

# Association of dietary sodium intake with atherogenesis in experimental diabetes and with cardiovascular disease in patients with Type 1 diabetes

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## Abstract

It is recommended that individuals with diabetes restrict their dietary sodium intake. However, although salt intake is correlated with BP (blood pressure), it also partly determines the activation state of the RAAS (renin–angiotensin–aldosterone system), a key mediator of diabetes-associated atherosclerosis. *apoE* KO (apolipoprotein E knockout) mice were allocated for the induction of diabetes with streptozotocin or citrate buffer (controls) and further randomized to isocaloric diets containing 0.05%, 0.3% or 3.1% sodium with or without the ACEi [ACE (angiotensin-converting enzyme) inhibitor] perindopril. After 6 weeks of study, plaque accumulation was quantified and markers of atherogenesis were assessed using RT–PCR (reverse transcription–PCR) and ELISA. The association of sodium intake and adverse cardiovascular and mortality outcomes were explored in 2648 adults with Type 1 diabetes without prior CVD (cardiovascular disease) from the FinnDiane study. A 0.05% sodium diet was associated with increased plaque accumulation in diabetic *apoE* KO mice, associated with activation of the RAAS. By contrast, a diet containing 3.1% sodium suppressed atherogenesis associated with suppression of the RAAS, with an efficacy comparable with ACE inhibition. In adults with Type 1 diabetes, low sodium intake was also associated with an increased risk of all-cause mortality and new-onset cardiovascular events. However, high sodium intake was also associated with adverse outcomes, leading to a J-shaped relationship overall. Although BP lowering is an important goal for the management of diabetes, off-target actions to activate the RAAS may contribute to an observed lack of protection from cardiovascular complications in patients with Type 1 diabetes with low sodium intake.

**Key words:** apolipoprotein E, atherogenesis, cardiovascular disease, renin–angiotensin–aldosterone system, sodium intake, Type 1 diabetes

## INTRODUCTION

Diabetes is associated with augmented development and progression of atherosclerosis, which contributes to an increased incidence of CVD (cardiovascular disease) and premature mortality. Accelerated atherogenesis in diabetes is a complex process

in which a combination of pathogenic and facilitatory factors (hyperglycaemia, dyslipidaemia, shear stress, etc.) activates common molecular pathways that lead to the development and progression of atherosclerotic plaque. One of these is activation of the RAAS (renin–angiotensin–aldosterone system). We have previously shown that blockade of the RAAS is able to

**Abbreviations:** ACE, angiotensin-converting enzyme; ACEi, ACE inhibitor; AER, albumin excretion rate; AngII, angiotensin II; *apoE*, apolipoprotein E; BP, blood pressure; CKD, chronic kidney disease; CN, condition number; CVD, cardiovascular disease; eGFR, estimated glomerular filtration rate; ESRD, end-stage renal disease; HbA<sub>1c</sub>, glycated haemoglobin; IL-6, interleukin-6; JAM-A, junctional adhesion molecule A; KO, knockout; LDL, low-density lipoprotein; MCP-1, monocyte chemoattractant protein 1; NBF, neutral buffered formalin; RAAS, renin–angiotensin–aldosterone system; RT–PCR, reverse transcription–PCR; TAG, triacylglycerol; VCAM-1, vascular cell adhesion molecule 1; VIF, variance inflation factor.

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prevent diabetes-associated plaque accumulation in atherosclerosis-prone *apoE* KO (apolipoprotein E knockout)-mice, independent of its actions on BP (blood pressure) [1,2]. Similar, BP-independent effects on cardiovascular outcomes have been ascribed to RAAS blockade in clinical studies of diabetic patients [3].

One of the most important regulators of RAAS activity is dietary sodium intake. Even modest salt restriction is associated with activation of RAAS in humans [4] as a means to retain more sodium and maintain vascular homeostasis. Despite this, nutritional guidelines advocate that all adults with diabetes should restrict their dietary intake of salt to less than 65 mmol/day [5], equating to a greater than 3-fold reduction on current intake levels in most patients. The target of these recommendations is salt-induced hypertension, which is an important contributor to CVD in patients with diabetes. However, although BP lowering is an important health goal, the balance of benefits and risks that may arise from a strategy of salt restriction in patients with diabetes is unclear. Indeed, the 'off target' or pleiotropic actions of salt restriction [4], including RAAS activation, have the potential to negate any beneficial effects of lowering the BP and may be one possible explanation for the lack of positive and even paradoxical findings from observational studies in subjects with diabetes [6,7].

Given our findings that RAAS activation is an important component of diabetes-associated atherosclerosis [1,2], and that salt intake modifies RAAS activation [4,8], we hypothesized that dietary sodium intake would also modify atherosclerosis associated with experimental diabetes. In addition, to explore the potential clinical relevance of this hypothesis, we also examined the association between sodium intake and adverse cardiovascular and mortality outcomes in 2648 patients with Type 1 diabetes without prior CVD from the large nationwide multi-centre cohort of Finnish adults with Type 1 diabetes (the FinnDiane study) [9–11].

## MATERIALS AND METHODS

### Animal models

*apoE* KO mice bred on to a c57bl6 background were sourced and generated in house, as described previously [1,2]. In these studies, male mice aged 10 weeks and weighing between 20 and 25 g were allocated induction of diabetes as per AMDCC (Animal Models of Diabetic Complications Consortium) protocols using five daily injections of streptozotocin [ $55 \text{ mg} \cdot (\text{kg of body weight})^{-1} \cdot \text{day}^{-1}$ ], with controls receiving citrate buffer alone. *apoE* KO mice used were further randomly allocated to receive an isocaloric diets with a low salt content (0.05% sodium), normal salt content (0.3% sodium) or a high salt content (3.1% sodium; Specialty Feeds). Control and diabetic *apoE* KO on a low-salt diet were further randomized to receive treatment with the ACEi [ACE (angiotensin-converting enzyme) inhibitor] perindopril (Servier) at a dose of  $2 \text{ mg} \cdot (\text{kg of body weight})^{-1} \cdot \text{day}^{-1}$  in drinking water. Each group contained at least 20 animals to facilitate structural and functional assays, detailed below.

After 6 weeks of study, all mice were placed in individual metabolic cages (Iffa Credo) for 24 h and their weight, water and food (sodium) intake and urine (sodium) output were documented. Fasting glucose was measured by glucometer (SensoCard; Point of Care Diagnostics). The sodium concentration was estimated in diluted urine on the same machine using an ion-sensitive electrode and the result adjusted for urinary output ( $\mu\text{mol}/\text{day}$ ). Systolic BP was measured by tail-cuff plethysmography in conscious, pre-warmed mice [12]. Animals were then killed using an intraperitoneal injection of Euthal (10 mg/kg) (Delvet Limited) followed by exsanguination via cardiac puncture. Total cholesterol and TAG (triacylglycerol) were measured in fasting plasma samples using a COBAS INTEGRA 400 auto-analyser (Roche Diagnostics). Plasma aldosterone was measured using a commercial RIA (proSearch). Aortae were collected and placed in 10% (v/v) NBF (neutral buffered formalin) and quantified for lesion area before being processed for subsequent immunohistochemical analysis or snap frozen and stored at  $-70^\circ\text{C}$  for subsequent RNA extraction. All experiments were approved by the animal ethics committee of the Alfred Medical Research Precinct and conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

### Plaque area quantification

Plaque area was quantified as described previously [1,13]. In brief, aortae were excised from the carcass, separated from the heart, cleaned of excess fat, blood and connective tissue and trimmed 1 mm below the left subclavian artery to yield the aortic arch. This was placed in an individual cassette and immersed in 10% (v/v) NBF solution for 24 h. After this individual aorta cassettes were rinsed in 80% (v/v) ethanol followed by 60 min rinsing under running tap water, followed by application of Sudan IV-Herxheimer's solution (0.5%; Gurr, BDH) for 30 min. Aortas were then re-immersed in 10% (v/v) NBF and subjected to *en face* plaque analysis within 24 h post staining. This involved dissecting the arch longitudinally and flat-mounting it on to pink wax. Images of the aortas were captured with an Olympus U-TVO digital camera using an Olympus SZX10 dissecting microscope. Each image was analysed with Q-Image Pro software (Olympus) using RGB thresholding to define lesion areas, and expressed as the percentage total surface area staining red (positive for Sudan IV).

### Quantitative real-time PCR

Gene expression of the adhesion molecules and pro-inflammatory cytokines were assessed in aortic and liver homogenates by quantitative real-time RT-PCR (reverse transcription-PCR) [14]. This was performed using the TaqMan system based on real-time detection of accumulated fluorescence (ABI Prism 7700, PerkinElmer) as described previously by our group [14]. Gene expression was normalized to 18S mRNA and reported as ratios compared with the level of expression in untreated control mice, which were given an arbitrary value of 1.

### Circulating inflammatory markers

To measure various adhesion molecules, serum soluble VCAM-1 (vascular cell adhesion molecule 1), soluble ICAM-1

(intercellular adhesion molecule 1), MCP-1 (monocyte chemoattractant protein 1) and IL-6 (interleukin-6) were measured using commercial ELISA kits (Quantakine; R&D Biosystems) performed according to the manufacturer's instructions.

### Studies in Finnish adults with Type 1 diabetes

The present study is part of the ongoing Finnish Diabetic Nephropathy (FinnDiane) Study, with the aim to identify genetic, clinical and environmental risk factors for diabetic nephropathy in patients with Type 1 diabetes [9–11]. Type 1 diabetes was defined as an onset of diabetes before the age of 40 years and permanent insulin treatment initiated within 1 year of diagnosis. For this study, outcomes were ascertained in patients in the FinnDiane prospective cohort with Type 1 diabetes without pre-existing CVD or ESRD (end-stage renal disease), in whom urinary sodium was estimated in a 24-h urine sample at baseline ( $n = 2648$ ). These baseline assessments were performed between 1995 and 2006. The ethical committees of all participating centres approved the study protocol. Written informed consent was obtained from each patient and the study was performed in accordance with the Declaration of Helsinki as revised in 2000.

### Cohort characteristics

At baseline, all patients underwent a thorough clinical investigation in connection with a regular patient visit to their attending physician. Data on medication and diabetic complications were registered with the use of a standardized questionnaire, which was completed by the physician based on medical files. BP was measured twice in the sitting position after a 10 min rest and the average of these two measurements were used in the analysis. Height, weight and waist/hip ratio were recorded and blood was drawn for the measurements of HbA<sub>1c</sub> (glycated haemoglobin), lipids and creatinine. CVD was defined as a history of myocardial infarction, a coronary artery procedure (by-pass surgery or angioplasty), stroke or a peripheral artery procedure (by-pass surgery or angioplasty), which was verified from the medical files. HbA<sub>1c</sub> and creatinine were determined by standardized assays at each centre. Serum lipid and lipoprotein concentrations were analysed centrally by automated enzymatic methods (Hoffmann-LaRoche). In addition, the urinary sodium concentration in 24-h urine collections was measured with an ion-selective electrode (Roche Diagnostics).

### Ascertainment of outcomes

Deaths from any cause through to 17 March 2010 were identified via a search of the Finnish National Death Registry and centre databases. All deaths were confirmed with death certificate data. In each case, vitality status was verified from the Finnish National Death Registry and cause of death classified on the basis of death certificates. Cardiovascular events, coronary heart events and strokes were ascertained on the basis of ICD (International Classification of Diseases) discharge codes.

### Statistical analysis

Continuous data are expressed as means  $\pm$  S.E.M. Differences in the mean among groups were compared using two-way ANOVA. Pair-wise multiple comparisons were made with the Student–

Newman–Keuls post-hoc analysis to detect significant differences between groups.  $P < 0.05$  was considered statistically significant.

In the present study, we aimed to identify the predictors of the cumulative incidence of new CVD events and mortality in individuals with Type 1 diabetes. To evaluate the independent predictors of outcomes, we used multivariate Cox proportional-hazards models. All variables known to be aetiologically associated with outcomes were included in the final model, along with any variables associated with mortality in univariate analyses with a  $P$  value of less than 0.01. Model selection from candidate variables was accomplished by minimization of the Akaike and Bayesian information criteria [15]. Overall, Cox model fit was assessed by: (i) approximation of cumulative Cox–Snell residuals to  $(-\log)$  Kaplan–Meier estimates, residual plots and specific testing of the proportional hazards assumption [16]; and (ii) Harrell's C statistic [17] and 'added-variable' goodness-of-fit tests [18]. Cox model performance was adjudged by the explained variation using 5000 bootstrap repetitions of the whole dataset, adjusting for covariates [19]. The potential for multiple collinearity was tested using the VIF (variance inflation factor) and CN (condition number), where  $VIF < 10$  and  $CN < 30$  are desirable [20].

## RESULTS

### Metabolic and BP levels in *apoE* KO mice

The induction of diabetes in *apoE* KO mice resulted in increased glucose levels and a modest further increase in circulating lipid levels. Diabetic mice had higher food and water intake than non-diabetic animals, and weight gain was reduced. Changes in the intake of dietary sodium had no effect on lipid or glucose levels, weight gain or feeding behaviour in control or diabetic mice. Circulating lipids levels were elevated in all groups of *apoE* KO mice (Table 1).

Dietary sodium intake and excretion were positively associated with systolic BP in *apoE* KO mice, as measured by tail cuff plethysmography (Table 1). A high-salt diet resulted in 8 mmHg increase in BP in diabetic *apoE* KO mice and 5 mmHg increase in wild-type controls. However, a low sodium intake did not significantly lower the systolic BP in any group when compared with mice receiving normal chow. This is consistent with the observation that BP levels in mice bred on to a C57 background are not especially salt sensitive. Treatment with the ACEi perindopril lowered the BP and attenuated sodium retention in *apoE* KO mice fed on a low-salt diet, consistent with its blockade of the RAAS. There was no difference in the daily sodium excretion between control *apoE* KO mice and diabetic *apoE* KO mice in mice on normal chow. However, sodium excretion was higher in diabetic mice on a high-sodium diet, reflecting their greater food and sodium intake.

### Activation of the RAAS in *apoE* KO mice

The intake of dietary sodium was also correlated with circulating levels of AngII (angiotensin II), such that AngII levels were increased in mice on a low-salt diet, and reduced in mice on a

**Table 1** General parametersResults are means  $\pm$  S.E.M. \*  $P < 0.05$  compared with *apoE* KO mice fed on a normal chow; †  $P < 0.01$  compared with mice fed on a low-salt diet.

| Mouse model                 | Sodium excretion ( $\mu$ mol/day) | Body weight (g) | Daily food intake ( $\mu$ g/m <sup>2</sup> ) | Daily water intake (ml/m <sup>2</sup> ) | Plasma glucose (mM) | Total cholesterol (mM) | TAG (mM)       | Systolic BP (mmHg) | AngII (pg/ml) | Aldosterone (pg/ml) |
|-----------------------------|-----------------------------------|-----------------|--|---|---------------------|------------------------|----------------|--------------------|---------------|---------------------|
| <i>apoE</i> KO              |                                   |                 |  |   |                     |                        |                |                    |               |                     |
| Low-salt diet               | 7 $\pm$ 2*                        | 27 $\pm$ 1      | 4 $\pm$ 1                                    | 4 $\pm$ 1                               | 11 $\pm$ 1          | 10.6 $\pm$ 0.9         | 1.4 $\pm$ 0.1  | 95 $\pm$ 2         | 854 $\pm$ 88* | 230 $\pm$ 25*       |
| Low-salt diet + perindopril | 49 $\pm$ 7†                       | 27 $\pm$ 1      | 3 $\pm$ 1                                    | 4 $\pm$ 1                               | 11 $\pm$ 1          | 10.8 $\pm$ 1.0         | 1.4 $\pm$ 0.1  | 85 $\pm$ 4†        | 18 $\pm$ 6†   | <20†                |
| Normal chow                 | 40 $\pm$ 7†                       | 27 $\pm$ 1      | 3 $\pm$ 1                                    | 4 $\pm$ 1                               | 12 $\pm$ 1          | 10.6 $\pm$ 0.8         | 1.5 $\pm$ 0.1  | 97 $\pm$ 3         | 115 $\pm$ 11† | 97 $\pm$ 16†        |
| High-salt diet              | 722 $\pm$ 206†                    | 28 $\pm$ 1      | 5 $\pm$ 1                                    | 5 $\pm$ 1                               | 12 $\pm$ 2          | 10.3 $\pm$ 0.9         | 1.5 $\pm$ 0.2  | 102 $\pm$ 3†       | 25 $\pm$ 3†   | <20†                |
| Diabetic <i>apoE</i> KO     |                                   |                 |  |   |                     |                        |                |                    |               |                     |
| Low-salt diet               | 3 $\pm$ 1*                        | 21 $\pm$ 1*     | 6 $\pm$ 1*                                   | 17 $\pm$ 2*                             | 26 $\pm$ 2*         | 16.4 $\pm$ 1.4*        | 2.3 $\pm$ 0.3* | 100 $\pm$ 2        | 804 $\pm$ 59* | 471 $\pm$ 26*       |
| Low-salt diet + perindopril | 55 $\pm$ 9†                       | 22 $\pm$ 1*     | 6 $\pm$ 1*                                   | 18 $\pm$ 2*                             | 25 $\pm$ 2*         | 16.2 $\pm$ 1.4*        | 2.2 $\pm$ 0.3* | 88 $\pm$ 4*        | 26 $\pm$ 6†   | <20†                |
| Normal chow                 | 49 $\pm$ 14†                      | 22 $\pm$ 1*     | 6 $\pm$ 1*                                   | 16 $\pm$ 2*                             | 24 $\pm$ 2*         | 17.2 $\pm$ 1.2*        | 2.1 $\pm$ 0.3* | 99 $\pm$ 3         | 285 $\pm$ 36† | 259 $\pm$ 28†       |
| High-salt diet              | 1183 $\pm$ 146†                   | 23 $\pm$ 1*     | 5 $\pm$ 1*                                   | 17 $\pm$ 3*                             | 26 $\pm$ 2*         | 16.6 $\pm$ 1.6*        | 1.8 $\pm$ 0.2* | 107 $\pm$ 3*       | 18 $\pm$ 9†   | 54 $\pm$ 12†        |

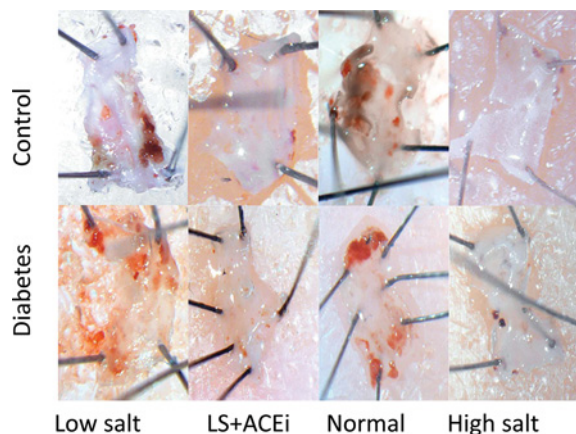
high-sodium diet (Table 1). The intake of dietary sodium was also correlated with circulating aldosterone levels, a marker of systemic RAAS activation (Table 1), such that circulating aldosterone levels were increased in mice on a low-salt diet, and undetectable (<20 pg/ml) in mice on a high-salt diet when compared with those receiving normal chow, as previously described [21]. AngII and aldosterone levels were also suppressed by ACE inhibition as described previously [22]. In *apoE* KO mice with diabetes, AngII and aldosterone levels were higher than in control *apoE* KO mice, consistent with the known activation of the systemic RAAS associated with experimental diabetes. The degree of RAAS activation associated with diabetes was similar to that observed in non-diabetic *apoE* KO mice fed on a low-salt diet (Table 1).

### Effect of dietary salt intake on plaque accumulation in diabetic *apoE* KO mice

Plaque accumulation, quantified as the percentage area of the aortic arch stained red with Sudan IV (Figure 1), was significantly increased in diabetic *apoE* KO mice when compared with *apoE* KO mice when both groups received normal chow containing 0.3% sodium, as previously described [1,2,23]. A low-salt (0.05% sodium) diet also increased plaque accumulation in both *apoE* KO mice and diabetic *apoE* KO mice when compared with mice receiving normal chow ( $P < 0.001$ , Figure 2). Indeed, a low-sodium diet was a more potent inducer of atherosclerosis than uncontrolled hyperglycaemia in this model. Notably, an additive effect of diabetes on atherogenesis was only observed in mice on normal chow, although diabetic mice on a low- or high-salt diet showed the same plaque burden as non-diabetic mice receiving the same (low or high) diet (Figure 2). Plaque accumulation associated with a low-sodium diet was prevented by the ACEi perindopril in both diabetic and non-diabetic *apoE* KO mice. Alongside suppression of the RAAS, intake of a diet high in sodium (3.1% sodium) for 6 weeks resulted in a reduction in plaque accumulation in both *apoE* KO mice and diabetic *apoE* KO mice when compared with *apoE* KO mice on normal chow (Figure 2). Although significant, the effect was small, as *apoE* KO mice on normal chow have accumulated only limited plaque by 14 weeks of age. However, a high-salt diet was more effective, in absolute terms, in reducing plaque accumulation in diabetic mice than the ACEi perindopril (Figure 2).

### Effect of dietary sodium intake on inflammation in the diabetic aorta

To explore the potential mechanisms by which sodium impacts on atherogenesis, the expression of a number of vascular adhesion molecules and inflammatory cytokines were determined in the aortae of diabetic *apoE* KO mice. Notably the expression of TNF $\alpha$  (tumour necrosis factor  $\alpha$ ), IL-6, MCP-1, VCAM-1 and the pro-inflammatory mediator JAM-A (junctional adhesion molecule A) were increased in diabetic mice fed on a low-sodium diet. In addition, expression of the leucocyte marker CD11c, and the T-cell markers, CD3 and MHC II were also increased, denoting the presence of vascular inflammation (Figure 3). By contrast, a high salt intake was also associated with reduced gene expression of pro-inflammatory



**Figure 1** Representative staining for Sudan IV in atherosclerotic plaque from the aortic arch in control and diabetic mice fed on a low-salt, normal chow or high-salt diet or a low-salt diet with the ACEi perindopril (LS + ACEi)

cytokines and adhesion molecules in the aorta, when compared with diabetic *apoE* KO mice receiving normal chow (Figure 3a). Treatment with the ACEi perindopril also attenuated increases in inflammatory mediators in the aorta. Changes in the expression of inflammatory mediators induced by a low-salt diet in non-diabetic *apoE* KO mice have been previously published elsewhere [8].

### Sodium intake in Finnish adults with Type 1 diabetes

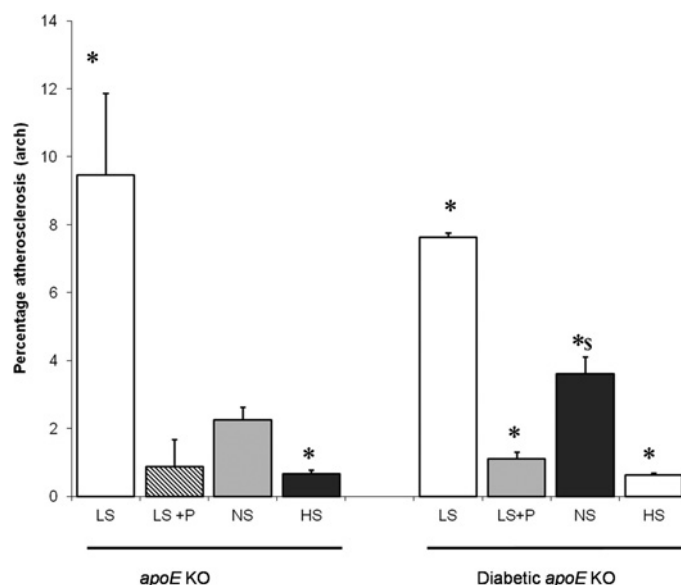
The FinnDiane cohort in whom 24 h urinary sodium excretion was estimated comprised 2648 adult patients with Type 1 diabetes without prior CVD or ESRD. The cohort characteristics at

baseline have been previously described in detail [9–11] and are summarized in Table 2. The mean urinary sodium excretion in these patients was 150 mmol/day, similar to that reported in the Finnish general population [24,25].

During a median follow-up of 10 years, 176 deaths were recorded (6.6%). Urinary sodium excretion was significantly associated with all-cause mortality. This association was nonlinear, such that individuals with the highest daily urinary sodium excretion, as well as the lowest excretion had reduced cumulative survival (Figure 4a). After adjusting for parameters associated with daily urinary sodium excretion, as well as other factors independently associated with all-cause mortality including age, gender, glycaemic control, the presence and severity of CKD (chronic kidney disease) [eGFR (estimated glomerular filtration rate) and AER (albumin excretion rate)], lipid levels (total cholesterol and TAG), urinary sodium excretion remained significantly associated with all-cause mortality on multivariate Cox regression analysis. There were no significant interactions. Notably, the relationship between sodium intake and mortality was independent of severity of CKD at baseline, the presence or absence of hypertension, its absolute level or its mode of treatment including the use of diuretics or RAAS blockers.

### Cardiovascular events and sodium intake in adults with Type 1 diabetes

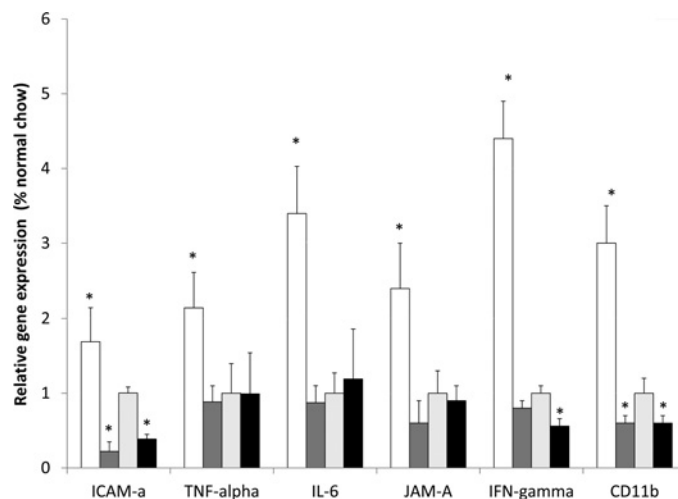
During follow-up 275 patients experienced a cardiovascular event for the first time, including 218 new coronary events and 75 new cerebrovascular events. Urinary sodium excretion was also associated with the incidence of a new CVD event, after adjusting for other risk factors. Again, this association was nonlinear, such that individuals with the highest daily urinary sodium excretion, as well as the lowest excretion, had reduced cumulative survival (Figure 4b). After adjusting for parameters associated with

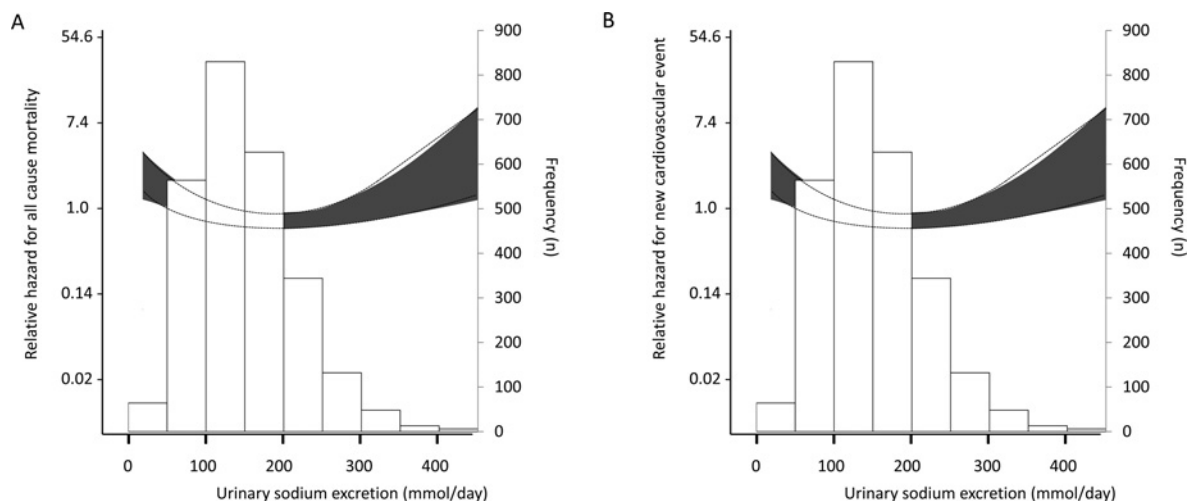


**Figure 2** Quantification of the percentage surface area of aortic arch stained red with Sudan IV. Data show means  $\pm$  S.E.M.,  $n = 8/\text{group}$ . \* $P < 0.01$  compared with *apoE* KO mice fed on normal chow (NS); § $P < 0.01$  compared with *apoE* KO mice fed on normal chow. LS, low salt; HS, high salt; LS + P, low salt plus perindopril.

**Table 2** Baseline clinical characteristics of adults with Type 1 diabetes from the FinnDiane study without pre-existing CVD, stratified according to their 24-h urinary sodium excretion\* $P < 0.05$  compared with the middle quartiles as determined by univariate analysis.

|   | Lower quartile<br>( $<102$ mmol/day) | Middle quartiles<br>( $102$ – $187$ mmol/day) | Upper quartile<br>( $>187$ mmol/day) |
|---|--------------------------------------|---|--------------------------------------|
| Age (years)                                   | $37 \pm 13^*$                        | $38 \pm 12$                                   | $39 \pm 12$                          |
| Male gender (%)                               | $32.5^*$                             | 48  | $71^*$                               |
| Duration of diabetes (years)                  | $21 \pm 12$                          | $21 \pm 12$                                   | $20 \pm 11^*$                        |
| Insulin dose (units/kg)                       | $0.7 \pm 0.2$                        | $0.7 \pm 0.2$                                 | $0.7 \pm 0.2$                        |
| HbA <sub>1c</sub> (%)                         | $8.4 \pm 1.5$                        | $8.4 \pm 1.5$                                 | $8.4 \pm 1.4$                        |
| Body mass index (kg/m <sup>2</sup> )          | $24.6 \pm 3.4$                       | $24.9 \pm 3.5$                                | $26.0 \pm 3.5$                       |
| Hypertension (%)                              | 44                                   | 48  | $54^*$                               |
| Systolic BP (mmHg)                            | $131 \pm 18$                         | $132 \pm 18$                                  | $134 \pm 18^*$                       |
| Diastolic BP (mmHg)                           | $78 \pm 9^*$                         | $79 \pm 9$                                    | $80 \pm 10^*$                        |
| Anti-hypertensive medication use (%)          | 34                                   | 37  | 39                                   |
| ACEi  | 24                                   | 26  | 28                                   |
| Angiotensin receptor blocker                  | 4                                    | 5   | 6                                    |
| Calcium channel blocker                       | 6                                    | 8   | 9                                    |
| $\beta$ -Blocker                              | 7                                    | 7   | 6                                    |
| Diuretic                                      | 7                                    | 9   | 9                                    |
| Lipid-lowering therapy                        | 9                                    | 7   | $10^*$                               |
| Total cholesterol (mmol/l)                    | $4.98 \pm 0.91$                      | $4.96 \pm 1.00$                               | $5.01 \pm 0.97$                      |
| Low-density lipoprotein cholesterol (mmol/l)  | $3.07 \pm 0.78$                      | $3.07 \pm 0.87$                               | $3.15 \pm 0.89$                      |
| High-density lipoprotein cholesterol (mmol/l) | $1.34 \pm 0.37$                      | $1.33 \pm 0.37$                               | $1.28 \pm 0.36^*$                    |
| TAG (mmol/l)                                  | $1.26 \pm 0.91$                      | $1.23 \pm 0.85$                               | $1.32 \pm 0.91^*$                    |
| Any retinopathy (%)                           | 51                                   | 50  | 51                                   |
| Retinopathy requiring laser therapy (%)       | 29                                   | 31  | 28                                   |
| Current smoker (%)                            | 22                                   | 24  | 27                                   |
| Microalbuminuria (%)                          | 16                                   | 16  | 18                                   |
| Macroalbuminuria (%)                          | 14                                   | 15  | 14                                   |
| eGFR (ml/min per 1.73 m <sup>2</sup> )        | $84 \pm 23$                          | $85 \pm 22$                                   | $88 \pm 21^*$                        |

**Figure 3** Gene expression profile expression of adhesion molecules and pro-inflammatory cytokines in the aortae of diabetic *apoE* KO mice fed on a diet low in salt (0.05% sodium; white columns) compared with normal chow (0.3% sodium; grey columns) or a high-salt diet (3.1% sodium; black columns) compared with normal chow, as measured by real-time RT-PCRResults are means  $\pm$  S.E.M.,  $n = 8$ /group. \* $P < 0.01$  compared with mice fed on normal chow.



**Figure 4** Non-linear relationship between sodium intake and all-cause mortality (a) and cardiovascular events (b) after adjusting for other risk factors

The knot for the cubic spline was situated at a sodium excretion of 102  $\mu\text{mol/day}$  for mortality (a) and 141  $\mu\text{mol/day}$  for new onset CVD (b).

daily urinary sodium excretion, as well as other factors independently associated with all-cause mortality including age, gender, glycaemic control, duration of diabetes, smoking, the presence and severity of CKD (eGFR and AER), TAG levels, urinary sodium excretion remained significantly associated with incident CVD events on multivariate Cox regression analysis. There were no significant interactions between predictive variables ( $P > 0.1$ ). Sodium intake was not significantly associated with stroke or new coronary events, after adjusting for other risk factors, when these outcomes were analysed on their own (results not shown). Notably BP parameters were not associated with CVD or mortality in the final prediction model, because of the goodness of fit and model assumption criteria. In addition, the addition of hypertension/BP/hypertensive medication to the model did not change the effect estimate for sodium intake (results not shown), consistent with the BP independent effects of sodium intake observed in the animal model. Similarly, social economical status or education status also did not confound analyses.

## DISCUSSION

Dietary salt intake is a key regulator of RAAS activity. A low-salt diet increases systemic activation of the RAAS, whereas a diet high in sodium suppresses the RAAS [4,8]. In atherosclerosis-prone *apoE* KO mice, and in other susceptible models [26], RAAS activation is pro-atherogenic [13,27], whereas RAAS blockade has vasculo-protective effects beyond its actions on systemic BP [1,2,23]. Consistent with this paradigm, we show in the present study for the first time that a diet high in salt both reduces activity of the RAAS, and attenuates plaque accumulation in diabetic *apoE* KO mice, as or more effectively as an ACEi. By contrast, a low-salt diet increases diabetes-associated atherogenesis and vascular inflammation in this mouse model, associated with com-

pensatory renal sodium retention and increased RAAS activity (Figures 1 and 2).

A key limitation of our experimental research is its reliance on the diabetic *apoE* KO mouse model. Although it is the most widely utilized model for the study of early atherosclerosis, and the sequential events involved in lesion formation from initial fatty streaks to complex atheroma are strikingly similar to those in humans, it remains contentious as to what extent this model reflects atherogenesis in a human context. Certainly, plaque rupture and/or thrombosis, two common features of human atherosclerosis, are only infrequently observed in *apoE* KO mice. The model is also dominated by elevated VLDL [very LDL (low-density lipoprotein)]-cholesterol levels, similar to combined dyslipidaemia found in humans usually as part of the metabolic syndrome or diabetes. However, pro-atherogenic effects of a low-sodium diet has also been described in LDL-receptor-KO mice [26]. We have also only studied our mice for 6 weeks, allowing us to focus on the early pathogenic changes initiated by RAAS activation, such as inflammation and adhesion. Although these early vascular changes are invariably correlated with subsequent plaque accumulation, plaque accumulation in this short-term model is only modest and longer-term studies are still required to confirm these findings.

Another potential limitation of this experimental work is the range of salt intake employed in our study, which was deliberately used to modify RAAS activation without ill effects on mice behaviour or health. Previous studies including our own have suggested that a low sodium intake accelerates atherosclerosis in *apoE* KO mice [8,34], largely by increasing RAAS activation. However, the 8–10-fold difference in plasma aldosterone levels achieved between low- and high-sodium diets in our mice is larger than the approximately 5-fold difference observed in clinical trials of high- and low-sodium diets in humans [35]. As the relationship between atherosclerosis and RAAS activation is continuous in both humans and mice, we speculate that lesser

changes in salt intake, as achieved in clinical trials, may also influence the same pathophysiological pathways, albeit to a lesser extent and more slowly.

Although these findings are clear in a murine model of diabetic atherosclerosis, the role of sodium intake in humans with Type 1 diabetes is more complicated. Sodium retention contributes to the development of hypertension in diabetic patients [28,29] and/or a reduced response to antihypertensive interventions [30,31]. Hypertension is a key risk factor in the development and progression of CVD. It has been argued that the clear benefits of additional BP lowering demands a low-sodium diet in all patients with diabetes [5]. However, consistent BP lowering associated with sodium restriction is not observed in all patients with diabetes, with both increases and decreases in BP observed in different individuals, with no net effect on systemic BP overall [32]. In addition, we have previously been unable to observe any association between sodium intake and BP indices in patients with either Type 1 or Type 2 diabetes after adjusting for age and gender, with or without antihypertensive treatment [6,7]. At the same time, RAAS activation is clearly increased with sodium restriction in diabetic patients [33], and the wide clinical utility and effects of drugs that block the RAAS [3] stand as testament to the importance of this pathway in diabetes, beyond its actions on BP regulation.

To explore the potential clinical relevance of the paradoxical association between sodium intake and atherosclerosis observed in diabetic mice, we also examined the association of sodium intake with cardiovascular and mortality outcomes in patients with Type 1 diabetes without prior CVD. Notably, there was a J-shaped relationship between sodium intake and adverse outcomes, such that mortality and new onset CVD were both paradoxically increased in individuals with a low sodium intake, and also modestly increased in those on a high salt intake. A similar J-shaped association between sodium intake and mortality was also observed in the ONTARGET study of non-diabetic patients at risk of CVD [36]. Staessen and co-workers also reported an increase in mortality associated with a low sodium intake, but no overall effect on CV events in a non-diabetic cohort with similar age to those in FinnDiane [37]. We speculate that this complex relationship between sodium intake and adverse outcomes reflects the sum of atherogenic effects of BP as well as off-target effects, such as those demonstrated in our experimental model.

The strengths of our clinical study include its very large cohort of individuals with Type 1 diabetes, a high participation rate, access to subsidized care (75–100% of costs) and contemporary treatment regimens, including a range of insulin regimens, statins, blockers of the RAS (rennin–angiotensin system), and self-monitoring technologies where appropriate. We used 24 h urinary sodium excretion to estimate sodium intake, a method regarded as the best way to estimate dietary sodium intake and substantially more effective than dietary recall [38]. Our daily sodium excretion levels are also similar to those previously reported in the general Finnish population [24,25].

Interpretation of our study may be limited because variability in dietary sodium excretion may be associated with other factors including indication, avidity of other health-related behaviours and/or differences in diet composition, processing and

preparation that may themselves impact on adverse outcomes in diabetic individuals. We have adjusted for known confounders in our multivariate analysis. However, as dietary components are often interrelated it cannot be excluded that other (unknown) dietary components, related to salt intake and CVD, may have confounded the results of our epidemiological study. Nonetheless, our findings are consistent with our experimental data in which such variables have been eliminated. Finally, we have not measured markers of RAAS activity in our patients with Type 1 diabetes. However, previous meta-analyses and trials have clearly demonstrated a relationship between sodium intake and RAAS activation in humans, including those with Type 1 diabetes [4,33].

In summary, in the diabetic *apoE* KO mouse model of atherosclerosis any reduction in dietary salt intake (from high to normal or normal to low) is pro-atherogenic due to activation of the RAAS. Indeed, in this model, a low-salt diet was a more powerful inducer of atherosclerosis than diabetes alone, possibly because of the absolute commitment of the RAAS to activation which is associated with salt restriction. In contrast, a high-salt diet suppressed RAAS-dependent atherogenesis to a similar or greater extent as an ACEi. Although BP lowering is an important health goal for the management of diabetes, the balance of benefits and risks that may arise out of encouraging adults to reduce their salt intake is likely to be variable and extend beyond BP. In our observational cohort, protective effects of a reduced sodium intake were not observed in patients with Type 1 diabetes without CVD. Indeed, there appears to be a modest and independent increase in all-cause mortality and cardiovascular events associated with achieving target levels of sodium intake. Although causality remains to be established, these findings further support the calls for caution before applying salt restriction to all patients with Type 1 diabetes, as clinical outcomes may be more complicated or even paradoxical in certain settings.

## CLINICAL PERSPECTIVES

- Although dietary sodium restriction is widely recommended to patients with diabetes to improve BP control, it may also activate neurohormonal pathways that contribute to atherosclerosis.
- In the present paper, we show that a low-salt diet augments atherosclerosis associated with diabetes in *apoE* KO mice, whereas a high-salt diet suppresses plaque accumulation, with an efficacy comparable with ACE inhibition. In adults with Type 1 diabetes, low sodium intake was also associated with an increased risk of all-cause mortality and new-onset cardiovascular events.
- Taken together, such data suggest that the association between sodium intake and cardiovascular outcomes in diabetes is more complicated than it simply being a cause of high BP.

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## AUTHOR CONTRIBUTION

Merlin Thomas conceived the project, developed the hypothesis, designed the experiments, co-ordinated and directed the project and wrote the paper. Raelene Pickering performed the ELISA



analysis. Despina Tsorotes performed the RT-PCR analysis, ELISA and Cobas analysis. RT-PCR analysis and aortic plaque quantification were performed by Chris Tikellis. Carol Forsblom, Valma Harjutsalo Lena Thorn, Aila Ahola, Johan Wadén, Nina Tolonen, Markku Saraheimo, Daniel Gordin and Per-Henrik Groop collected the data. Merlin Thomas and John Moran performed statistical analyses. Merlin Thomas, Per-Henrik Groop and Mark Cooper wrote and edited the paper. Merlin Thomas is the guarantor of this work and, as such, had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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