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The effect of a high-protein, high-sodium diet on calcium and bone metabolism in postmenopausal women and its interaction with vitamin D receptor genotype

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The influence of a high-Na, high-protein (calciuric) diet on Ca and bone metabolism was investigated in postmenopausal women (aged 50–67 years) who were stratified by vitamin D receptor (VDR) genotype. In a crossover trial, twenty-four women were randomly assigned to a diet high in protein (90 g/d) and Na (180 mmol/d) or a diet adequate in protein (70 g/d) and low in Na (65 mmol/d) for 4 weeks, followed by crossover to the alternative dietary regimen for a further 4 weeks. Dietary Ca intake was maintained at usual intakes (about 20 mmol (800 mg)/d). Urinary Na, K, Ca, N and type I collagen cross-linked N-telopeptide (NTx; a marker of bone resorption), plasma parathyroid hormone (PTH), serum 25-hydroxycholecalciferol ($25(OH)D_3$), 1,25-dihydroxycholecalciferol ($1,25(OH)_2D_3$), osteocalcin and bone-specific alkaline phosphatase (B-Alkphase) were measured in 24 h urine samples and fasting blood samples collected at the end of each dietary period. The calciuric diet significantly ($P<0{\cdot}05$) increased mean urinary Na, N, K, Ca and NTx (by 19 %) compared with the basal diet, but had no effect on circulating $25(OH)D_3$, $1,25(OH)_2D_3$, PTH, osteocalcin or B-Alkphase in the total group (n 24). There were no differences in serum markers or urinary minerals between the basal and calciuric diet in either VDR genotype groups. While the calciuric diet significantly increased urinary NTx (by 25·6 %, $P<0{\cdot}01$) in the $f+$ VDR group (n 10; carrying one or more (f) *Fok* I alleles), it had no effect in the $f-$ VDR group (n 14; not carrying any *Fok* I alleles). It is concluded that the Na- and protein-induced urinary Ca loss is compensated for by increased bone resorption and that this response may be influenced by VDR genotype.

Sodium: Protein: Bone metabolism: Vitamin D receptor: Postmenopausal women

Various dietary factors have been suggested as risk factors for osteoporosis, including high protein and high salt intakes (Cohen & Roe, 2000). Increasing Na intake within the usual dietary range is associated with increased urinary Ca loss (calciuria) (Shortt *et al.* 1988; Itoh & Suyama, 1996; Ginty *et al.* 1998*b*; Sellmeyer *et al.* 2002; for reviews, see Shortt & Flynn, 1990; Massey & Whiting, 1996). It has been estimated that a 100 mmol increment in daily Na intake is associated with an average additional loss of urinary Ca of approximately 1 mmol in free-living normocalciuric healthy populations (Nordin *et al.* 1993). A calciuric effect is also associated with high-protein diets (Allen *et al.* 1979*a*,*b*; Schuette *et al.* 1980; Hegsted *et al.* 1981; Mahalko *et al.* 1983; Pannemans *et al.* 1997; for review, see Kerstetter & Allen, 1994). It has been estimated that a doubling of dietary protein intake increases daily urinary Ca excretion by approximately 50 % (Walker & Linkswiler, 1972; Heaney, 1993). Furthermore, there is some evidence from animal studies that the calciuric effects of high Na and high protein intakes may be additive (Goulding & Campbell, 1984; Chan & Swaminathan, 1994). However, it is not known to what extent Na- (and/or protein-) induced calciuria is compensated for by increased absorption of dietary Ca and/or reduced endogenous Ca losses or to what extent this urinary Ca is derived from resorption of bone (Shortt & Flynn, 1990; Itoh *et al.* 1998).

There is evidence that increasing intakes of both Na and protein significantly increases urinary-based biochemical markers of bone resorption, and this has been associated with increased risk of hip fracture (Garnero *et al.* 1996, 2000). For example, Sellmeyer *et al.* (2002) recently reported that when postmenopausal women, adapted to a low-Na diet (87 mmol/d) for 3 weeks, were switched to a high-Na diet (225 mmol/d) diet for a further 4 weeks, urinary type I collagen cross-linked N-telopeptide (NTx) levels

Abbreviations: Dpyr, deoxypyridinoline; NTx, type I collagen cross-linked N-telopeptide; PTH, parathyroid hormone; $1,25(OH)_2D_3$, 1,25-dihydroxycholecalciferol; $25(OH)D_3$, 25-hydroxycholecalciferol.

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were significantly increased. Similarly, Evans *et al.* (1997) reported that urinary excretion of deoxypyridinoline (Dpyr) was greater following 7 d on a high-Na diet (300 mmol/d) than a low-Na diet (50 mmol/d) for postmenopausal but not premenopausal women. Shortt & Flynn (1990) have suggested that postmenopausal women are a group whose adaptive mechanisms for increasing Ca absorption in response to Na-induced calciuria may be incomplete, and thus the Ca needed to buffer the additional loss in urine may be derived from bone resorption. However, Lietz *et al.* (1997) found no effect of increasing Na intake for 8 d on urinary Dpyr levels in postmenopausal women. Increasing dietary protein is associated with increased urinary hydroxyproline (Schuette *et al.* 1981; Schuette & Linkswiler, 1982) and NTx excretion (Kerstetter *et al.* 1999) in young adult men and women respectively. However, Shapses *et al.* (1995) found no effect of increasing dietary protein on urinary Dpyr excretion in young adults. The effect of a high protein intake on bone turnover in postmenopausal women has not been reported. Furthermore, to date there has been no study of the effect of high dietary Na in combination with high protein intake on urinary Ca excretion and biomarkers of Ca and bone metabolism in human subjects.

Recent evidence suggests that the increased susceptibility to bone loss associated with the vitamin D receptor (VDR) genotype in postmenopausal women may be mediated, at least in part, through processes which are diet related. For example, Rapuri *et al.* (2001) recently showed that genetic variation in the VDR gene interacts with high caffeine intake (another diet-derived calciuric factor) in determining the rate of bone loss in postmenopausal women.

Thus, the aims of the present study were first, to investigate the effect of a high-Na, high-protein (i.e. a calciuric) diet on urinary Ca excretion and biomarkers of Ca and bone metabolism in postmenopausal women, and second, to investigate the possible interaction between this calciuric diet and VDR genotype on the rate of bone turnover in postmenopausal women.

Methods

Subjects

Twenty-six free-living postmenopausal women (mean age 57·1 (range 50–67) years) were recruited in the region of Cork, Republic of Ireland. A pre-study screening to assess basic blood chemistry was performed to ensure that the results showed no relevant clinical deviations from standard biochemical reference ranges for healthy adults. The mean height, weight, BMI and number of years post-menopause are provided in Table 1. The subjects were apparently healthy, without any history of bone or articular disease, and with no intake of medicine that could affect bone or cartilage metabolism. Additional exclusion criteria included renal damage, chronic illness or established familial history of hypertension, and taking hormone replacement therapy or nutritional supplements. Subjects were requested to avoid vigorous exercise and excessive alcohol intake for the duration of the study.

Table 1. Characteristics of the group of apparently healthy postmenopausal women (*n* 26) selected for the high-sodium, high-protein intervention trial

(Mean values, standard deviations and ranges)

	Mean	SD	Range
Age (years)	57·1	5·1	50–67
Years since menopause (years)	6·1	4·8	2–20
Height (m)	1·65	0·06	1·53–1·75
Weight (kg)	65·3	11·1	49–86
BMI (kg/m^2)	23·9	3·7	18·5–32·3

Ethical considerations

Before participation in this study, all subjects signed an informed consent document approved by the Clinical Research Ethics Committee of the Cork Teaching Hospitals.

Design

The study consisted of a randomized crossover trial of the effect of a 'high' Na (180 mmol/d), 'high' protein (90 g/d) intake (i.e. calciuric diet) *v.* a 'low' Na (65 mmol/d), 'usual' protein (70 g/d) intake (basal diet) for 4 weeks on the metabolism of Ca and bone in postmenopausal women.

The dietary intervention trial was designed in two successive dietary periods, each of 4 weeks. Subjects were assigned randomly to the calciuric diet (i.e. high in Na and protein) or the basal diet (i.e. adequate in protein and low in Na) for 4 weeks, followed by crossover to the alternative dietary regimen for a further 4 weeks (Fig. 1). The calciuric diet was based on the basal diet (Shortt *et al.* 1988; Ginty *et al.* 1998*b*), to which appropriate quantities of protein and Na were added while maintaining Ca intake at usual levels (about 20 mmol (800 mg)/d). The additional Na was added through dietary means as described previously by Ginty *et al.* (1998*b*). The additional protein was in the form of protein-rich bread (see later) that also provided extra Na.

As the intervention took at least 2 months to complete; subjects were supplemented throughout with 5 μg cholecalciferol/d, commencing 1 month before the first intervention, in order to avoid seasonal changes in vitamin D status. Subjects were instructed to collect consecutive 24 h urine samples for the last 2 d of each dietary period and during the week immediately preceding the trial (baseline). In addition, after an overnight fast, a blood sample (25 ml) was taken at 09.00 hours on the morning of the last day of each dietary period and during the baseline week immediately preceding the trial. Blood pressure was measured on the first day and days 7, 14, 21 and 28 of each dietary period and during the baseline week. Four-day (including weekend) estimated diet records for each subject were obtained by a trained investigator during each treatment period and during the baseline week immediately preceding the trial.

Bread preparation

A high-protein, high-Na bread was manufactured in the experimental bakery at University College, Cork, Republic

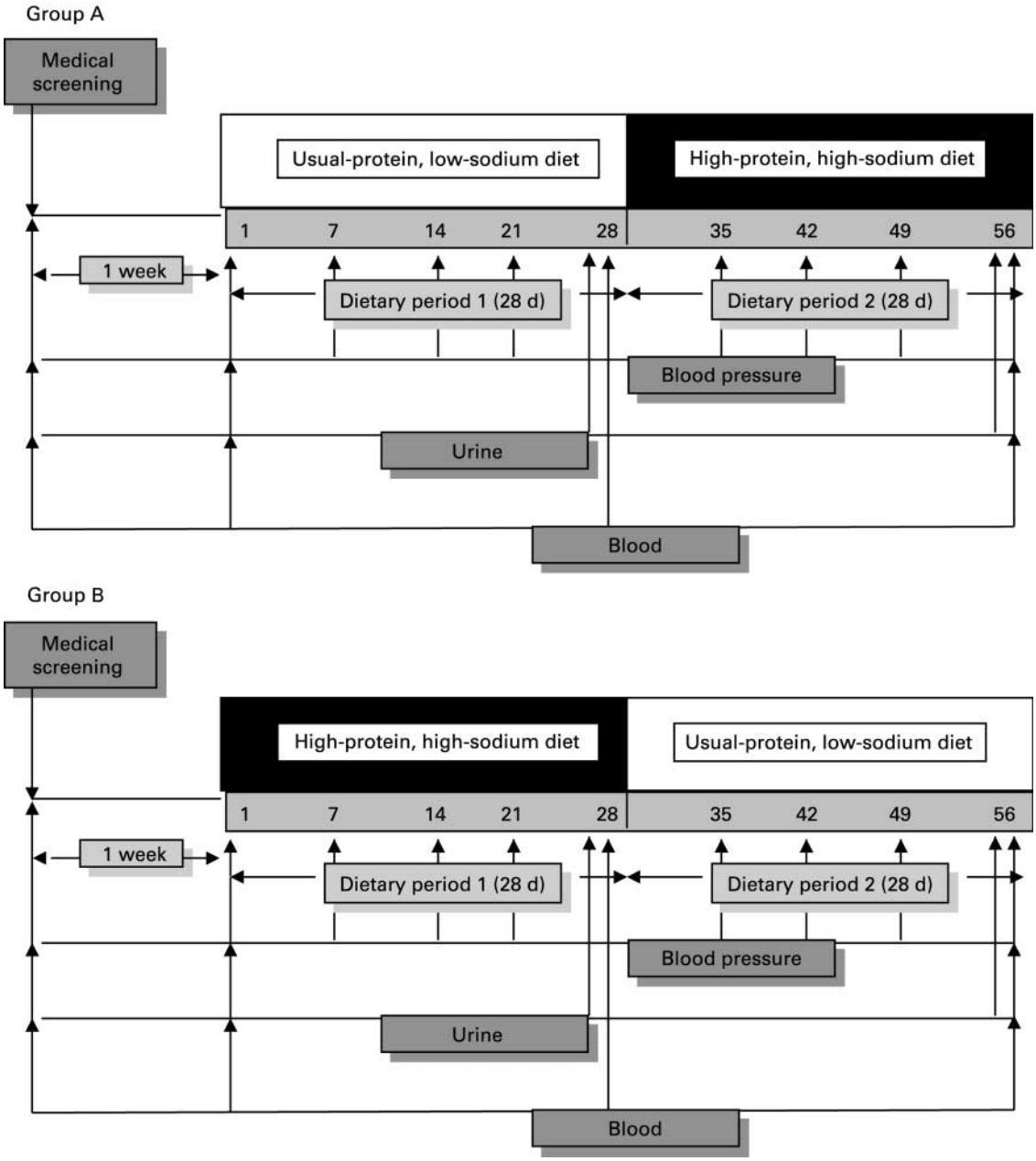

Fig. 1. Experimental design.

of Ireland. The bread was made from dough of the following composition: sodium caseinate (Dairygold, Mitchelstown, Co. Cork, Republic of Ireland) 136 g; wheat gluten (Gluvital 21000; Cerestar, Eridania Beghin-Say, Germany) 136 g; spray-dried egg white powder (Sanova Foods A/S, Odense, Denmark) 30 g; salt (Saxa; RHM Foods, Glasnevin, Co. Dublin, Republic of Ireland) 18 g, stoneground coarse wholemeal flour (Odlums, Kennedy Quay, Cork, Republic of Ireland) 227 g, bakers' flour (Odlums) 68 g, granulated sugar (Siucra; Irish Sugar plc., Co. Carlow, Republic of Ireland) 9 g, instant dry yeast (Mauripan; Burns Philip, Stillorgan, Co. Dublin, Ireland) 9 g, sunflower oil (Flora; Van Den Berg Foods Ltd, Crawley, Sussex, UK) 17 ml, dough emulsifier (sodium stearoyl 2-lactylate; Quest International Irl Ltd, Carrigaline, Co. Cork, Republic of Ireland) 1·5; water 455 ml. The high-protein, high-Na bread contained (g/kg bread): protein 244·0, Na 8·2, P 2·5, K 1·6, Ca 0·3.

A normal-protein, low-Na bread was also manufactured from dough of the following composition: stoneground coarse wholemeal flour (Odlums) 500 g; bakers' flour (Odlums) 167 g; salt (Saxa; RHM Foods) 6 g, granulated sugar (Siucra; Irish Sugar plc.) 42 g; instant dry yeast (Mauripan;) 21 g; sunflower oil (Flora; Van Den Berg Foods Ltd) 42 ml, dough emulsifier (sodium stearoyl 2-lactylate; Quest International Irl Ltd) 3·3 g; water 333 ml. The normal-protein, low-Na bread contained (g/kg bread): protein 83·0, Na 3·8, P 1·1, K 2·5, Ca 0·4.

Blood pressure measurements

Blood pressure (systolic and diastolic) was measured three times in succession in the seated position after the subjects sat quietly for at least 5 min in the morning, using an Omron HEM-705CP fully automatic digital blood-pressure monitor (Omron Healthcare Europe B.V., Hoofddorp, The Netherlands), which was validated by the British Hypertension Society.

Dietary analysis

Food intakes were estimated from 4 d estimated diet record data and quantified using average food portion sizes (Ministry of Agriculture, Fisheries & Food, 1997) and a photographic album (PJ Robson, unpublished results). Nutrient intakes were calculated using the computer package WISP© (Tinuviel Software, Warrington, Ches., UK). WISP© uses *McCance and Widdowson's Composition of Foods* (5th ed.) food nutrient database (Holland *et al.* 1995) and supplemental volumes (Holland *et al.* 1988, 1989, 1991, 1992, 1993, 1996; Chan *et al.* 1994, 1995, 1996).

Collection and preparation of samples

Subjects were supplied with suitable collection containers for urine samples and asked to make 24 h collections at baseline and for the last two consecutive days of each dietary period. The volumes of 24 h urine collections were recorded and portions of urine were acidified using 0·36 M-HCl and stored at −20°C from the morning of collection until required for analysis. Blood was collected by venepuncture into vacutainer tubes containing either no additive or K_3EDTA and processed to serum and plasma respectively; the samples were immediately stored at −70°C until required. Whole blood samples were used on the day of collection for DNA isolation by a Wizard® genomic DNA purification kit (Promega Corporation, Madison, WI, USA). The isolated DNA was then stored at −20°C until required for analysis.

Experimental techniques

DNA analysis. Restriction fragment length polymorphisms in the VDR gene were determined by PCR followed by digestion of the amplified PCR product with the *Fok* I restriction endonuclease as described by Gross *et al.* (1996). Following restriction endonuclease digestion, VDR genotypes were determined by ethidium bromide–u.v.-B illumination of the fragments separated on agarose (20 g/l) gel. Homozygous absence of the *Fok* I restriction fragment length polymorphism site (designated *FF*) resulted in two fragments of 265 bp and sixty-nine bp, while homozygous presence of the site (*ff*) resulted in two fragments of 196 and sixty-nine bp. Heterozygotes (*Ff*) exhibited all three fragments. Subjects carrying one or more *f* (*Fok* I) alleles were designated *f* + (i.e. *Ff* and *ff* VDR genotypes), while those not carrying an *f* allele were designated *f* − (i.e. *FF* VDR genotype). This VDR genotype stratification approach has recently been used by Tofteng *et al.* (2002), who showed that the presence of an *f* VDR allele was associated with lower bone mineral density in Danish postmenopausal women compared with those without the *f* allele.

Urinary type I collagen cross-linked N-telopeptides. NTx was measured in the urine samples by an ELISA (Osteomark®, Ostex International, Inc., WA, USA). The intra-assay CV was 5 %. Inter-assay variation was avoided by analysing all samples from an individual in the same run.

Urinary creatinine. Creatinine was determined in urine samples using a diagnostic kit (Metra Creatinine Assay Kit, catalogue no. 8009; Quidel Corporation, San Diego, CA, USA). The intra-assay CV was 1·6 %. Inter-assay variation was avoided by analysing all samples from an individual in the same run.

Urinary calcium, sodium and potassium. Ca was analysed in duplicate in urine samples by atomic absorption spectrophotometry (Spectr AA-600; Varian Australia Ltd, St Helens Victoria, Australia) after appropriate dilution with $LaCl_3$ solution (5 g/l; BDH Ltd, Poole, Dorset, UK). A range of Ca standards was used to obtain a Ca calibration curve. The intra- and inter-assay CV for Ca were 2·8 and 7·8 % respectively. Na and K were determined in the urine samples by flame photometry (Jenway PFP7; ACB Ltd, Dublin, Republic of Ireland) using appropriate Na and K standards. The intra- and inter-assay CV for Na were 3·8 and 6·9 %, and for K were 4·5 and 9·3 % respectively.

Urinary nitrogen level. N was measured in urine samples using a modification of the Kjeldahl method (Association of Official Analytical Chemists, 1995). The intra-assay CV was 7·6 % and inter-assay variation was avoided by analysing all samples from an individual in the same run.

Serum osteocalcin and bone-specific alkaline phosphatase. Bone-specific alkaline phosphatase levels were measured in serum samples using a recently developed ELISA (Alkphase-B™; MetraBiosystems Inc., Mountain View, CA, USA). The intra-assay CV was 4·5 %. Osteocalcin levels were measured in serum samples using an ELISA (N-MID™; Osteometer Biotech, Osteopark, Denmark). The intra-assay CV was 11 %. Inter-assay variation for both serum osteocalcin and bone-specific alkaline phosphatase was avoided by analysing all samples from an individual in the same run.

Plasma parathyroid hormone. Samples were analysed in duplicate for intact parathyroid hormone (PTH) with a chemiluminescent immunometric assay (IMMULITE®; Diagnostic Products Corporation, Los Angeles, CA, USA). The intra-assay CV for this assay was 5·8 %. Inter-assay variation was avoided by analysing all samples from an individual in the same run.

Serum 25-hydroxycholecalciferol. Serum proteins were precipitated with ethanol, and deproteinized serum was subsequently applied to a monofunctional saline (MF) C_{18} solid-phase extraction column (Isolute®; International Sorbent Technology, Glamorgan, Wales, UK) for elution of the 25-hydroxycholecalciferol (25(OH)D_3) fraction with ethylacetate–*n*-heptane. The extracted 25(OH)D_3 was injected onto an HPLC-system (Waters, Milford, MA, USA) equipped

with a 600 controller and pump, a refrigerated 717 Plus Autosampler, a 996 Diode Array Detector (set at 220–320 nm) for detection, and a 2487 Absorbance Detector (set at 265 nm) for quantification. The HPLC column used for separation was a cyano (Luna; Phenomenex, Torrance, CA, USA) in which $25(OH)D_3$ and 1,25-dihydroxycholecalciferol ($1,25(OH)_2D_3$) were eluted separately with 2-propanol–*n*-heptane. However, none of the samples contained $25(OH)D_3$; the intra-assay CV for $25(OH)D_3$ was 6·0 %. The accuracy of the analysis was monitored by participation in the vitamin D external quality assessment scheme (DEQAS; Charing Cross Hospital, London, UK).

Serum 1,25-dihydroxycholecalciferol. A radioimmunoassay (65100E; DiaSorin, Stillwater, MN, USA) was used for the quantification of $1,25(OH)_2D_3$ levels. The manufacturer's method was modified slightly as solid-phase extraction was performed on MF C_{18} columns (Isolute®; International Sorbent Technology). The intra-assay CV for this assay was 12·6 %. The accuracy of the analysis was monitored by participation in the vitamin D external quality assessment scheme (DEQAS; Charing Cross Hospital, London, UK).

Statistical analysis

Values are presented as means and standard deviations. Data for all variables were normally distributed (except for bone-specific alkaline phosphatase in the *f* − VDR group) as determined by the method of Kolmogorov & Smirnov and allowed for parametric tests of significance. Data for bone-specific alkaline phosphatase in the *f* − VDR group were log-transformed prior to statistical analysis, to achieve a near-normal distribution. Differences in age, height, weight, BMI and years since menopause between the two VDR genotype groups were examined by unpaired Student's *t* tests. Nutrient intakes between each dietary period and between both genotype groups were compared using paired and unpaired Student's *t* tests respectively. Differences between the two dietary regimens in biochemical indices of bone turnover and urinary minerals were analysed in the total group and in the two VDR genotype groupings separately by the appropriate analysis for a crossover trial with continuous data as described by Jones & Kenward (1989); two-sample *t* tests and ANOVA were used to test hypotheses about direct treatments effects (i.e. calciuric diet), carry-over effects and interactions.

Results

Baseline blood pressures in all twenty-six postmenopausal women were within the World Health Organization (1999) targets (i.e. <140 and <90 mmHg for systolic and diastolic blood pressure respectively). Daily urinary Na output by these women while on their habitual diet ranged between 37 and 178 mmol/d.

All twenty-six women completed the dietary intervention study. However, two subjects had no increase in daily urinary Na or N output on the high-Na, high-protein diet and, therefore, were considered to be protocol violators and were excluded from the analysis.

Dietary intakes of energy, Ca, Mg, P, K or fibre (NSP) were similar during the two dietary regimens (Table 2). Dietary intakes of caffeine were similar during the two dietary regimens (306 (SD 126) mg on the basal diet *v.* 301 (SD 157) mg on the calciuric diet; $P=0{\cdot}840$). There was no significant difference in body weight between the two dietary regimens (63·5 (SD 10·6) kg on the basal diet *v.* 63·6 (SD 10·4) kg on the calciuric diet; $P=0{\cdot}542$).

The effects of increasing Na and protein intake from the basal diet to the calciuric diet for 28 d on urinary and serum biochemical variables, and on blood pressure are shown in Table 3. There were no significant differences in systolic or diastolic blood pressure of the women between the two diets. Compared with the basal diet, significant increases were observed on the calciuric diet for mean urinary Na, Ca, K and N.

There were no significant differences between the two diets for mean urinary creatinine or mean serum concentrations of $25(OH)D_3$ or $1,25(OH)_2D_3$ or mean plasma PTH (Table 3). Urinary excretion of NTx/creatinine was significantly ($P<0{\cdot}05$) higher when subjects were fed the calciuric diet compared with the basal diet (Table 3). There were no significant differences between the two diets for mean levels of serum biochemical markers of bone formation (serum bone-specific alkaline phosphatase or osteocalcin) (Table 3).

There were no significant differences between the *f* − and *f* + VDR genotype groups for age, weight, height or BMI (Table 4) or for baseline biochemical indices or

Table 2. Intakes of selected nutrients from the final 4 d during low-sodium, usual-protein and high-sodium, high-protein dietary periods for apparently healthy postmenopausal women (*n* 24)*

(Mean values and standard deviations)

Dietary period…	Low-Na, usual-protein		High-Na, high-protein		
Nutrient	Mean	SD	Mean	SD	Statistical significance of effect: *P*
Energy (kJ/d)	8061	3094	8132	2300	0·944
Fibre (NSP) (g/d)†	17·8	5·0	18·2	5·8	0·773
Ca (mg/d)	733	221	689	176	0·395
P (mg/d)	1362	364	1404	469	0·789
K (mg/d)	3469	1640	3437	764	0·945
Mg (mg/d)	346	109	342	80	0·917

* For details of subjects and procedures, see Table 1 and p. 42.
† Total NSP measured according to Englyst & Cummings (1988).

Table 3. Urinary and serum biochemical variables and blood pressure in apparently healthy postmenopausal women (*n* 24) during the low-sodium, usual-protein and high-sodium, high-protein dietary periods*

(Mean values and standard deviations)

Diet period...	Low-Na, usual-protein (65 mmol Na + 70 g protein/d)		High-Na, high-protein (180 mmol Na + 90 g protein/d)		Statistical significance of effect: *P*†
	Mean	SD	Mean	SD	
Urine:					
Na (mmol/d)	60·3	26·0	131·0	37·6	<0·0001
K (mmol/d)	66·9	13·1	70·6	13·8	<0·0001
Ca (mmol/d)	3·91	1·39	4·72	1·66	<0·001
N (mmol/d)	736	189	859	148	<0·001
Creatinine (mmol/d)	8·8	1·6	8·6	1·6	0·239
NTx (nmol BCE/mmol creatinine)	34·9	9·8	41·6	12·6	0·012
Blood:					
Serum 25(OH)D_3 (nmol/l)	70·9	13·6	70·8	13·6	0·979
Serum 1,25$(OH)_2D_3$ (pmol/l)	119·9	21·1	125·5	29·2	0·373
Serum B-alkphase (U/l)	27·3	7·6	28·4	8·8	0·120
Serum osteocalcin (μg/l)	26·6	9·0	26·3	9·2	0·773
Plasma PTH (pmol/l)	3·1	1·4	3·1	1·2	0·984
Blood pressure:					
Systolic (mmHg)	116	13	114	11	0·576
Diastolic (mmHg)	74	7	75	8	0·802

NTx, type I collagen cross-linked N-telopeptides; BCE, bone collagen equivalents; 25(OH)D_3, 25-hydroxycholecalciferol; 1,25$(OH)_2D_3$, 1,25-dihydroxycholecalciferol; B-alkphase, bone-specific alkaline phosphatase; PTH, parathyroid hormone.

* For details of subjects and procedures, see Table 1 and p. 42

† Direct treatment effect (i.e. calciuric diet) was analysed for each biochemical index and blood pressure by two-sample *t* tests of within-group differences after testing for carry-over effects. (No significant carry-over effects were found.)

blood pressure (results not shown). In addition, there were no significant differences in the dietary intakes of energy, Ca, P, K, Mg, caffeine or fibre between the calciuric and basal diets in either VDR genotype groups, or between the VDR genotype groups consuming either the calciuric or basal diets (results not shown).

There were no significant differences in serum osteocalcin, bone-specific alkaline phosphatase or vitamin D metabolites, or plasma PTH between the basal and calciuric diets in either VDR genotype groups (Table 5).

Compared with the basal diet, significant increases were observed on the calciuric diet for mean urinary Na, Ca, K and N in both VDR genotype groups (Table 5). Urinary excretion of NTx/creatinine was significantly ($P<0·01$) higher in subjects carrying one or more *f* VDR alleles when consuming the calciuric diet compared with the basal diet (Table 5). On the other hand, there was no effect of the calciuric diet on urinary NTx/creatinine in subjects not carrying a *f* VDR allele (Table 5).

Discussion

In the present study, the average increase in urinary Ca in response to increased dietary Na and protein in the twenty-four postmenopausal women who took part in the dietary intervention trial was 0·81 mmol/d. It is well established that increasing dietary Na intake within the usual range of dietary intakes increases urinary Ca excretion (Shortt & Flynn, 1990; Ginty *et al.* 1998*b*). This dependence of urinary Ca excretion on urinary Na excretion has been attributed to the existence of linked or common re-absorption pathways for both ions in the convoluted portion of the proximal tubule and thick ascending loop of Henle (Shortt & Flynn, 1990). There is also considerable evidence that increasing dietary protein intake within the usual range of dietary intakes increases urinary Ca excretion (for reviews, see Kerstetter & Allen, 1990, 1994; Heaney, 1993, 1998; Massey, 1998). Protein-induced calciuria has been attributed to an increased glomerular filtration rate

Table 4. Characteristics of the vitamin D receptor (VDR) genotype groups of apparently healthy postmenopausal women (*n* 24)*†

(Mean values and standard deviations)

	f− (*n* 14)		*f*+ (*n* 10)		
	Mean	SD	Mean	SD	Statistical significance of effect: *P*‡
Age (years)	57·0	5·6	56·9	5·2	0·965
Years since menopause (years)	5·8	4·6	7·1	5·4	0·504
Height (m)	1·64	0·06	1·66	0·04	0·425
Weight (kg)	62·3	10·2	66·0	10·7	0·393
BMI (kg/m^2)	23·0	3·4	23·9	3·5	0·595

* For details of subjects, see Table 1.

† For details of VDR genotype groups, see p. 44.

‡ Comparison of means between *f*− and *f*+ VDR genotype groups using unpaired Student's *t* test.

Table 5. Urinary and serum biochemical variables and blood pressure in apparently healthy postmenopausal women in both vitamin D receptor (VDR) genotype groups during low-sodium, usual-protein and high-sodium, high-protein dietary periods†‡
(Mean values and standard deviations)

VDR genotype group...	*f* − (*n* 14)				*f* + (*n* 10)			
Diet period...	Low-Na, usual-protein		High-Na, high-protein		Low-Na, usual-protein		High-Na, high-protein	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Urine:								
Na (mmol/d)	60·2	26·3	132·2**	40·0	60·5	27·1	129·3**	36·0
K (mmol/d)	65·8	20·2	72·4**	14·8	69·0	15·4	73·8**	26·5
Ca (mmol/d)	3·54	1·26	4·63**	1·57	3·56	1·33	4·36**	1·24
N (mmol/d)	749	208	880**	166	718	165	830**	121
Creatinine (mmol/d)	8·6	1·4	8·3	1·5	9·1	1·8	9·0	1·7
NTx (nmol BCE/mmol creatinine)	34·3	9·0	38·9	12·3	34·4	12·6	43·2**	12·5
Blood:								
Serum 25(OH)D_3 (nmol/l)	68·4	13·5	70·0	15·4	74·2	13·8	71·8	11·6
Serum 1,25$(OH)_2D_3$ (nmol/l)	112	23	113	28	132	10	144	19
Serum B-alkphase (U/l)	25·7	9·4	27·1	10·4	27·5	5·5	27·6	6·1
Serum osteocalcin (μg/l)	26·6	7·1	25·8	8·9	26·6	11·6	26·9	10·2
Plasma PTH (pmol/l)	2·9	1·2	3·0	1·3	3·3	1·6	3·2	1·0

NTx, type I collagen cross-linked N-telopeptides; BCE, bone collagen equivalents; 25(OH)D_3, 25-hydroxycholecalciferol; 1,25$(OH)_2D_3$, 1,25-dihydroxycholecalciferol; B-alkphase, bone-specific alkaline phosphatase; PTH, parathyroid hormone.
Direct treatment effect (i.e. calciuric diet) was analysed for each biochemical index within a VDR genotype group by two sample *t* tests of within group differences after testing for carry-over effects: $^{**}P<0{\cdot}01$. (No significant carry-over effects were found.)
† For details of subjects and procedures, see Table 1 and p. 42.
‡ For details of VDR genotype groups, see p. 44.

and a decreased tubular reabsorption of Ca, caused by increased excretion of sulfate derived from catabolism of S-containing amino acids native to the protein (Kim & Linkswiler, 1979; Schuette *et al.* 1980). There are no comparable results in the literature from trials with human subjects on the combined effect of increased Na and protein intake on urinary Ca excretion in adults. There is some evidence from animal studies that the calciuric effects of high Na and high protein intakes may be additive (Goulding & Campbell, 1984; Chan & Swaminathan, 1994). To place the Na and/or protein-induced calciuria observed in the present study in the context of bone health, it has been estimated that a net deficit of only 1 mmol Ca/d would result in losing one-third of the Ca contained in the typical adult skeleton in just over 20 years, unless a compensatory increase in the efficiency of intestinal Ca absorption and/or decrease in endogenous loss of Ca occurred (Shortt & Flynn, 1990; Sellmeyer *et al.* 2002).

The increase in urinary Ca was not influenced by other dietary factors (e.g. Ca, Mg, P, K or fibre) that have been reported to influence urinary Ca excretion (Heaney & Recker, 1982; Massey & Wise, 1984), since the intakes of these were similar for the basal and calciuric diets.

In the present study, urinary excretion of K was significantly higher when subjects consumed the calciuric diet compared with the basal diet. While there are no comparable results in the literature from trials with human subjects on the combined effect of increased Na and protein intake on urinary K excretion, previous studies of postmenopausal women have reported a lack of effect of increasing dietary Na alone on K excretion (Zarkadas *et al.* 1989; Lietz *et al.* 1997; Sellmeyer *et al.* 2002). However, other studies have reported an increased urinary K excretion with increasing Na intake in adults. For example, Castenmiller *et al.* (1985) showed that increasing Na intake in adult men significantly increased urinary K as well as urinary Ca. Bell *et al.* (1992) reported that increasing the Na content of a control diet fed to healthy young women increased urinary K, while Ginty *et al.* (1998*a*) found a significant positive correlation between urinary Na and K in a group of healthy young adults.

In the present study, increasing the dietary Na and protein significantly increased the urinary excretion of N ($P<0{\cdot}001$). This is in agreement with the findings of several studies in which urinary N increased significantly in adults when dietary protein was increased (Draper *et al.* 1991; Kerstetter *et al.*, 1998).

Urinary NTx excretion was about 19 % higher in postmenopausal women in the present study when consuming the calciuric diet compared with the basal diet. NTx is considered a sensitive and specific marker of bone resorption (Rosen *et al.* 1994). While there are no comparable results from trials with human subjects in the literature on the combined effect of increased Na and protein intake on bone resorption, the effect of increased Na intake on bone resorption was investigated in postmenopausal women (Evans *et al.* 1997; Lietz *et al.* 1997; Sellmeyer *et al.* 2002). In one crossover trial, fourteen postmenopausal women were randomized to a fixed diet containing 20 mmol (800 mg) Ca/d and either 60 or 170 mmol Na/d for 8 d and then crossed-over to the alternative diet for a further 8 d (Lietz *et al.* 1997). Urinary Dpyr excretion was unaffected by Na intake. In a second crossover trial, eleven postmenopausal women (with a mean usual Ca intake of 18 mmol (741 mg)/d) were randomized to a low (50 mmol/d) or high (300 mmol/d) Na diet for 7 d and then crossed-over to the alternative diet for a further 7 d (Evans *et al.* 1997). There was a significant increase (27 %, $P=0{\cdot}024$) in urinary Dpyr excretion on the high-Na diet. Finally, Sellmeyer *et al.* (2002) recently reported

that when postmenopausal women, who received a daily supplement of 12 mmol (500 mg) Ca in addition to their usual diet and who were adapted to a low-Na diet (87 mmol/d) for 3 weeks, were switched to a high-Na (225 mmol/d) diet for a further 4 weeks, urinary NTx levels were significantly increased (by 23 %, $P<0{\cdot}001$).

There are a few studies that have investigated the influence of increased protein intake on the rate of bone resorption, but none that have investigated this association in postmenopausal women. Schuette *et al.* (1981) reported that urinary hydroxyproline was significantly increased (by 32 %, $P<0{\cdot}05$) in sixteen young men when the protein content of the diet (with a fixed dietary Ca level, 12 mmol (500 mg)/d) was increased from about 50 to about 150 g/d for 12 d (Schuette *et al.* 1981). However, the suitability of urinary hydroxyproline as a marker of bone resorption has been questioned because of its lack of specificity and sensitivity (Lietz *et al.* 1997). Kerstetter *et al.* (1999) reported that increasing the protein content of the diet (with a fixed dietary Ca level of 20 mmol (800 mg)/d) from 45 to 129 g/d for 4 d led to an increased urinary excretion of NTx (by 47 %, $P<0{\cdot}05$) in healthy young women. However, increasing the protein content of the diet from 63 to 129 g/d for 4 d had no effect on urinary NTx levels in that study. Shapses *et al.* (1995) found that urinary hydroxyproline, pyridinoline and Dpyr were unaffected in seven young men and eight young women by increasing dietary protein content from 0·44 to 2·71 g/kg body weight per d for 5 d. However, in that study, dietary Ca was increased from 11 (423 mg) to 40 mmol (1589 mg)/d concomitant with the increase in dietary protein, potentially obscuring an effect of dietary protein on bone resorption (Kerstetter *et al.* 1999).

The calciuric diet in the present study had no effect on serum osteocalcin or bone-specific alkaline phosphatase levels. McParland *et al.* (1989) reported that in addition to an increased rate of bone resorption (as shown by increased urinary hydroxyproline excretion), serum osteocalcin levels were also increased in postmenopausal women supplemented with 100 mmol Na/d for 10 d. However, another Na intervention study reported a lack of effect of increasing Na intake on serum osteocalcin in postmenopausal women (Evans *et al.* 1997). The lack of effect in the present study as well as in that of Evans *et al.* (1997), however, could possibly be due to their relative short duration: bone formation, although coupled to resorption, is separated by approximately 6 weeks (Eriksen *et al.* 1984).

The increased rate of bone resorption observed in postmenopausal women in the present study may be as a consequence of a lack of, or an incomplete, intestinal adaptation to the Na and/or protein-induced calciuria. Breslau *et al.* (1985) found no increase in intestinal Ca absorption in seven osteoporotic postmenopausal women when Na intake was increased from 10 to 250 mmol/d while dietary Ca was maintained at 10 mmol/d. Two other studies have reported a lack of effect of increasing dietary Na on Ca absorption, using Sr absorption as an index in postmenopausal women (McParland *et al.* 1989; Evans *et al.* 1997); however, the precision of this method may have been too low to detect a change. Similarly, increasing the protein intake in postmenopausal women (Draper *et al.* 1991) or elderly adults (Dawson-Hughes & Harris, 2002) appears to have no effect on the efficiency of intestinal Ca absorption. In the present study, there was no effect of the calciuric diet on serum $1,25(OH)_2D_3$ levels, an important mediator of the adaptive component of Ca absorption. This is in agreement with the findings of some studies that found no effect of increasing dietary protein (Schuette *et al.* 1980) or Na (Breslau *et al.* 1982; McParland *et al.* 1989) on circulating $1,25(OH)_2D_3$ levels in postmenopausal women and older adults. There is evidence that serum PTH is increased in association with Na-induced calciuria (McCarron *et al.* 1981; Breslau *et al.* 1982). There was no significant effect of the calciuric diet on plasma PTH levels in postmenopausal women in the present study. This is in agreement with the findings of some Na-loading studies (Evans *et al.* 1997; Lietz *et al.* 1997; Sellmeyer *et al.* 2002) and some studies of increased protein intake (Schuette *et al.* 1980, 1981; Draper *et al.* 1991) in postmenopausal women. However, interpretation of studies that examine PTH changes are made difficult by differences in sensitivity of the radioimmunoassays used, the pulsatile nature of serum PTH concentrations, the half-life of intact PTH (4 min) and the possible haemodilution effect of Na loading (Jubiz *et al.* 1972; Slatopolsky *et al.* 1982; Garel *et al.* 1987; Chan *et al.* 1992, for review, see Massey & Whiting, 1996).

In the present study, postmenopausal women carrying an *f* VDR allele had significantly elevated levels of urinary NTx (by about 25·6 %) when consuming the calciuric diet compared with that during the basal dietary period, whereas those without the *f* allele had no increase in urinary NTx when placed on the calciuric diet. Interestingly, the increment in urinary Ca with additional Na and protein was of a similar magnitude in the two VDR genotype groups. Furthermore, there were no differences in the responses of the other serum or urinary variables to the calciuric diet between the genotype groups. There were also no differences in dietary factors (e.g. Ca, Mg, P, K, caffeine or fibre), which have been reported to influence Ca and bone metabolism between the genotype groups. It would appear from these findings that the subjects carrying one or more *f* VDR alleles were less able to adapt to the calciuria than those without the *f* allele. It is possible that in subjects without the *f* allele(s) the Na- and protein-induced urinary Ca loss is compensated for by increased Ca absorption and/or reduced endogenous Ca loss rather than increased bone resorption, whereas the reverse may be true in those carrying the *f* allele. Ames *et al.* (1999) showed that children (White and Mexican-American, aged 7·5–12·0 years) with the *FF* (i.e. $f-$) VDR genotype had 52·9 % ($P<0{\cdot}02$) and 29·6 % ($P=0{\cdot}08$) greater Ca absorption as compared with children with the *ff* and *Ff* (i.e. $f+$) VDR genotypes respectively. Therefore, the *f* VDR allele may modulate the adaptive capacity of intestinal Ca absorption in response to dietary calciuric factors. The mechanism by which this occurs is not clear and warrants further investigation. VDR molecules encoded by the *f* allele initiate translation from an upstream ATG (at the site of the *Fok* I polymorphism) and are three amino acids longer than the *F* allele products, and moreover,

the protein encoded by the *f* allele produced 1·7-fold lesser transactivation of transcription from a promoter containing a vitamin D-responsive element than did the *F* allele product (Arai *et al.* 1997).

The influence of another dietary calciuric factor, i.e. high caffeine intake (>300 mg/d), on skeletal integrity has recently been shown to interact with the VDR genotype. Rapuri *et al.* (2001) reported that postmenopausal women with the *tt* genetic variant of VDR appeared to be at a greater risk for the deleterious effect a high caffeine intake on vertebral bone loss over 3 years compared with women with the *TT* VDR genotype.

Finally, there was no effect of a high Na (and high protein) diet over 4 weeks on body weight in the present study. While weight gains resulting from an increase in plasma volume in response to abrupt increases in dietary NaCl (an additional 235–240 mmol Na/d) were reported in some short-term (7 d) studies (Kurtz *et al.* 1987; Morris *et al.* 1999), Zarkadas *et al.* (1989) found no effect of increasing salt intake (an additional 51 and 102 mmol Na/d) on body weight in postmenopausal women over 5 d. The apparent lack of effect in the present study could be due to the relatively low level of NaCl supplementation administered (an additional 115 mmol Na/d) or because the subjects had achieved Na balance by the end of the 4 weeks (it usually takes 3–5 d for complete restoration of NaCl balance after altering NaCl intake), as suggested by Zarkadas *et al.* (1989).

In conclusion, the effect of the calciuric diet on biochemical markers of bone resorption in postmenopausal women in the present study suggests that the Na- and protein-induced urinary Ca loss is compensated for by increased bone resorption rather than by a complete adaptation at the level of intestinal Ca absorption and/or endogenous Ca loss in women who carry one or more of the *f* VDR alleles. This group represents over half of all postmenopausal women. Until such time as an individual's genotype are know, it may be prudent to make dietary recommendations based on the assumption that each person may have a genetic susceptibility to osteoporosis. Therefore, it would also seem prudent to recommend moderate protein and Na intake for postmenopausal women.

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