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ORIGINAL RESEARCH

Relationship of Sodium Intake With Granulocytes, Renal and Cardiovascular Outcomes in the Prospective EPIC-Norfolk Cohort

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BACKGROUND: Experimental studies show that high-sodium intake affects the innate immune system, among others with increased circulating granulocytes. Whether this relationship exists on a population level and whether this relates to disease outcomes is unclear. We aimed to test the hypotheses that (1) sodium intake is associated with granulocytes on a population level; (2) granulocytes are associated with the presence of hypertension and both cardiovascular and renal outcomes; and (3) the relation between high-sodium intake and these outcomes is mediated by granulocytes.

METHODS AND RESULTS: We performed an analysis in 13 804 participants from the prospective EPIC (European Prospective Investigation into Cancer)-Norfolk cohort, with a mean age of 58 years and median follow-up of 19.3 years. Analyses were carried out using calculated estimated sodium intake and sodium-to-potassium ratios from spot urines at baseline. The main outcomes were hypertension at baseline, and composite cardiovascular (mortality or cardiovascular events) and renal (mortality or renal events) outcomes during follow-up. Sodium intake and urine sodium-to-potassium ratio were positively associated with circulating granulocyte concentrations after adjustment for confounders (β=0.03; P=0.028 and β=0.06; P<0.001, respectively). Granulocytes significantly mediated the associations of, respectively, sodium intake and urine sodium-to-potassium ratio with hypertension at baseline, and cardiovascular and renal outcomes.

CONCLUSIONS: Sodium intake is positively associated with circulating granulocyte concentrations, and higher granulocyte concentrations associate with worse long-term cardiovascular and renal outcomes. Given the recently established immune-modulating effects of sodium and the role of immune cells in both cardiovascular and renal disease, causality for this pathway may need consideration in further studies.

Key Words: cardiovascular ■ granulocytes ■ hypertension ■ renal ■ sodium

High sodium intake has been associated with adverse outcomes, including hypertension, cardiovascular disease, renal disease, and all-cause mortality.[1–3] Underlying causal mechanisms are likely multifactorial and, other than effects on the extracellular fluid compartment, involve neural, hormonal, and oxidative stress-related pathways.[4] In past decades, it became increasingly clear that sodium also has immune-modulating properties, likely also playing a role in associated deleterious health outcomes.[5–7] High sodium consumption has differential effects on leukocyte subsets with regard to their absolute numbers as well as activation state, involving pro-inflammatory effects on monocytes, macrophages,

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CLINICAL PERSPECTIVE

What Is New?

- Sodium intake has a positive association with peripheral granulocyte concentrations.
- Granulocyte concentrations are associated with worse long-term cardiovascular and renal outcomes.

What Are the Clinical Implications?

- Given the experimentally established immune-modulating effects of sodium, it deserves further clinical exploration whether these immune changes serve as a causal link between high sodium intake and worse long-term health outcomes.

Nonstandard Abbreviations and Acronyms

BMI	body mass index
BP	blood pressure
CKD	chronic kidney disease
CKD-EPI	Chronic Kidney Disease Epidemiology Collaboration

and T-cells, among others.[5,7–12] Less is known about the effects on the most abundant leukocyte subset in humans, namely neutrophilic granulocytes. In our randomized controlled trial investigating the effect of a one-to-two-week high-sodium diet on healthy males, circulating neutrophil counts increased by ≈20%—an effect that has not been linked to the deleterious effects of sodium consumption to date.[10] Only recently, granulocyte counts (neutrophil counts and the neutrophil/lymphocyte ratio in particular) have been associated with hypertension, cardiovascular disease, renal outcomes, and all-cause mortality.[13–20] Whether increased sodium consumption underlies this association is unknown. We hypothesized that (1) sodium intake is associated with granulocytes on a population level; (2) granulocytes are associated with the presence of hypertension and both cardiovascular and renal outcomes; and (3) the relation between high sodium intake and these outcomes may be mediated by granulocytes. We tested these hypotheses in the EPIC (European Prospective Investigation into Cancer)-Norfolk population-based prospective study.

METHODS

The data underlying this article were provided by the Epidemiology Unit of Cambridge University with permission. Data will be shared upon request with the corresponding author with permission of this party.

Study Design

We performed an analysis in the EPIC-Norfolk population-based prospective population study. This cohort included 25 639 men and women between 40 to 79 years old residing in Norfolk, United Kingdom. Participants were recruited via general practice registers. Between 1993 and 1998, baseline visits were carried out, in which a variety of measurements was done by trained study nurses, including body weight and length, blood sampling, urine sampling, and blood pressure recording. During follow-up, several health checks were performed and outcomes were identified using national registries. We report results with follow-up up to March 31, 2016. The Norwich District Health Authority Ethics Committee approved the study and all participants provided written informed consent. The study was conducted in accordance with the Declaration of Helsinki. The data from the EPIC-Norfolk study were obtained after an In-Reach agreement was signed (ENDR004_2020). This report was written in accordance with the STROBE guidelines.[21]

Selection of Participants

Differential leukocyte concentrations were not measured in the entire cohort due to funding reasons. For the present study, we identified 17 670 participants that had differential leukocyte concentrations available together with the values needed for estimation of 24-hour urine sodium and potassium excretion by means of the Kawasaki formula (ie, spot urine levels of sodium and potassium and creatinine, sex, age, weight, and height).[22] Assessment of baseline characteristics did not show differences from the total cohort for this cohort selection. Participants with prevalent or incident cancer were excluded from the analyses, resulting in a cohort selection of n=13 804 participants. Chronic kidney disease at baseline was defined based on an estimated glomerular filtration rate (CKD-EPI) of <60 mL/min per 1.73 m^2.

Biochemical Analyses

Random spot urine specimens were obtained from the participants, which were stored at –20 °C without a preservative. Between 1998 and 2002, the urine samples were thawed and assayed for sodium and potassium levels with flame photometry (IL 943; Instrumentation Lab, Warrington, UK) and for creatinine levels (Roche Cobas Mira Plus analyzer). These levels were used to estimate 24-hour urine sodium and potassium excretion by means of the Kawasaki formula.[22] The estimated 24-hour urine sodium excretion was regarded as a proxy for sodium intake. Additionally, we

performed analyses using the urine sodium/potassium ratio, as recent evidence shows it may be a better predictor for hypertension and cardiovascular disease compared to urine sodium concentration alone and is less subject to bias because it does not depend on body weight and urine creatinine.[23,24] For leukocyte measurements, non-fasting venous blood samples were stored overnight at room temperature and subsequently transferred to the EPIC Norfolk laboratory in Attleborough, UK, where leukocyte differentiation was carried out using an MD18 haematology analyzer (Coulter Corporation, Miami, FL, USA). Experimental details have been described previously.[20] Circulating granulocyte, monocyte, and lymphocyte concentrations were expressed as a percentage of total blood volume. For measurements of other laboratory values, samples were stored at 4 °C and assayed at the Department of Clinical Biochemistry, University of Cambridge, Cambridge, UK.

Outcomes of Interest

Primary outcomes were hypertension at baseline, the composite of mortality and cardiovascular events, and the composite of mortality and renal events. Hypertension was defined as use of antihypertensive drugs or blood pressure >140/90 mm Hg at baseline. Blood pressure was measured with a validated noninvasive blood pressure monitor (Accutorr, Datascope, Mahwah, NJ, USA) after the participant had been seated for 5 minutes.[25] The mean of 2 readings was used for analysis. Cardiovascular events were defined as hospitalizations during follow-up with cardiovascular disease coded as the underlying cause (*International Classification of Diseases, Tenth Revision* [*ICD-10*] codes I10-I79, which include ischemic heart disease, peripheral artery disease, aortic aneurysm, aortic stenosis, heart failure, and cerebrovascular accident). Study participants with a history of cardiovascular disease were excluded for this analysis (n=556). Renal events were defined as hospitalizations during follow-up due to kidney disease coded as the underlying cause (ICD-10 codes N00-N19 or N25-N29). The secondary outcome was all-cause mortality. Vital status was ascertained for the entire cohort at the UK Office of National Statistics.

Statistical Analysis

Continuous variables are reported as mean with standard deviation, and categorical variables were reported as frequencies and percentages. Multiple linear regression was performed to examine the relation between sodium intake or urine sodium-to-potassium levels and granulocytes. Additionally, the relation with other leukocyte subsets (ie, monocytes and lymphocytes) was explored. The distribution of data was assessed with visual inspection of Q-Q plots. In case of non-normally distributed data, data were log-transformed before they were included in the regression analysis. An interaction test between urine sodium and urine potassium was carried out. Also, to explore the presence of potential sex-specific differences, formal interaction tests with sex were performed. Logistic regression models and cox proportional hazards models were used to examine the relation between granulocytes and hypertension at baseline and the long-term health outcomes of interest, as appropriate. The proportional hazard assumption was checked using formal statistical tests and graphic plots of Schoenfeld residuals. Mediation analyses were performed to explore whether there could be a mediating effect of granulocytes (M) on the relation of urine sodium or sodium-to-potassium (X) with the health outcomes of interest (Y) (Figure). When relationships between X→M and M→Y emerge from separate regression models and clinical or experimental knowledge supports a causal sequence of X→M→Y, mediation analyses can be used to formally test whether M could statistically serve as a mediator between X and Y.[26] Different methodological schools exist regarding these analyses, each advocating specific arguments against or in support of certain analytical methods.[26–28] We performed mediation analyses with a structural equation (SEM) approach, as this approach estimates all associations simultaneously and does not rely on the assumption that the separate associations are independent, in contrast to a regression-based approach.[26–28] The added value of these analyses is that they incorporate 3 regression models or pathways (X→Y, X→M, M→Y) into one model, and give a statistical probability about whether an indirect mediating pathway may be present (X→M→Y) as well as provide a quantitative estimate on their effect relative to the direct pathway (X→Y).[28] The SEM approach comes with its own assumptions, that may mainly involve linearity between several pathways (X→Y, X→M, M→Y), absence of confounding on all 3 pathways, reliability of measurements, and temporality.[26–28] Bootstrapping with 5000 samples was used to calculate percentile 95% CIs (which are non-symmetric and therefore better reflect the sampling distributions of the conditional indirect effects) for significance testing. The observed coefficients were used to calculate the proportion of mediation (a*b/c) (Figure). All analyses were adjusted for sex, age, body mass index, smoking status, alcohol use, diabetes, total cholesterol, baseline chronic kidney disease, and antihypertensive drug use (the latter was not used in the analyses with hypertension at baseline as the outcome), based on literature and clinical rationale. Analyses using estimated sodium intake as an independent variable were additionally adjusted for estimated potassium intake. As a sensitivity analysis, all analyses were furthermore adjusted for CRP (C-reactive protein) levels. Statistical analyses were

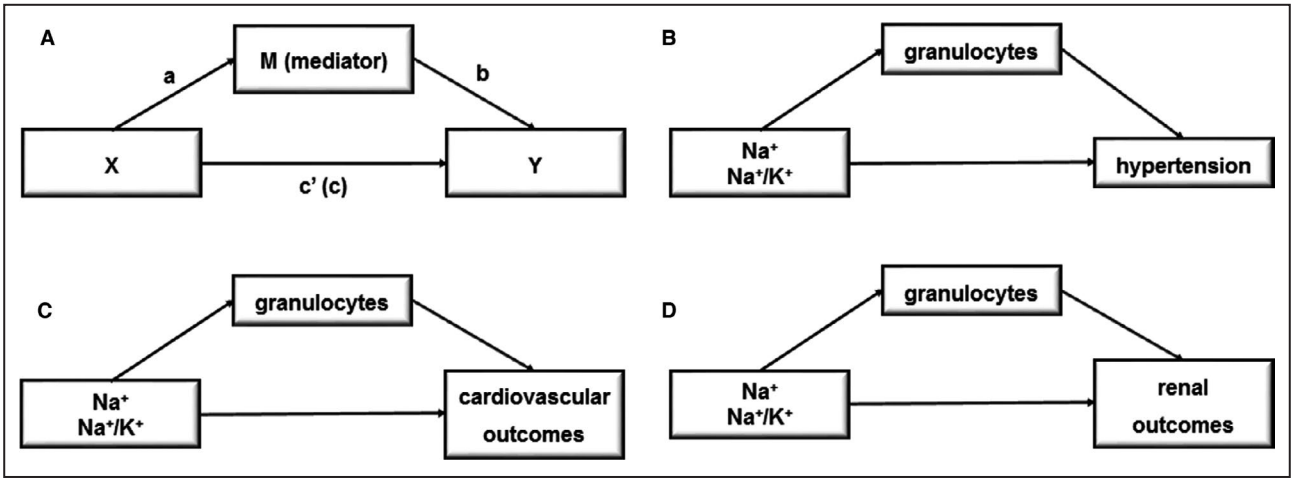

Figure. Mediation analyses.
A, Schematic depiction of the relation between X, M, and Y in the mediation analyses. The proportion of mediation (%) is calculated by a*b/c (=a*b/(a*b + c') *100). **B**, Hypertension was determined at baseline. **C**, The composite cardiovascular outcome involves cardiovascular events and mortality. **D**, The composite renal outcome involves renal disease events and mortality. Na^+ indicates sodium; and K^+, potassium.

conducted using SPSS (version 26.0, SPSS Inc.) and Stata (version 15.1, StataCorp). A value of $P<0.05$ was considered significant.

RESULTS

After application of inclusion and exclusion criteria, data from 13 804 subjects were available for analysis. Baseline characteristics are depicted in Table 1 and Table 2. Hypertension was present in 6426 participants (46.6%) at baseline. During a median follow-up time of 19.3 years, cardiovascular outcomes occurred in 7579 participants (54.9%) and renal outcomes in 3442 participants (24.9%). All-cause mortality at the end of follow-up was 21.3% (2941 participants).

Estimated Sodium Intake, Urine Sodium-to-Potassium Levels, and Granulocytes

Both estimated sodium intake and urine sodium-to-potassium levels showed a significant positive association with granulocyte concentrations after adjustment for potential confounders (β=0.03; $P=0.028$ and β=0.06; $P<0.001$, respectively) (Table 3). The association between sodium intake and granulocytes was not significant when no adjustment for potassium intake was made. Estimated potassium intake appeared to have a negative association with granulocytes, in the unadjusted as well as the adjusted models (Table S1). There were no significant interactions between estimated sodium intake and potassium intake in the models, or between sex and the independent variable of interest. There were no associations of sodium intake and urine sodium-to-potassium levels with lymphocytes, whereas urine sodium and urine sodium-to-potassium levels showed a significant negative association with monocytes (Table S2 and S3). Sensitivity analyses showed that additional adjustment for CRP in the models did not materially affect the results.

Granulocytes, Hypertension, and Risk of Cardiovascular and Renal Outcomes

Table 4 depicts Odds and Hazard ratios with 95% CIs for the association between granulocytes and the specified outcomes. Granulocytes are significantly associated with hypertension at baseline and with composite cardiovascular and renal outcomes in follow-up (all $P<0.001$). One unit increase of granulocytes increases the relative risk of hypertension with 19% (16%–23%), and cardiovascular and renal outcomes with 7% (6%–9%) and 13% (10%–16%), respectively. There was also an association between granulocytes and all-cause mortality ($P<0.001$). Risk on all-cause mortality increases with 14% (11%–17%) per one unit increase in circulation granulocyte concentration. There was no interaction between granulocytes and sex in the models (Table 4). Monocytes were not associated with hypertension or worse long-term outcomes, lymphocytes were associated with hypertension at baseline but not with other outcomes (Table S4 and S5). Sensitivity analyses showed that additional adjustment for CRP in the models did not materially affect the results.

Table 1. Baseline Characteristics Stratified on Estimated 24-Hours Urine Na^+

	All	Tertiles of estimated sodium intake			
	n=13 804	<168 mmol n=4601	168–220 mmol n=4602	>220 mmol n=4601	*P* value
Male, n (%)	6201 (44.9)	1389 (30.2)	2125 (46.2)	2687 (58.4)	<0.001
European descent, n (%)	13 698 (99.6)	4563 (99.2)	4568 (99.3)	4567 (99.3)	0.18
Age, y	58.2 (9.3)	59.3 (9.5)	58.0 (9.3)	57.3 (9.1)	<0.001
BMI, kg/m^2	26.2 (3.9)	25.8 (3.8)	26.1 (3.8)	26.8 (3.9)	<0.001
Smoking					<0.001
Current	1555 (11.3)	509 (11.1)	516 (11.3)	530 (11.6)	
Past	5648 (41.2)	1765 (38.6)	1863 (40.8)	2020 (44.2)	
Never	6508 (47.5)	2300 (50.3)	2192 (48.0)	2016 (44.2)	
Alcohol use, (grams/d)*	4.7 (0.8–11.0)	4.0 (0.8–10.2)	4.7 (0.8–11.4)	4.9 (0.8–11.8)	<0.001
Systolic BP, mm Hg	135 (18)	133 (18)	134 (17)	137 (18)	<0.001
Diastolic BP, mm Hg	82 (11)	81 (11)	82 (11)	84 (11)	<0.001
Diabetes n (%)	306 (2.2)	88 (1.9)	99 (2.2)	119 (2.6)	0.08
Hypertension n (%)	6426 (46.6)	2004 (43.6)	2037 (44.3)	2385 (51.8)	<0.001
Antihypertensive drugs n (%)	2390 (17.3)	844 (18.3)	690 (15.0)	856 (18.6)	<0.001
CKD n (%)	1765 (12.8)	838 (18.2)	536 (11.6)	391 (8.5)	<0.001
Total cholesterol mmol/L	6.17 (1.2)	6.21 (1.2)	6.15 (1.2)	6.15 (1.2)	0.03
CRP, pg/mL*	1.5 (0.7–3.1)	1.5 (0.7–3.3)	1.4 (0.7–2.9)	1.5 (0.7–3.0)	<0.001
Leukocytes (%)	6.5 (1.7)	6.5 (1.7)	6.5 (1.7)	6.5 (1.7)	0.48
Granulocytes	3.97 (1.38)	3.98 (1.41)	3.95 (1.38)	3.97 (1.34)	0.63
Monocytes	0.52 (0.36)	0.54 (0.39)	0.51 (0.35)	0.49 (0.33)	<0.001
Lymphocytes	2.00 (0.62)	1.99 (0.62)	2.01 (0.62)	2.02 (0.61)	0.13
eGFR (CKD-EPI)	74.3 (15.7)	70.5 (14.9)	74.6 (15.4)	77.8 (16.0)	<0.001
Estimated 24-hour urine K^+, mmol/24 h	68.9 (17.4)	59.9 (13.4)	68.2 (14.4)	78.6 (18.4)	<0.001

Leukocytes were presented as percentages (%) of total blood volume. Data are depicted as mean (SD) or median (IQR)*. Data comparing tertiles were tested with a one-way ANOVA for continuous variables (after log transformation in case of non-parametrically distributed variables) and a Chi-square test for categorical variables. BMI indicates body mass index; BP, blood pressure; CKD, chronic kidney disease; CRP, C-reactive protein; eGFR, estimated glomerular filtration rate; and K^+, potassium.

Granulocytes Mediate the Association Between Sodium Intake and Urine Sodium-to-Potassium Levels With Worse Outcomes

Granulocytes significantly mediated the relation between sodium intake and urine sodium-to-potassium levels with hypertension at baseline, long-term composite cardiovascular and renal outcomes, and all-cause mortality (Table 5). The proportion of the relation between sodium intake and urine sodium-to-potassium levels and outcomes that was mediated by granulocytes (ie, mediated proportion) was highest for cardiovascular outcomes (11.8% for estimated sodium intake and 17.6% for urine sodium-to-potassium levels). Overall, there was a higher mediation proportion by granulocytes in the analyses using urine sodium-to-potassium levels (7.0%–17.6%) than for estimated sodium intake (3.6%–11.8%).

DISCUSSION

We demonstrate in a large prospective cohort that estimated sodium intake and urine sodium-to-potassium ratios are independently and positively associated with granulocyte concentrations. Granulocyte concentrations show a positive association with hypertension at baseline, and are prospectively associated with the risk of cardiovascular and renal outcomes during 19 years of follow-up. As a by-finding, we revealed an independent negative association between estimated potassium intake and granulocytes that may exceed the effects of sodium, which merits further exploration.

This study is the first (to our knowledge) to incorporate evidence from small-scaled intervention studies on the immune-modulating properties of sodium into a population study, enabling exploration of associated deleterious health outcomes. As emphasized, our analyses cannot prove a causal pathway, nor its

Table 2. Baseline Characteristics Stratified on Estimated 24-Hours Urine Na^+/K^+

	All	Tertiles of estimated 24-hours urine Na^+/K^+			
	n=13 804	<2.5 n=4595	2.5–3.2 n=4608	>3.2 n=4601	*P* value
Male, n (%)	6201 (44.9)	1696 (36.9)	2126 (46.1)	2379 (51.7)	<0.001
European descent, n (%)	13 698 (99.6)	4561 (99.3)	4563 (99.0)	4574 (99.4)	0.51
Age, y	58.2 (9.3)	58.3 (9.2)	58.1 (9.3)	58.2 (9.4)	0.63
BMI, kg/m^2	26.2 (3.9)	25.9 (3.8)	26.2 (3.9)	26.5 (4.0)	<0.001
Smoking					<0.001
Current	1555 (11.3)	4319 (9.5)	537 (11.7)	587 (12.8)	
Past	5648 (41.2)	1811 (39.7)	1933 (42.2)	1904 (41.7)	
Never	6508 (47.5)	2318 (50.8)	2112 (46.1)	2078 (45.5)	
Alcohol use (grams/d)*	4.7 (0.8–11.0)	5.2 (0.8–12.0)	4.7 (0.8–11.3)	3.4 (0.8–10.1)	<0.001
Systolic BP, mm Hg	135 (18)	132 (17)	134 (18)	137 (19)	<0.001
Diastolic BP, mm Hg	82 (11)	81 (11)	82 (11)	84 (11)	<0.001
Diabetes n (%)	306 (2.2)	93 (2.0)	103 (2.2)	110 (2.4)	0.49
Hypertension n (%)	6426 (46.6)	2004 (43.6)	2057 (44.6)	2365 (51.4)	<0.001
Antihypertensive drugs n (%)	2390 (17.3)	836 (18.2)	712 (15.5)	842 (18.3)	<0.001
CKD n (%)	1765 (12.8)	711 (15.5)	563 (12.2)	491 (10.7)	<0.001
Total cholesterol, mmol/L	6.17 (1.2)	6.18 (1.2)	6.19 (1.2)	6.15 (1.2)	0.14
CRP (pg/mL)*	1.5 (0.7–3.1)	1.4 (0.7–3.0)	1.4 (0.7–3.0)	1.5 (0.7–3.3)	0.15
Leukocytes (%)	6.5 (1.7)	6.4 (1.6)	6.5 (1.7)	6.6 (1.7)	<0.001
Granulocytes	3.97 (1.38)	3.89 (1.34)	3.96 (1.38)	4.06 (1.41)	<0.001
Monocytes	0.52 (0.36)	0.53 (0.37)	0.52 (0.36)	0.50 (0.34)	0.001
Lymphocytes	2.00 (0.62)	1.99 (0.59)	2.01 (0.63)	2.01 (0.63)	0.47
eGFR (CKD-EPI)	74.3 (15.7)	72.7 (15.4)	74.4 (15.5)	75.7 (16.0)	<0.001

Leukocytes were presented as percentages (%) of total blood volume. Data are depicted as mean (SD) or median (IQR)*. Data comparing tertiles were tested with a one-way ANOVA for continuous variables (after log transformation in case of non-parametrically distributed variables) and a Chi-square test for categorical variables. BMI indicates body mass index; BP, blood pressure; CKD, chronic kidney disease; CRP, C-reactive protein; eGFR, estimated glomerular filtration rate; K^+, potassium; and Na^+, sodium.

direction nor sequence. The current hypothesized pathway is based on multiple experimental and mechanistic studies, showing that 1–2 week high-sodium interventions induce various effects on leukocyte subsets (in number as well as activation state),[5,10–12,29] and revealing a causal role for leukocyte subsets and particularly granulocytes with regard to development and progression of cardiovascular and renal disease.[17,30] Statistically, granulocyte concentrations were able to serve as a potential mediator in our models, fitting the above-mentioned mechanistic ideas, but again, associations do not mean causality or causality could exist in other directions. Especially for the hypertension outcome reverse causality cannot be excluded, as this was measured at baseline (not enough data points were available during follow-up).

Mechanisms underlying salt-induced granulocyte increases are not yet identified and deserve further exploration. Amongst others, sympathetic activation can induce neutrophilia,[31] and likely contributes to sodium-induced granulocyte increases given the sympathetic-stimulating effect of sodium.[32] However, sodium may also have direct effects on proliferation of hematopoietic stem cells through metabolic changes, as has been established for hyperglycemia and hypercholesterolemia.[17] As said, the negative association between granulocytes and estimated potassium intake that we observed is noteworthy, touching upon the hypothesized anti-inflammatory effects of potassium, and should be investigated in further detail.[33] Also, the negative association between sodium intake and urine sodium-to-potassium levels with monocytes was unexpected given the sodium-induced increases of monocytes in previously conducted dietary intervention trials, and motivates exploration of short-term versus long-term effects of sodium on leukocyte subsets as well as consideration of potential unmeasured confounders in this observational cohort which may bias the observed association.[10,29,34] Mean (SD) estimated daily sodium intake in this cohort equaled 199 (67) mmol, which corresponds to 4.7 grams of sodium (±11.8 grams of salt [NaCl]) and is more than double the amount that is recommended by WHO guidelines (2 grams of sodium, or 5 grams of salt).[1] As such, the

Table 3. Relationship of Urine Na^+ and Urine Na^+/K^+ With Granulocytes at Baseline Visit

Granulocytes	Standardized coefficient (β)	t	*P* value
Urine Na^+			
Model 1	−0.010	−1.181	0.24
Model 2*	0.039	3.917	<0.001
Model 3*,†	0.025	2.193	0.028
Urine Na^+/K^+			
Model 1	0.064	7.484	<0.001
Model 2	0.060	7.003	<0.001
Model 3†	0.056	5.786	<0.001

Model 1: crude analysis. Model 2: adjusted for sex and age. Model 3: adjusted for sex, age, BMI, smoking status, alcohol use, diabetes, total cholesterol, antihypertensive drug use and baseline chronic kidney disease. Models using urine Na^+ were additionally adjusted for urine K^+.

*There was no significant interaction between urine Na^+ and urine K^+ (model 2: P=0.13; model 3: P=0.13).

†There was no significant interaction between urine Na^+ and sex (P=0.38) or urine Na^+/K^+ and sex (P=0.76). Results were obtained using linear regression models. Na^+, sodium. K^+, potassium.

Table 4. Relationship Between Granulocytes at Baseline With Hypertension at Baseline and Long-Term Deleterious Outcomes During Follow-up

Granulocytes	Odd's ratio (95% CI)	*P* value
Hypertension baseline		
Model 1	1.151 (1.123–1.180)	<0.001
Model 2	1.180 (1.149–1.212)	<0.001
Model 3*	1.193 (1.155–1.232)	<0.001
Granulocytes	**Hazard ratio (95% CI)**	***P* value**
Cardiovascular outcomes		
Model 1	1.071 (1.054–1.089)	<0.001
Model 2	1.101 (1.083–1.120)	<0.001
Model 3*	1.074 (1.055–1.093)	<0.001
Renal outcomes		
Model 1	1.136 (1.111–1.161)	<0.001
Model 2	1.171 (1.144–1.199)	<0.001
Model 3*	1.130 (1.103–1.158)	<0.001
All-cause mortality		
Model 1	1.150 (1.123–1.178)	<0.001
Model 2	1.187 (1.157–1.217)	<0.001
Model 3*	1.144 (1.114–1.174)	<0.001

Model 1: crude analysis. Model 2: adjusted for sex and age. Model 3: adjusted for sex, age, BMI, smoking status, alcohol use, diabetes, total cholesterol, baseline chronic kidney disease and antihypertensive drug use (the latter was not used in the analyses with hypertension at baseline as the outcome). Results were obtained with logistic regression or Cox proportional hazards model, as appropriate, and Odds ratio and Hazard ratios are given for one unit increase in circulating granulocyte concentrations.

*There were no interactions between sex and granulocytes (all P>0.05).

modest increase in granulocytes (eg, 4% in the highest Na^+/K^+ tertile compared to the lowest tertile) found in this cohort reflects a comparison of rather high sodium intakes (lowest tertile with estimated sodium intake of <168 mmol and highest tertile of estimated sodium intake >220 mmol). A ±20% increase was found in a dietary intervention study comparing extremely low and high sodium intakes (<32 mmol versus >324 mmol).[10]

Although the pro-inflammatory effect of sodium on innate immune cells that was found in short-term intervention trials was hypothesized to play a role in long-term deleterious outcomes of sodium, to date, this had not been established in longitudinal studies. The underlying question is—if the sequence of the present hypothesized causal pathway is followed—whether the mediation of the relation between sodium intake and deleterious outcomes by granulocytes represents a role for (low grade) inflammation or other phenomena, like sympathetic activation. This is important to establish since it changes the pathophysiological explanation and subsequent potential therapeutic targets.[35] A recent review discusses the emerging evidence on mechanisms underlying the link between neutrophils and cardiovascular inflammation, which involves interaction with monocytes and macrophages and direct chemotaxic effects.[17] Monocytes showed an association with the composite cardiovascular outcome in this cohort but not with hypertension, renal outcomes, or all-cause mortality. Previous studies in the EPIC-Norfolk cohort explored the relation of different leukocyte subsets with coronary artery disease and incident heart failure (respectively) and could only find an association with granulocytes.[20,36] The relation between urine sodium-to-potassium ratio with deleterious health outcomes was stronger than with sodium intake alone. This agrees with an increasing body of evidence suggesting that the urine sodium-to-potassium ratio is a better prognostic factor regarding worse outcomes than estimated sodium intake alone.[23] The mediated proportion (not exceeding ≈20%, depending on the type of outcome) likely both reflects that the multifactorial nature of the link between sodium intake and cardiovascular and renal long-term outcomes is multifactorial, and/or the fact that certain parameters are probably imprecise reflections of reality (eg, the use of spot urine for estimations of dietary intake). Lastly, although experimental animal studies showed that high sodium diet affects adaptive immune cells—involving induction of Th17 cells and inhibition of regulatory T-cells—no associations between sodium intake and total lymphocyte concentrations were observed in this cohort.[37,38]

Strengths of the present analysis include the large number of participants and durations of follow-up. We are—to our knowledge—the first to translate findings from small-scale experimental studies on the immune-modulating effects of sodium to a population study, and

Table 5. Mediation Analyses Between Urine Na^+(X) / Urine Na^+/K^+ (X), Granulocytes (M), and Deleterious Outcomes (Y)

^{X} Urine Na^+ ^{M} Granulocytes	Standardized coefficient (β) (bootstrapped percentile 95% CI)	Mediated proportion
^{Y} Hypertension baseline		
Indirect effect$^{(X\rightarrow M\rightarrow Y)}$	0.001 (0.0004 to 0.002)	3.6% (0.9 to 6.4)
Direct effect$^{(X\rightarrow Y)}$	0.038 (0.027 to 0.049)	
^{Y} Cardiovascular outcomes		
Indirect effect$^{(X\rightarrow M\rightarrow Y)}$	0.0005 (0.00002 to 0.001)	11.8% (2.7 to 22.9)
Direct effect$^{(X\rightarrow Y)}$	0.003 (−0.007 to 0.012)	
^{Y} Renal outcomes		
Indirect effect$^{(X\rightarrow M\rightarrow Y)}$	0.0006 (0.0001 to 0.001)	6.6% (1.8 to 11.8)
Direct effect$^{(X\rightarrow Y)}$	0.007 (−0.0006 to 0.014)	
^{Y} All-cause mortality		
Indirect effect$^{(X\rightarrow M\rightarrow Y)}$	0.0006 (0.00008 to 0.001)	6.7% (1.9 to 11.8)
Direct effect$^{(X\rightarrow Y)}$	0.009 (0.002 to 0.018)	
^{X} Urine Na^+/K^+ ^{M} Granulocytes	**Standardized coefficient (β) (bootstrapped percentile 95% CI)**	**Mediated proportion**
^{Y} Hypertension baseline		
Indirect effect$^{(X\rightarrow M\rightarrow Y)}$	0.003 (0.002 to 0.004)	7.0% (4.5 to 9.7)
Direct effect $^{(X\rightarrow Y)}$	0.024 (0.015 to 0.032)	
^{Y} Cardiovascular outcomes		
Indirect effect$^{(X\rightarrow M\rightarrow Y)}$	0.001 (0.0006 to 0.002)	17.6% (9.5 to 26.8)
Direct effect$^{(X\rightarrow Y)}$	0.003 (−0.005 to 0.010)	
^{Y} Renal outcomes		
Indirect effect$^{(X\rightarrow M\rightarrow Y)}$	0.002 (0.001 to 0.002)	11.1% (7.0 to 15.9)
Direct effect$^{(X\rightarrow Y)}$	0.009 (0.002 to 0.016)	
^{Y} All-cause mortality		
Indirect effect$^{(X\rightarrow M\rightarrow Y)}$	0.001 (0.0009 to 0.002)	12.6% (7.8 to 18.0)
Direct effect$^{(X\rightarrow Y)}$	0.010 (0.003 to 0.017)	

The mediated proportion displays the percentage of mediation of the indirect pathway relative to the total (indirect + direct) pathway. Percentile 95% CIs were calculated with 5000 bootstrap samples. Na^+ indicates sodium; and K^+, potassium.

to incorporate this relationship into analyses assessing long-term health outcomes associated with high sodium consumption. Our analyses press the need for further mechanistic and interventional research on the potential causal link between sodium consumption, immunological changes and long-term worse health outcomes, as they cannot—as emphasized earlier in our discussion—in themselves prove this pathway. Although theoretically, ideally, the temporal relation should support causation (ie, M is measured at a later moment than X, and Y is measured at a later moment than M), the feasibility for this research set-up may be questioned. Since there is no sodium intervention, and sodium intake is estimated on one point in time (X), we would not expect to see a change in granulocytes (M) at a later time point. Rather, temporality of X→M in our analyses is hypothesized based on mechanistic evidence, which of course comes with limitations that are touched upon throughout the manuscript. For the current mediation analysis we found it appropriate to assume linearity, absence of confounding (the analyses were adjusted for potential confounders, although it may be obvious that (unmeasured) confounding can never be excluded with certainty), and acceptable reliability of measurements (the measurement errors of urine sodium and urine sodium-to-potassium-ratio are further touched upon). Our analysis is limited by the fact that estimation of 24-hour urine sodium excretion from spot urine samples with the Kawasaki formula is known to have its pitfalls, especially for individual estimates. However, to date, there is no usable alternative to investigate the effects of sodium in large cohort studies, since collection of multiple 24-hour urine collections of thousands of individuals may be unfeasible. Mean estimates for 24-hour sodium and potassium excretions derived from spot urine samples closely resembled the actual values from 24-hour urine collections obtained in a subsample (n=340) of this cohort, and significantly correlated with the intakes as estimated from 7-day food diaries.[39] Also, we performed additional analyses using the urine sodium-to-potassium ratio. The sodium-to-potassium ratio may be subject to less bias since spot urine sodium-to-potassium ratios show very strong correlations with 24-hour urine sodium-to-potassium ratios (higher than the correlations between spot urine sodium and 24-hour urine sodium), and appears more relevant with regard to worse clinical outcomes.[23,24] Furthermore, the spot urines in the EPIC-Norfolk were collected randomly, while the Kawasaki formula was developed and validated for second morning urine samples specifically (collected after the first voiding upon awakening).[22] Nevertheless, in a recent comparison between random spot urine samples and 24-hour samples, the Kawasaki formula still appeared to be less biased for sodium estimations than formulas validated for random collections (ie, Tanaka and INTERSALT).[40–42] When replacing the 24-hour sodium estimations derived from the Kawasaki formula by those derived from the INTERSALT and Tanaka formula, the associations between sodium and granulocytes remained present (data not shown). Lastly, as the vast majority of this cohort is from European descent, we recommend investigating these findings in other ethnicities, especially given the known but still unexplained differences in salt sensitivity.[43]

In conclusion, we demonstrate an association of estimated sodium intake and urine sodium-to-potassium levels with granulocytes on population level, and a subsequent association of granulocytes with worse long-term cardiovascular and renal outcomes. Potassium

intake unexpectedly showed an inverse association with granulocytes, which merits further investigation. Given the available experimental evidence on the immune-modulating effects of sodium as well as the notion that granulocytes and other leukocyte subsets have a causal role in cardiovascular and renal disease, future studies need to investigate this potential causal pathway.

ARTICLE INFORMATION

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Disclosures

None.

Supplemental Material

Table S1-S5

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SUPPLEMENTAL MATERIAL

Table S1. Relationship of urine K^+ with granulocytes at baseline visit.

Granulocytes	Standardized coefficient (β)	t	P-value
Urine K^+			
Model 1	-0.088	-10.389	<0.001
Model 2A	-0.099	-11.482	<0.001
Model 2B*	-0.119	-11.910	<0.001
Model 3*	-0.115	-10.268	<0.001

Model 1: crude analysis. Model 2A: adjusted for sex and age. Model 2B: adjusted for sex, age, and urine Na^+. Model 3: adjusted for sex, age, BMI, smoking status, alcohol use, diabetes, total cholesterol, antihypertensive drug use, baseline chronic kidney disease, and urine Na^+. Data were tested with linear regression. *There was no significant interaction between urine Na^+ and urine K^+ (model 2B: P=0.13; model 3: P=0.13). Na^+, sodium. K^+, potassium.

Table S2. Relationship of urine Na^+ and urine Na^+/K^+ with monocytes at baseline visit.

Monocytes	Standardized coefficient (β)	t	P-value
Urine Na^+			
Model 1	-0.059	-6.917	<0.001
Model 2A	-0.061	-7.018	<0.001
Model 2B	-0.053	-5.221	<0.001
Model 3	-0.062	-5.344	<0.001
Urine Na^+/K^+			
Model 1	-0.030	-3.540	<0.001
Model 2A	-0.033	-2.940	0.003
Model 3	-0.037	-3.772	<0.001

Model 1: crude analysis. Model 2A: adjusted for sex and age. Model 2B: adjusted for sex, age, and urine K^+ (the latter not in the analyses using urine Na^+/K^+). Model 3: adjusted for sex, age, BMI, smoking status, alcohol use, diabetes, total cholesterol, antihypertensive drug use, baseline chronic kidney disease and urine K^+ (the latter not in the analyses using urine Na^+/K^+). Data were tested with linear regression. Na^+, sodium. K^+, potassium.

Table S3. Relationship of urine Na^+ and urine Na^+/K^+ with lymphocytes at baseline visit.

Lymphocytes	Standardized coefficient (β)	t	P-value
Urine Na^+			
Model 1	0.016	1.824	0.068
Model 2A	0.012	1.414	0.157
Model 2B	0.016	1.565	0.118
Model 3	0.003	0.267	0.790
Urine Na^+/K^+			
Model 1	0.015	1.805	0.071
Model 2A	0.014	1.620	0.105
Model 3	0.010	1.041	0.298

Model 1: crude analysis. Model 2A: adjusted for sex and age. Model 2B: adjusted for sex, age, and urine K^+ (the latter not in the analyses using urine Na^+/K^+). Model 3: adjusted for sex, age, BMI, smoking status, alcohol use, diabetes, total cholesterol, antihypertensive drug use, baseline chronic kidney disease and urine K^+ (the latter not in the analyses using urine Na^+/K^+). Data were tested with linear regression. Na^+, sodium. K^+, potassium.

Table S4. Relationship between monocytes at baseline with hypertension at baseline and long-term deleterious outcomes during follow-up.

Monocytes	Odd's ratio (95% CI)	P-value
Hypertension baseline*		
Model 1	1.268 (1.155 – 1.393)	<0.001
Model 2	1.082 (0.977 – 1.198)	0.129
Model 3	1.090 (0.979 – 1.214)	0.115
Monocytes	**Hazard ratio (95% CI)**	**P-value**
Cardiovascular outcomes		
Model 1	1.271 (1.196 – 1.350)	<0.001
Model 2	1.103 (1.035 – 1.176)	0.003
Model 3	1.048 (0.981 – 1.119)	0.167
Renal outcomes		
Model 1	1.407 (1.296 – 1.526)	<0.001
Model 2	1.135 (1.039 – 1.241)	0.005
Model 3	1.063 (0.969 – 1.165)	0.198
All-cause mortality		
Model 1	1.440 (1.320 – 1.572)	<0.001
Model 2	1.155 (1.051 – 1.270)	0.003
Model 3	1.079 (0.978 – 1.191)	0.129

Model 1: crude analysis. Model 2: adjusted for sex and age. Model 3: adjusted for sex, age, BMI, smoking status, alcohol use, diabetes, total cholesterol, baseline chronic kidney disease and antihypertensive drug use (the latter not in the analyses with hypertension at baseline as the dependent variable). Data were tested with a Cox proportional hazards model. *For hypertension at baseline as the dependent variable, data were tested using logistic regression.

Table S5. Relationship between lymphocytes at baseline with hypertension at baseline and long-term deleterious outcomes during follow-up.

Lymphocytes	Odd's ratio (95% CI)	P-value
Hypertension baseline*		
Model 1	1.227 (1.161 – 1.297)	<0.001
Model 2	1.285 (1.210 – 1.364)	<0.001
Model 3	1.182 (1.108 – 1.260)	<0.001
Monocytes	**Hazard ratio (95% CI)**	**P-value**
Cardiovascular outcomes		
Model 1	1.089 (1.051 – 1.129)	<0.001
Model 2	1.079 (1.043 – 1.116)	<0.001
Model 3	1.020 (0.982 – 1.058)	0.304
Renal outcomes		
Model 1	1.015 (0.961 – 1.072)	0.596
Model 2	1.063 (1.011 – 1.118)	0.017
Model 3	0.999 (0.945 – 1.057)	0.971
All-cause mortality		
Model 1	0.993 (0.935 – 1.054)	0.807
Model 2	1.051 (0.994 – 1.111)	0.079
Model 3	0.989 (0.931 – 1.052)	0.735

Model 1: crude analysis. Model 2: adjusted for sex and age. Model 3: adjusted for sex, age, BMI, smoking status, alcohol use, diabetes, total cholesterol, baseline chronic kidney disease and antihypertensive drug use (the latter not in the analyses with hypertension at baseline as the dependent variable). Data were tested with a Cox proportional hazards model. *For hypertension at baseline as the dependent variable, data were tested using logistic regression.