has been restricted due to the lack of appropriate agents, but we now have novel agent; febuxostat, which can be used effectively even in CKD patients. Thus, we need further investigations about the role of febuxostat in the progression of CKD. Although the reduction of uric acid itself may be protective for CKD patients, uric acid-independent actions of XOR inhibitor may play significant roles on the progression of CKD or CVD. It may eventually support the idea to apply XOR inhibitors not only to hyperuricemic, but also to non-hyperuricemic subjects to modulate these UA-independent actions of XOR.

In conclusion, our results show that XOR activity contributes to the progression of renal interstitial injury by modulating oxidative stress and ER stress. Our observations support the current pathological concept that, in addition to hyperuricemia, increased XO activity itself may play important roles in the progressive renal injury, and a novel XOR inhibitor, febuxostat, may be a therapeutic tool for progressive renal injury.

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ORIGINAL ARTICLE
Vascular Biology and Vascular Medicine

# Smoking Promotes Subclinical Atherosclerosis in Apparently Healthy Men

## – 2-Year Ultrasonographic Follow-up –

Shoji Sanada, MD, PhD; Makoto Nishida, MD, PhD; Kouki Ishii; Toshiki Moriyama, MD, PhD; Issei Komuro, MD, PhD; Keiko Yamauchi-Takihara, MD, PhD

***Background:*** Smoking is a major risk factor for cardiovascular disease. Also, inflammatory activation and metabolic disorder are the mediators of smoking-induced atherosclerotic progression. The aim of the present study was to investigate whether current smoking and smoking cessation alter inflammatory or metabolic status and affect subclinical atherosclerosis in apparently healthy men.

***Methods and Results:*** Classical risk factors and smoking habit were evaluated in 354 men who completed health examinations annually without any current medications. Carotid intima-media thickness (IMT) was followed for 27.1±4.5 months. At baseline, both maximum and mean IMT significantly changed during 2-year follow-up. They tended to increase along with progression of smoking habit, with significantly greater maximum IMT in current smokers compared with never smokers. Both maximum and mean IMT significantly changed during 2-year follow-up, and tended to increase with progression of smoking habit, with maximum IMT being greatest for current smokers. Past smokers tended to have greater IMT increase than never smokers. Among smoking habit and some atherosclerotic risk markers that showed significant correlation with maximum IMT increase, stepwise regression showed that smoking habit and serum low-density lipoprotein-cholesterol (LDL-C) level were the only independent predictors.

***Conclusions:*** Significant 2-year progression of subclinical atherosclerosis was associated with continuous smoking and LDL-C. This was only partly moderated in past smokers despite complete reversal of inflammatory activation, suggesting another crucial factor for inhibiting accelerated progression of subclinical atherosclerosis in men. (*Circ J* 2012; **76:** 2884–2891)

**Key Words:** Inflammation; Intima-media thickness; Metabolic syndrome; Progression; Smoking cessation

Previous epidemiological studies had proposed numerous risk factors for cardiovascular disease (CVD), such as hypertension, diabetes, and hyperlipidemia,[1] all of which comprise metabolic syndrome (MetS).[2] Also, it has been reported that lower plasma adiponectin[3] is an independent risk factor for CVD.[4] Furthermore, serum high-sensitivity C-reactive protein (hs-CRP) level is recognized as an independent predictor of CVD,[1] and serum interleukin-6 (IL-6) level is associated with increased incidence of CVD,[5] implicating inflammatory responses in the incidence of CVD.

Meanwhile, smoking has also emerged as an important risk factor for CVD,[6] and the inflammatory responses as well as impairment of MetS are thought to be involved in the underlying mechanisms of atherosclerosis development,[7,8] the leading cause of CVD.[9] Therefore, in addition to recovery from MetS through reduction of body weight or salt intake,[3,6] smoking cessation is generally and strongly recommended in current anti-atherosclerotic lifestyle improvement.[6] The impact, however, of smoking cessation on reduction of atherosclerotic changes and, if so, which mechanism confers the improvement, is not fully identified.

Recently, non-invasive measurements of arterial intima-media thickness (IMT) have been widely used for assessment of subclinical arterial alterations, and have demonstrated that this is a predictor of CVD.[9,10] In addition, the association of traditional risk factors with IMT (mainly maximum IMT) has been well examined.[11–13]

In the present study, to elucidate whether smoking cessation reduces or reverses the progression of atherosclerosis, and to explore what underlying mechanisms might be associated with

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**Table 1. Baseline Clinical Subject Characteristics**

| | All | Smoking habit | | | P-value |
|---|---|---|---|---|---|
| | | Never | Past | Current | |
| n | 354 | 195 (55) | 84 (24) | 75 (21) | |
| Age (years) | 48.5±5.7 | 47.7±5.6 | 49.6±5.7 | 49.4±5.5 | 0.009 |
| BMI (kg/m²) | 23.2±2.8 | 23.1±2.7 | 23.0±2.4 | 23.8±3.3 | 0.09 |
| Waist (cm) | 82.6±7.7 | 81.9±7.7 | 82.1±6.5 | 85.3±8.6 | 0.004 |
| SBP (mmHg) | 122±14 | 121±13 | 122±15 | 123±15 | 0.42 |
| DBP (mmHg) | 79±11 | 79±10 | 80±12 | 80±12 | 0.52 |
| UA (mg/dl) | 6.0±1.2 | 6.0±1.1 | 6.2±1.1 | 5.9±1.3 | 0.25 |
| TG (mg/dl) | 118±104 | 110±81 | 109±62 | 147±172 | 0.02 |
| HDL-C (mg/dl) | 56±14 | 57±15 | 57±12 | 55±14 | 0.74 |
| LDL-C (mg/dl) | 128±29 | 126±27 | 129±33 | 130±28 | 0.68 |
| FPG (mg/dl) | 92±12 | 91±12 | 94±11 | 90±10 | 0.18 |
| $HbA_{1c}$, % | 5.0±0.5 | 5.0±0.5 | 5.0±0.5 | 5.0±0.4 | 0.68 |
| Max. IMT (mm) | 0.922±0.502 | 0.877±0.471 | 0.958±0.474 | 1.001±0.597 | 0.15 |
| Mean IMT (mm) | 0.682±0.170 | 0.662±0.156 | 0.712±0.198 | 0.699±0.168 | 0.05 |
| Presence of plaque | 38 (10.7) | 16 (8.2) | 12 (14.3) | 10 (13.3) | 0.23 |

Data given as n (%) or mean±SD.
BMI, body mass index; DBP, diastolic blood pressure; FPG, fasting plasma glucose; HDL-C, high-density lipoprotein cholesterol; IMT, intima-media thickness; LDL-C, low-density lipoprotein cholesterol; SBP, systolic blood pressure; TG, triglycerides; UA, uric acid.

this reduction, we evaluated the associations of MetS parameters as well as inflammatory markers with IMT and their relationship with smoking habit in drug-naïve apparently healthy subjects.

## Methods

### Subjects

The subjects were the men who underwent health examinations in the Osaka University Health Care Center during 2005–2007. Apparently healthy Japanese men (n=354), 40–59 years of age, who completed an annual visit for medical checkup in 3 consecutive years, did not take any chronic or frequent medicine from at least 1 year before the first visit to the end of follow-up, did not suffer acute illness within 2 weeks before each visit and successfully underwent carotid ultrasonography in the first and the third visits were consecutively included. Informed consent was obtained from all subjects prior to participation in the study following approval of the study by the Ethics Committee of Osaka University. Because blood tests for hs-CRP, IL-6, and adiponectin concentration were beyond routine annual medical checkup, these tests were also performed in samples from 89 men (42/29/18 in never, past and current smokers, respectively) who participated in this study and who also agreed in writing to additional investigational measurements.

### Definition of Past Smoker and Smoking Cessation Period

Smoking habit for each participant was primarily obtained from the mark in the check boxes sorting them into never, current or past smokers, as well as complementary descriptions determining the duration of smoking period in the interview sheet at annual medical checkup. For the past smokers, because the smoking cessation periods were not directly queried on the interview sheet, all the past interview sheet records for each individual were surveyed and the duration of smoking cessation defined as the period starting from the first year after the smoking habit changed from current to past smoker. If it was the case that all the past records indicated past smoking habit or the record was not available, we then referred to a formula of [(Age, years old)–(duration of smoking period, years)–20] year(s), based on the directly acquired data via the interview, to estimate the smoking cessation period.

**Table 2. Smoking Status**

| | Past smoker, n (%) | Current smoker, n (%) |
|---|---|---|
| **Smoking period (years)** | | |
| 1–5 | 20 (23.8) | 0 (0) |
| 6–10 | 24 (28.6) | 2 (2.7) |
| 11–20 | 25 (29.8) | 15 (20.3) |
| 21– | 15 (17.9) | 57 (77.0) |
| **No. cigarettes** | | |
| 1–10 | 23 (34.3) | 14 (18.7) |
| 11–20 | 29 (43.3) | 31 (41.3) |
| 21–40 | 13 (19.4) | 29 (38.7) |
| 41– | 2 (3.0) | 1 (1.3) |
| **Smoking cessation period (years)** | | |
| 2–5 | 1 (1.2) | |
| 6–10 | 12 (14.3) | |
| 11–20 | 33 (39.3) | |
| 21–30 | 27 (32.1) | |
| 31– | 11 (13.1) | |

### Risk Factor Assessment

Information on medical history, use of medicines and personal smoking habit were obtained via questionnaire, and was reconfirmed in expert interview by trained nurses. Waist circumference at the umbilical level was measured in the late exhalation phase in standing position.

### Laboratory Measurements

Serum was collected from subjects after overnight fasting and kept at ≤–20°C until assay. Serum hs-CRP, IL-6 and adipo-

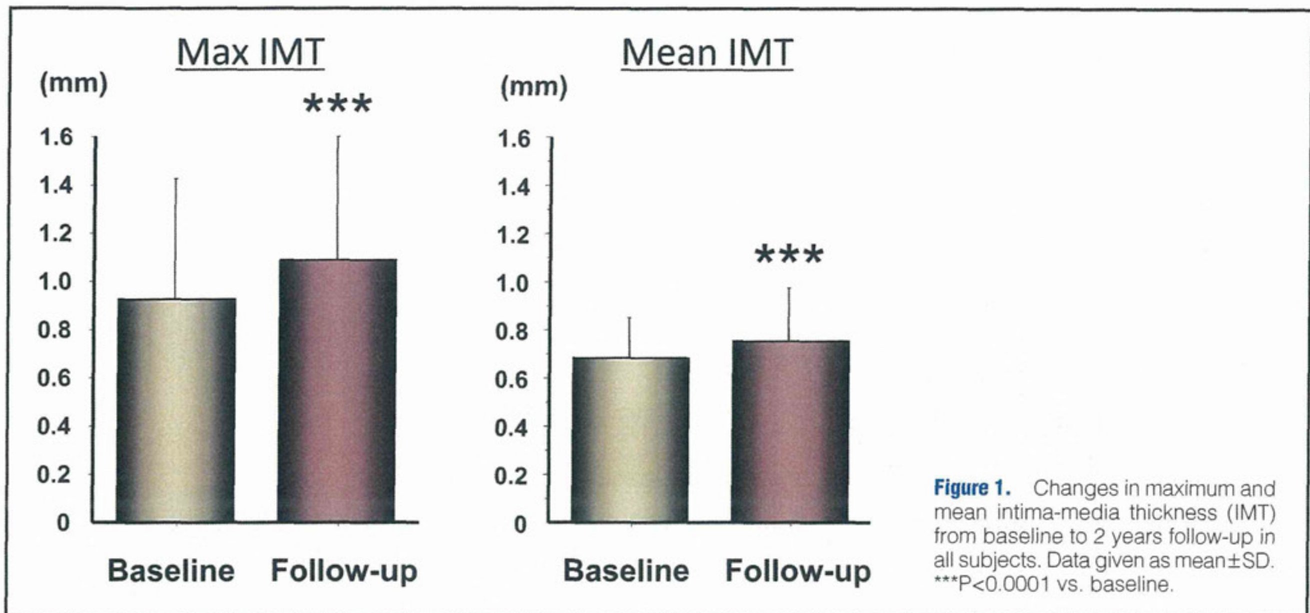


**Figure 1.** Changes in maximum and mean intima-media thickness (IMT) from baseline to 2 years follow-up in all subjects. Data given as mean±SD. ***P<0.0001 vs. baseline.

**Table 3. Risk Factors and 2-Year Increase of IMT**

| | Delta-maximum IMT | | Delta-mean IMT | |
|---|---|---|---|---|
| | r | P-value | r | P-value |
| Age | 0.041 | 0.44 | 0.063 | 0.24 |
| BMI | 0.035 | 0.51 | 0.023 | 0.67 |
| Waist | 0.042 | 0.43 | 0.013 | 0.80 |
| SBP | 0.114 | 0.031 | 0.097 | 0.068 |
| DBP | 0.025 | 0.64 | 0.073 | 0.17 |
| UA | 0.079 | 0.14 | 0.110 | 0.039 |
| TG | 0.108 | 0.042 | 0.019 | 0.72 |
| HDL-C | –0.029 | 0.58 | 0.052 | 0.33 |
| LDL-C | 0.142 | 0.009 | 0.155 | 0.004 |
| FPG | 0.087 | 0.10 | 0.092 | 0.082 |
| $HbA_{1c}$ | 0.108 | 0.042 | 0.118 | 0.026 |
| Smoking habit | 0.130 | 0.015 | 0.096 | 0.071 |

Abbreviations as in Table 1.

nectin concentration were measured as described previously.[14,15] Briefly, they were measured using an immunoenzyme assay, a chemiluminescent enzyme immunoassay (CLEIA) and a sandwich enzyme-linked immunosorbent assay (ELISA) system, respectively.

The mean interclass coefficient of variation (CV) of hs-CRP, IL-6, and adiponectin measurements (n=40) in the assays before this study were 1.1%, 4.5%, and 1.2%, respectively. Kits from the same lots were used in this study to maintain reliability of measurement.

### Evaluation of Carotid Atherosclerosis

All ultrasound examinations were performed by a single well-trained sonographer (K.I.) who regularly participated in quality control measurement sessions and was totally blinded to all clinical information, using LOGIQ 5 (GE Yokogawa Medical Systems, Tokyo, Japan) with an 8.8-MHz linear transducer. Three different longitudinal images (anterior oblique, lateral, and posterior oblique) of the left common carotid artery (CAA) of a 1.0–1.5-cm section at the distal end of the CCA proximal to the carotid bulb were obtained as described previously,[14,15] complying with validated protocols. In addition, transverse images were then obtained to confirm the accuracy of longitudinal images. After examination, the best longitudinal images were analyzed for each individual. Maximum and mean IMT was obtained using computer software that automatically traces the intima-media edge of the far wall. The presence of plaque was defined as detection of a focal structure encroaching into the arterial lumen of at least 0.5 mm or 50% of the surrounding IMT or having a thickness of ≥1.5 mm, in concordance with a previous report.[16]

### Statistical Analysis

Data were analyzed using SPSS 14.0 (Chicago, IL, USA). Pearson's correlation coefficients were calculated for variables with skewed distribution after logarithmic transformation. Stepwise multiple regression analysis was conducted using the enter method. ANOVA with modified Bonferroni's post-hoc test was used to assess differences between groups based on category. In order to analyze correlation of smoking with the progression of IMT, current, past and never smokers were scored as 1, 0.5 and 0, respectively, and the sum of this score was used, together with IMT progression within 2 years in each individual. P<0.05 was considered statistically significant.

## Results

### Baseline Demographics

Clinical characteristics of the study subjects are summarized in **Table 1**. With regard to risk factors, age was significantly older, and waist circumference and serum triglyceride (TG) level significantly higher as smoking habit progressed in men, whereas no significant differences were seen in body mass index (BMI), blood pressure (BP), uric acid (UA), high-density lipoprotein-cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), fasting plasma glucose (FPG) and $HbA_{1c}$. As shown in **Table 1**, both maximum and mean IMT tended to increase as smoking habit progressed, reaching significance in mean IMT. Plaques as defined in a previous report[16] were