The cardiovascular system in familial hypocalciuric hypercalcemia: a cross-sectional study on physiological effects of inactivating variants in the calcium-sensing receptor gene

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Abstract

Objective: Loss-of-function variants in the gene encoding the calcium-sensing receptor (CASR) result in familial hypocalciuric hypercalcemia (FHH), causing hypercalcemia with high normal or elevated parathyroid hormone levels. The CASR may also influence electrolyte and water homeostasis. It is unknown whether FHH affects cardiovascular health. We, therefore investigated whether FHH is associated with changes in the regulation of the cardiovascular system by measuring 24-h blood pressure (BP), arterial stiffness and vasoactive hormones.

Design: Cross-sectional study comparing 50 patients with FHH to age- and gender-matched controls.

Results: Studied subjects (69% women) had a mean age of 56 years. A similar number of patients and controls (33%) were on treatment with antihypertensive drugs. Overall, no differences were found between groups in 24-h ambulatory BP or pulse wave velocity. However, compared with controls, diastolic BP during nighttime was lower in FHH females (60 ± 5 vs 66 ± 9 mmHg, $P < 0.01$) and higher in FHH males (69 ± 6 vs 64 ± 5 mmHg, $P = 0.02$). FHH was associated with a significantly higher plasma osmolality ($P < 0.01$), higher plasma levels of vasopressin ($P < 0.01$) and a higher renal excretion of epithelial sodium channels (ENaCs) ($P = 0.03$), whereas urine aquaporin-2 and plasma sodium, aldosterone and renin did not differ between groups. FHH patients had a lower urinary volume with an increased osmolality if analyses were restricted to those not on treatments with antihypertensive drugs.

Conclusions: FHH does not seem to be associated with an increased risk of CVD.

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Introduction

The calcium-sensing receptor (CASR) was first described by Brown *et al.* (1) in 1993. Today, the receptor is known to be expressed in many different tissues including the parathyroid glands, bone, kidney and the cardiovascular

system (2). Its major function is to sense even small changes in plasma-ionized calcium (Ca^{2+}) concentrations and to couple this information to intracellular signaling pathways that modify parathyroid hormone (PTH)

secretion and renal cation handling (3). Loss-of-function variants in the gene encoding the CASR on chromosome 3q21.1 are known to cause familial hypocalciuric hypercalcemia (FHH) type 1 (OMIM #145980). The condition is inherited in an autosomal dominant manner and causes an increase in the set point of the parathyroid glands, i.e. the plasma Ca^{2+} concentration at which PTH secretion is half-maximal. Accordingly, higher than normal plasma Ca^{2+} levels are necessary to inhibit the release of PTH, resulting in hypercalcemia associated with inappropriately normal or elevated plasma levels of PTH. The degree of hypercalcemia depends on how severely the genetic variant affects the function of the CASR (4). Similarly, calcium reabsorption is increased in the renal tubules causing a relatively low renal calcium excretion (5, 6).

There is considerable physiologic and genetic evidence indicating that the CASR takes part in the renal salt and water regulation by influencing the function of the renal outer medullary potassium channel (ROMK), the expression of aquaporin-2 (AQP2) and the secretion of renin (7). It is presumably due to the action of the CASR that renal calcium excretion is increased in response to a high calcium load. Furthermore, the receptor causes a decreased blood pressure (BP) following a high dietary intake of calcium in spontaneously hypertensive rats and humans with hypertension (8, 9). Finally, it has been shown that calcimimetics may exert a hypotensive effect which is believed to be mediated through their effects on the CASR in uremic rats (10), spontaneously hypertensive rats (11), and in kidney transplant humans (12).

The CASR is also expressed by cells in the cardiovascular system including the intimal and medial layers of large elastic arteries (13, 14). Loss-of-function of the CASR has been suggested to be involved in the development of vessel wall calcification which may be inhibited by treatment with calcimimetics (15). Conflicting results have, however, been reported on the potential role of the CASR on risk of CVD. By comparing 2561 patients with coronary artery disease (CAD) with 698 controls, März *et al.* (16) reported an association between the common A986S CASR polymorphism and risk of angiographic CAD, previous myocardial infarction and cardiovascular mortality. In contrast, Babinsky *et al.* (17) found no effects of six common CASR polymorphisms (including A986S) on risk of CAD in a cohort of 284 renal transplant patients. In FHH, it has been speculated whether BP regulation is affected (18, 19). It is well known that FHH causes a resistance to the diuretic effect of calcium, but the effect of the inactivating variant on sodium balance

and BP has not yet been fully elucidated (7). Moreover, in primary hyperparathyroidism (PHPT), the state of hyperparathyroid hypercalcemia is associated with an increased risk of cardiovascular diseases, including hypertension and increased arterial stiffness (20, 21). As the biochemical characteristics of FHH and PHPT are to some extent similar, it may be speculated whether the hyperparathyroid hypercalcemia in FHH affects risk of cardiovascular diseases.

The aim of this study is to investigate surrogate markers of cardiovascular health in terms of vasoactive hormones 24-h BP, and arterial stiffness in FHH compared with a sex- and age-matched control group.

Subjects and methods

Design and participants

In a cross-sectional study, we compared 50 FHH patients with 51 sex- and age-matched population-based controls aged 18–85 years. The design of the study has previously been detailed (22). In brief, patients with FHH were recruited from our out-patient clinic and all except one had a genetically verified diagnosis. The patient without a genetically verified diagnosis was included based on a family history of hypercalcemia and a low renal calcium excretion as determined by calculating the calcium–creatinine clearance ratio. Age- (± 2 years) and sex-matched controls were randomly selected from the general background population.

We excluded patients and controls with major medical or social problems including impaired renal function (plasma creatinine $> 125 \mu\text{mol/L}$), malignancies, untreated intestinal malabsorption, chronic disabling disease or prior hospital admission due to chronic drug or alcohol abuse. In addition, we excluded pregnant women and patients treated with drugs known to affect the CASR (lithium, strontium or cinacalcet), as well as subjects diagnosed with calcium metabolic disorders except osteoporosis.

All participants gave verbal and written informed consent and the study was conducted in accordance with the Declaration of Helsinki II. The study was approved by The Central Denmark Region Committees on Health Research Ethics (#M-2010-0296) and notified to The Danish Data Protection Agency (#2011-41-5733).

We asked participants to fill in a questionnaire concerning their state of health (Table 1). We assessed daily total calcium intake according to reported dietary intakes of milk, cheese, milk products and use of calcium

Table 1 Characteristics of patients with familial hypocalciuric hypercalcaemia (FHH) and their matched controls. Data is presented as *n* (%), mean $\pm$ (s.d.) or median with Interquartile (25; 75%) range.

	Controls (<i>n</i> =51)	FHH (<i>n</i> =50)	<i>P</i> -value
Age, years (range)	56 (23–82)	56 (23–82)	–
Females, <i>n</i> (%)	35 (68.6)	34 (68.0)	
Postmenopausal, <i>n</i> (%)	23 (65.7)	23 (68.0)	0.61
Anthropometrics, mean (s.d.)			
Height (cm)	170.1 (8.4)	168.7 (8.6)	0.43
Weight (kg)	77.0 (14.0)	75.7 (16.7)	0.66
Body mass index (kg/m ²)	26.6 (4.4)	26.4 (4.7)	0.84
Medication, <i>n</i> (%)	28 (54.9)	28 (56.0)	0.69
Cholesterol-lowering drugs	8 (15.7)	11 (22.0)	0.46
Any antihypertensive drug	17 (33.3)	17 (34.0)	0.94
Beta-blockers	7 (14)	6 (12)	0.80
Angiotensin-converting-enzyme inhibitors	8 (16)	6 (12)	0.59
Angiotensin receptor blockers	1 (2)	7 (14)	0.03
Calcium channel blockers	10 (20)	6 (12)	0.30
Diuretics	8 (16)	7 (14)	0.81
Calcium and vitamin D intake			
Use of vitamin-D supplements, <i>n</i> (%)	35 (68.6)	28 (56.0)	0.31
Vitamin-D intake from supplement (μ g/day)	7.5 (0.0; 20.0)	5.0 (0.0; 24)	0.54
User of calcium supplements, <i>n</i> (%)	23 (45.1)	13 (26.0)	0.09
Dietary calcium intake (mg/day)	1000 (800; 1300)	1000 (750; 1250)	0.74
Total calcium intake (mg/day [†])	1250 (970; 1750)	1050 (763; 1575)	0.28

[†]Intake from diet and supplements.

supplements (23). We measured height and body weight in light indoor clothing using the same scale (Seca, Sa-med, Kvistgaard, Denmark).

Biochemistry

Blood samples were collected in the morning after an overnight fast and following 1 h of bed rest. In addition, participants collected a 24-h urine sample. Blood samples were analyzed immediately or stored at -80°C until analysis. General biochemical parameters were analyzed by standard laboratory methods using Roche/Hitachi Cobas c-systems (Cobas c 501). Plasma total calcium (P-Ca) was corrected for individual variations in plasma albumin (24).

Plasma 25-hydroxyvitamin D (25OHD) was analyzed by isotope dilution liquid chromatography-tandem mass spectrometry (LC-MS/MC) (25). This method quantifies both 25OHD₂ and 25OHD₃. Calibrators traceable to NIST SRM 972 (Chromsystems, München, Germany) were used. Coefficient of variance (CV) values (%) for 25OHD₃ were 6.4 and 9.1% at levels of 66.5 and 21.1 nmol/L, and for 25OHD₂, the CV values were 8.8 and 9.4% at levels of 41.2 and 25.3 nmol/L respectively. We measured plasma-intact PTH by a second-generation assay using Cobas e601 immunoassay analyzers (Roche Diagnostics, Basel, Switzerland). The lower limit of detection of the assay is

0.127 pmol/L, with a CV of 3.3 and 2.7% at PTH levels of 3.7 and 26.6 pmol/L respectively.

Blood samples for measurements of vasoactive hormones were centrifuged for 10 min at 2200g and 4°C . Plasma was separated from blood cells and kept frozen until assayed. Plasma renin concentration was determined using an immunoradiometric assay from CIS Bio International, Gif-Sur-Yvette Cedex, France. Minimal detection level was 1 pg/mL. The CV values were 0.9–3.6% (intra-assay) and 3.7–5.0% (inter-assay) in the range of 4–263 pg/mL. Plasma aldosterone was determined by RIA using a kit from Demeditec Diagnostics GmbH, Kiel, Germany. Minimal detection level was 25 pmol/L. The CV values were 9.0% (interassay) and 8.5% (intra-assay). Vasopressin (AVP) were extracted from plasma with C₁₈ Sep-Pak (Water Associates, Milford, MA, USA), and subsequently determined by radioimmunoassay (26, 27). The antibody against AVP was a gift from Prof Jacques Dürr, Miami, FL, USA. Minimal detection level was 0.2 pmol/L. The CV values were 13% (interassay) and 9% (intra-assay).

Urine samples were kept frozen at -20°C until assayed. Urine aquaporin-2 (AQP2) was measured by radioimmunoassay as described previously (28, 29). Antibodies were raised in rabbits to a synthetic peptide corresponding to the 15 COOH-terminal amino acids in human AQP2 to which was added an NH₂-terminal

cysteine for conjugation and affinity purification. Minimal detection level was 34 pg/tube. The CV values were 11.7% (interassay) and 5.9% (intra-assay). The urinary excretion of epithelial sodium channels (ENaCs) was measured by radioimmunoassay as described previously (30). Antibodies were raised against the synthetic ENaC peptide in rabbits and affinity purified as described previously (31). Minimal detection level was 48 pg/tube. The CV values were 14% (interassay) and 6.7% (intra-assay).

Genetics

FHH patients were characterized by molecular genetic analysis of the *CASR* gene as described previously (4, 32). Sequences were aligned to GenBank reference sequence NM_000388.2. Findings were confirmed in a second analysis on separately drawn blood. Identified variants of the *CASR* within the group of FHH patients have been reported previously (22).

Blood pressure

Twenty-four-hour ambulatory BP (ABP) monitoring was performed using Spacelabs 90217 (Spacelabs Healthcare, Issaquah, Washington, USA). The measurements were scheduled at 20-min intervals during day and night. Day and night periods were defined based on subjects' diaries of awake and sleeping hours. During the recordings, the subjects were asked to attend their usual daily schedule, refraining from unusual activities and from excessive physical exercise. They were also instructed to keep their arm still at the time of each automated measurement, according to usual procedures.

Arterial stiffness

Arterial stiffness and central hemodynamics were assessed noninvasively with the SphygmoCor device (AtCor Medical, Sydney, Australia). Arterial stiffness was assessed by carotid-femoral pulse wave velocity (PWV), the gold-standard method for noninvasive assessment of arterial stiffness (33). Hemodynamics at the level of the aorta was assessed by pulse wave analysis (PWA). Before measurements, study subjects had abstained from smoking and intake of tea, coffee or other caffeine-containing beverages for at least 3 h before the examinations. At least 2 h passed between food intake and the examinations. Measurements of pulse wave velocity (PWV) were performed using an applanation tonometer

(Millar, SPT-301B, Houston, Texas, USA) and SphygmoCor equipment and software, version 8.0 (AtCor Medical, Sydney, Australia). After a minimum of 5 min of rest in the supine position, three office blood pressures were measured at the right arm with an appropriately sized cuff using a Riester Champion N automatic blood pressure monitor (Riester GmbH, Jungingen, Germany). The mean of three systolic and diastolic BP was used for calibration of the SphygmoCor for the PWV and PWA measurements. The carotid-femoral PWV was determined by sequential ECG referenced recordings of the pulse wave at the carotid and the femoral artery by the tonometer. The transit time was determined by the intersecting tangent algorithm method and the path length calculated by subtracting the distance between the carotid artery measurement site and sternal notch from the distance between the femoral artery site and the sternal notch (33), all measured directly by a tape measure. The mean of two PWV measurements was calculated for each patient. The peripheral pulse wave was assessed by applanation tonometry at the right radial artery, and the central pulse waves were derived by the transfer function of the SphygmoCor device. Only values with an operator index >80 were accepted for analysis.

Statistical analysis

We assessed differences between groups using χ^2 test for categorical variables and a two-sample *t*-test or Mann–Whitney *U*-test for continuous variables, as appropriate after testing for normal distributions. Correlations between variables were tested by bivariate correlation analysis (Pearson's or Spearman's correlation coefficients, as appropriate). In our primary analyses, we included all studied subjects. As some of the participants were on treatment with antihypertensive and cholesterol-lowering drugs, we also performed analyses including only untreated participants. Furthermore, males and females were compared separately. Results are reported as mean $\pm$ s.d. or as median with interquartile range (25 and 75% percentiles), as appropriate according to the distribution of data. Before initiating the study, we calculated that 45 participants were needed in each group in order to be able to detect a 9 mmHg difference in systolic blood pressure between groups, assuming a mean (s.d.) blood pressure of 130 (15) mmHg (80% power, $\alpha=0.05$). *Post hoc*, we found that 50 participants in each groups provided the study with a 92% statistical power to detect a between-group difference of 1.0 m/s in PWV, which was considered as clinically relevant, as previous studies have shown that the risk of total and cardiovascular risk increases with 15% for

every increase of 1 m/s in PWV (34). A two-sided *P*-value <0.05 was considered statistically significant. Statistical analyses were performed using the Statistical Package for Social Sciences (SPSS 21.0) for Windows (IBM).

Results

Characteristics of the FHH patients and their matched controls are shown in Table 1. Our participants had a mean age of 56 years. 69% were females. The groups did not differ in height, weight, BMI or total intake of calcium from diet and supplements (Table 1). A similar number of patients and controls were on treatment with cholesterol-lowering drugs and antihypertensive drugs (AHD). Table 1 also shows types of AHDs which in general did not differ between groups, except that patients in the FHH group were more frequently on treatment with an angiotensin receptor blocker than the controls (14% vs 2%, *P*<0.03). A similar number of

patients and controls were on treatment with one, two or three AHD (Table 1).

Biochemical variables

Compared with controls, patients with FHH had significantly higher plasma levels of calcium, magnesium and PTH, whereas plasma phosphate levels were lower (Table 2). Plasma 25OHD levels did not differ between groups. Plasma osmolality was higher in the FHH-group, which was also found if analysis was restricted to those not on treatment with AHD (288 ± 3 vs 290 ± 3 mmol/kg, *P*=0.03). Within the entire group of participants, urinary volume and osmolality (U-osm) did not differ between groups (Table 2). However, after excluding those taking AHD, FHH patients had a lower 24-h urinary volume (1802 ± 529 vs 2115 ± 55 mL, *P*=0.03) with a higher U-osm (518.6 ± 208.8 vs 421.5 ± 144.1 mmol/kg, *P*=0.04) compared with the controls. Other biochemical indices

Table 2 Biochemistry in familial hypocalciuric hypercalcaemia (FHH) patients and matched controls. Data is presented as mean $\pm$ s.d. or as median with interquartile (25; 75 percentiles) range.

	Reference interval	Control (n=51)	FHH (n=50)	P-value
Plasma				
Albumin (g/L)	34–48	37 $\pm$ 3	38 $\pm$ 2	0.11
Calcium, ionized (mmol/L)	1.18–1.32	1.24 $\pm$ 0.03	1.45 $\pm$ 0.12	<0.001
Calcium, total (mmol/L)	2.20–2.55	2.33 $\pm$ 0.09	2.69 $\pm$ 0.20	<0.001
Calcium, albumin adj (mmol/L)	2.18–2.52	2.50 $\pm$ 0.10	2.84 $\pm$ 0.20	<0.001
Phosphate (mmol/L)	0.71–1.53	1.03 $\pm$ 0.16	0.87 $\pm$ 0.15	<0.001
PTH (pmol/L)	1.6–6.9	3.9 (3.2; 4.8)	4.7 (3.7; 6.4)	0.01
25OHD (nmol/L)	50–150	52 $\pm$ 25	51.5 $\pm$ 21.4	0.92
Creatinine (μ mol/L)	45–105	69 $\pm$ 15	69 $\pm$ 13	0.93
Creatinine clearance (mL/min)	>90	123 $\pm$ 39	117 $\pm$ 31	0.44
Osmolality (mmol/kg)	280–300	289 $\pm$ 4	291 $\pm$ 3	<0.01
Potassium (mmol/L)	3.5–4.6	3.9 $\pm$ 0.3	4.0 $\pm$ 0.3	0.25
Sodium (mmol/L)	137–145	140 $\pm$ 2	140 $\pm$ 2	0.46
Total CO ₂ (mmol/L)	23–37	26 $\pm$ 2	27 $\pm$ 2	0.05
Total cholesterol (mmol/L)	<5	5.0 $\pm$ 1.0	5.0 $\pm$ 0.9	0.90
Total cholesterol* (mmol/L)	<5	5.1 $\pm$ 0.9	5.2 $\pm$ 0.9	0.80
HDL cholesterol (mmol/L)	>1.0	1.7 $\pm$ 0.4	1.5 $\pm$ 0.5	0.11
HDL cholesterol* (mmol/L)	>1.0	1.7 $\pm$ 0.4	1.5 $\pm$ 0.4	0.04
LDL cholesterol (mmol/L)	<3	2.8 $\pm$ 0.9	2.9 $\pm$ 0.9	0.79
LDL cholesterol* (mmol/L)	<3	3.0 $\pm$ 0.9	3.1 $\pm$ 0.8	0.38
Triglycerides (mmol/L)	<2	1.2 $\pm$ 0.6	1.3 $\pm$ 0.7	0.25
Triglycerides* (mmol/L)	<2	1.3 $\pm$ 0.7	1.1 $\pm$ 0.6	0.20
Urine				
Volume (mL/24 h)		2012 $\pm$ 562	1955 $\pm$ 623	0.64
Calcium (mmol/24 h)	2.0–9.0	3.6 (2.4; 5.6)	2.9 (1.9; 5.1)	0.12
Creatinine (mmol/24 h)	8–22	12 $\pm$ 4	12 $\pm$ 4	0.66
Osmolality (mmol/kg)	300–900	438 $\pm$ 153	475 $\pm$ 201	0.32
Sodium (mmol/24 h)	50–150	145 $\pm$ 63	159 $\pm$ 52	0.25
Potassium (mmol/24 h)	30–130	71 $\pm$ 25	68 $\pm$ 23	0.65

*After excluding participants receiving cholesterol-lowering drugs.

Table 3 Vasoactive biochemical agents in familial hypocalciuric hypercalcaemia (FHH) patients and matched controls. Data is presented as mean $\pm$ s.d. or median with interquartile (25; 75 percentiles) range.

	All			Male			Female		
	Control (n=51)	FHH (n=50)	P value	Control (n=16)	FHH (n=16)	P value	Control (n=35)	FHH (n=34)	P value
U-ENaC (pg/mL)	340 $\pm$ 153	426 $\pm$ 217	0.03	395 $\pm$ 164	469 $\pm$ 271	0.36	315 $\pm$ 143	407 $\pm$ 191	0.03
U-aquaporin (ng/mL)	0.85 (0.59; 1.13)	0.74 (0.62; 1.06)	0.76	1.1 (0.89; 1.28)	0.93 (0.64; 1.46)	0.65	0.75 (0.56; 0.97)	0.71 (0.61; 1.00)	0.79
S-aldosterone (pmol/L)	94 (53; 165)	87 (54; 140)	0.65	94 (57; 150)	107 (59; 148)	>0.99	93 (50; 170)	81 (53; 140)	0.62
P-renin (pg/mL)	4.2 (2.6; 8.0)	5 (3.4; 7.7)	0.49	5.7 (3.5; 9.2)	5.1 (3.9; 8.8)	0.90	3.9 (2.5; 6.2)	5.0 (3.0; 7.5)	0.34
P-vasopressin (pg/mL)	0.3 (0.1; 0.6)	0.5 (0.2; 0.9)	<0.01	0.5 (0.3; 1)	0.9 (0.3; 1.2)	0.18	0.2 (0.1; 0.4)	0.4 (0.2; 0.7)	0.01

shown in [Table 2](#) did not change after excluding those who received AHD (data not shown).

Plasma lipids and cholesterol status did not differ between groups. However, after exclusion of participants on treatment with cholesterol-lowering drugs, HDL cholesterol was lower in FHH patients ([Table 2](#)). Stratification by sex did not affect indices shown in [Table 2](#) (data not shown).

Markers of volume regulation

[Table 3](#) shows measured vasoactive biochemical markers. In the entire group of participants as well as within the group not on treatment with AHD, FHH patients had significantly higher plasma levels of AVP and a higher renal excretion of ENaC than controls, whereas urinary AQP2 and plasma levels of aldosterone and renin did not differ between groups.

After stratification by sex, AVP levels and renal ENaC excretion were higher in women but not in men ([Table 3](#)), which was also found if analyses were restricted to participants not on treatment with AHD (data not shown). Within the group of participants not on treatment with AHD, urinary ENaC excretion correlated positively with urinary AQP2 ($r=0.61$, $P<0.01$), urinary osmolality ($r=0.41$, $P<0.01$), plasma osmolality ($r=0.30$, $P=0.02$), ionized calcium levels ($r=0.36$, $P<0.01$) and PTH ($r=0.31$, $P=0.02$), but not with plasma levels of aldosterone, renin or AVP.

Blood pressure

Office BP and 24-h ABP did not differ between FHH and controls ([Table 4](#)). However, after stratification by sex, 24-h averages as well as day- and night-time systolic-, diastolic- and mean arterial (MAP) ABP was significantly lower in FHH females compared with controls ([Table 4](#)). If patients on treatment with AHD were excluded, the direction of the differences remained the same even though only diastolic BP at nighttime was statistically significantly lower in FHH females (61 ± 5 vs 66 ± 9 mmHg, $P<0.05$) ([Supplementary Table 1](#), see section on [supplementary data](#) given at the end of this article).

In FHH males, a significantly higher diastolic BP and MAP at nighttime were observed. This difference remained significant after exclusion of participants on treatment with AHD (nighttime diastolic BP 70 ± 4 vs 63 ± 5 mmHg, $P=0.01$ and MAP 84 ± 5 vs 78 ± 7 mmHg, $P<0.05$).

Table 4 Office blood pressure and 24-h ambulatory blood pressure (mmHg) and heart rate (per min) in patients with familial hypocalciuric hypercalcaemia (FHH) and matched controls. Data is presented as mean $\pm$ s.d. for all participants and also stratified by sex..

	All			Males			Females		
	Controls (n=36)	FHH (n=38)	P value	Controls (n=13)	FHH (n=13)	P value	Controls (n=36)	FHH (n=25)	P value
Office blood pressure									
Systolic	129 $\pm$ 14	129 $\pm$ 17	0.90	131 $\pm$ 11	136 $\pm$ 16	0.39	128 $\pm$ 16	126 $\pm$ 17	0.61
Diastolic	79 $\pm$ 10	79 $\pm$ 12	0.73	81 $\pm$ 8	89 $\pm$ 10	0.05	79 $\pm$ 11	75 $\pm$ 11	0.19
MAP	97 $\pm$ 11	97 $\pm$ 13	0.94	98 $\pm$ 9	105 $\pm$ 11	0.10	96 $\pm$ 12	94 $\pm$ 12	0.40
24-h measurement									
Systolic	123 $\pm$ 13	118 $\pm$ 12	0.10	123 $\pm$ 11	126 $\pm$ 13	0.60	123 $\pm$ 15	114 $\pm$ 10	0.02
Diastolic	75 $\pm$ 7	72 $\pm$ 9	0.15	75 $\pm$ 6	79 $\pm$ 9	0.15	76 $\pm$ 8	69 $\pm$ 7	<0.01
MAP	91 $\pm$ 8	88 $\pm$ 9	0.20	90 $\pm$ 7	94 $\pm$ 8	0.20	91 $\pm$ 9	85 $\pm$ 7	0.02
Heart rate	69 $\pm$ 9	69 $\pm$ 7	0.97	68 $\pm$ 9	71 $\pm$ 9	0.30	70 $\pm$ 9	68 $\pm$ 6	0.39
Day									
Systolic	128 $\pm$ 14	124 $\pm$ 13	0.19	129 $\pm$ 12	131 $\pm$ 15	0.67	128 $\pm$ 15	120 $\pm$ 10	0.05
Diastolic	79 $\pm$ 8	76 $\pm$ 9	0.23	79 $\pm$ 8	83 $\pm$ 9	0.24	79 $\pm$ 8	73 $\pm$ 7	0.02
MAP	95 $\pm$ 9	93 $\pm$ 9	0.23	95 $\pm$ 8	98 $\pm$ 9	0.31	95 $\pm$ 9	90 $\pm$ 7	0.02
Heart rate	72 $\pm$ 10	73 $\pm$ 8	0.87	70 $\pm$ 10	74 $\pm$ 10	0.31	73 $\pm$ 10	72 $\pm$ 6	0.50
Night									
Systolic	110 $\pm$ 13	106 $\pm$ 10	0.11	108 $\pm$ 10	112 $\pm$ 9	0.28	111 $\pm$ 15	102 $\pm$ 10	0.01
Diastolic	65 $\pm$ 8	63 $\pm$ 7	0.18	64 $\pm$ 5	69 $\pm$ 6	0.02	66 $\pm$ 9	60 $\pm$ 5	<0.01
MAP	81 $\pm$ 9	79 $\pm$ 8	0.28	79 $\pm$ 6	84 $\pm$ 6	0.04	82 $\pm$ 10	76 $\pm$ 7	0.02
Heart rate	62 $\pm$ 8	61 $\pm$ 7	0.63	60 $\pm$ 8	63 $\pm$ 8	0.26	63 $\pm$ 8	60 $\pm$ 6	0.11

MAP, mean arterial blood pressure.

In the FHH group, but not in the control group, we found significantly higher systolic and diastolic (24-h, day and night) BP in males vs females (Table 4). This was not changed by exclusion of patients on treatment with AHD ($P < 0.02$ for all comparisons).

Pulse wave analyses and pulse wave velocity

Overall, indices of arterial stiffness in terms of pulse wave velocity (PWV) and pulse wave analyses (PWA) did

not differ between FHH patients and controls (Table 5). Within the male group, FHH patients had a tendency for higher peripheral diastolic pressure (88.5 ± 10.2 vs 80.5 ± 7.5 , $P = 0.05$), central diastolic pressure (89.6 ± 10.2 vs 81.7 ± 7.5 , $P = 0.06$) and pulse wave velocity (7.6 ± 0.6 vs 6.9 ± 1.0 , $P = 0.09$). After exclusion of those on treatment with antihypertensive drugs, the tendency was no longer present ($P > 0.10$ for all analyses, Supplementary Table 1).

In females, after exclusion of participants on treatment with antihypertensive drugs, FHH patients compared with

Table 5 Pulse wave analysis and pulse wave velocity in patients with familial hypocalciuric hypercalcaemia (FHH) and their matched controls. Data is presented as mean $\pm$ s.d. for all participants and stratified by sex.

	All			Male			Female		
	Control (n=38)	FHH (n=39)	P value	Control (n=16)	FHH (n=16)	P value	Control (n=35)	FHH (n=34)	P value
Augmentation index (%)	23 $\pm$ 11	27 $\pm$ 11	0.13	16 $\pm$ 7	18 $\pm$ 12	0.69	26 $\pm$ 11	30 $\pm$ 9	0.12
Adjusted augmentation index*	19 $\pm$ 11	22 $\pm$ 9	0.16	11 $\pm$ 6	16 $\pm$ 10	0.21	22 $\pm$ 12	24 $\pm$ 8	0.35
Estimated aortic systolic BP (mmHg)	118 $\pm$ 14	119 $\pm$ 15	0.76	117 $\pm$ 11	124 $\pm$ 12	0.21	118 $\pm$ 15	117 $\pm$ 16	0.82
Estimated aortic diastolic BP (mmHg)	80 $\pm$ 10	80 $\pm$ 12	0.74	82 $\pm$ 8	90 $\pm$ 10	0.06	80 $\pm$ 11	76 $\pm$ 11	0.21
Estimated aortic mean pressure (mmHg)	97 $\pm$ 11	97 $\pm$ 13	0.94	98 $\pm$ 9	105 $\pm$ 11	0.10	96 $\pm$ 12	94 $\pm$ 12	0.40
Carotid-femoral pulse wave velocity (m/s)	7.5 $\pm$ 1.8	8.0 $\pm$ 1.9	0.32	6.9 $\pm$ 1	7.6 $\pm$ 0.6	0.09	7.8 $\pm$ 2	8.1 $\pm$ 2.1	0.58

*Adjusted for heart rate (Aix@HR75).

controls had a tendency for lower peripheral diastolic pressure (74 ± 10 vs 80 ± 10 mmHg, $P=0.08$) and central diastolic pressure (75 ± 10 vs 80 ± 10 mmHg, $P=0.09$).

In the FHH group, females had a significantly lower estimated aortic diastolic (but not systolic) BP compared with males (76 ± 11 vs 90 ± 10 mmHg, $P<0.01$), and this was not changed by exclusion of FHH patients on treatment with AHD ($P<0.01$), whereas measurements did not differ between sexes in the control group.

Discussion

To the best of our knowledge, this study is one of the first to investigate possible effects in humans of inactivating variants in the *CASR* on blood pressure and volume regulation. After exclusion of participants on therapy with AHD, our study showed higher plasma levels of AVP and a higher plasma and urinary osmolality, as well as a higher renal excretion of ENaC and a lower 24-h urinary volume in the FHH group compared with the controls. In addition, compared with their respective controls, males with FHH had a higher diastolic BP and MAP at nighttime, whereas FHH females had a lower DBP at nighttime. However, PWV and PWA did not differ between groups.

The *CASR* is considered to be primarily of importance to the regulation of calcium balance, but a number of studies have suggested that the receptor also may be involved in salt and water transport in the kidney. The *CASR* is localized to segments of the nephron that participate in urinary concentration. In principal cells of the collecting duct, *CASR* co-localizes with AQP2 in vesicles. Increased u-AQP2 reflects increased water transport from the tubular lumen to the intracellular space via the aquaporin2 water channels, located in the principal cells in the distal part of the nephron (28, 29, 35). Activation of the *CASR* causes a reduced AQP2 expression and AVP-stimulated insertion of AQP2 into the apical membrane (36). Consequently, activation of the *CASR* results in dilution of the urine and may through this mechanism reduce the risk of kidney stones in patients with hypercalcemia (37). Our findings agree to some extent with these observations as we found an increased urinary osmolality in FHH patients compared with controls after excluding participants on treatment with AHD. Accordingly, the inactivating variant of the *CASR* causes less-diluted urine. Apparently, patients with FHH are not at an increased risk of renal stones, which may be explained by the fact that inactivating variants increases tubular calcium reabsorption and calcium levels

in the tubular fluid are accordingly not increased (38). However, our study does not suggest an increased activity of AQP2 in FHH, as renal excretion of AQP2 did not differ between groups.

In accordance with our findings, previous studies have shown that individuals with inactivating variants of the *CASR* are able to concentrate their urine quite normally despite hypercalcemia (38). In a direct comparison between patients with FHH and PHPT, PHPT was associated with an impaired ability to concentrate urine compared with FHH (5). Similarly, nonparathyroid hypercalcemia is associated with a nephrogenic concentrating defect (39). Our findings support intact urinary concentration ability in FHH. Actually, urinary osmolality was increased in the FHH group after excluding the group of participants on treatment with antihypertensive drugs.

Our study showed an increased renal excretion of the epithelial sodium channel (ENaC) in FHH compared with controls. ENaC is responsible for the reabsorption of sodium through the apical membrane of the principal cells in the distal part of the nephron and plays a key role in controlling sodium balance, extracellular fluid volume and blood pressure. Although aldosterone is considered as the principal regulator of the ENaC, AVP has also been shown to activate ENaC. Similar to AQP2, the sodium transport via the epithelial sodium channels is supposed to be reflected by the level of urinary excretion of a protein fraction from the channels (35). In a recent study, activation of ENaC by AVP was shown to facilitate urine concentration and dilution of plasma (40). This may be of importance in FHH, as AQP2 levels did not differ between groups despite increased AVP levels with no differences between groups in plasma levels of sodium and aldosterone. However, we found no significant correlations between urinary ENaC and plasma levels of AVP. On the other hand, we found a significant positive correlation between ionized calcium levels and renal excretion of ENaC. Further studies are needed to determine whether the *CASR* is directly involved in the regulation of ENaC.

Our findings suggest diurnal differences in BP regulation in patients with FHH, with opposite effects in males and females. In females, FHH was associated with a lower 24-h, day and night BP compared with controls, whereas we found an opposite association in males, in whom FHH was associated with higher diastolic BP at night. In both the 24-h ABP measurements and the central diastolic BP measurements, we observed a significant difference between sexes in the FHH group with higher values in the FHH males. Accordingly, it seems that the

consequences of inactivating variants in the *CASR* may differ between males and females. We have previously reported similar gender differences on bone effects of FHH (41). Further studies are needed to elucidate such potential difference. The 24-h ABP data could indicate a tendency to lower cardiovascular risk in female FHH patients, yet the cross-sectional study design preclude conclusions in this regard.

The major strength of our study is the design with a balanced matching of FHH patients with controls and the fact that controls were randomly recruited from the general background population. All investigations were carried out by trained personnel. The fact that some of our participants were on treatment with drugs affecting measured indices contributes with some uncertainties on the physiological effects studied. We cannot exclude that further differences between groups had been found if all participants were investigated without being on drug treatment. However, we did not find it ethically acceptable to stop therapy in FHH patients and controls to investigate such effects. Instead, we stratified analyses according to treatment. In general, exclusion of participants on treatment with AHD did not change results to any major degree. Analyses of the *CASR* have revealed more than 100 different variants which are associated with FHH, and the degree of hypercalcemia in affected individuals varies between different variants (4, 42). We cannot exclude that the physiological effects of variants in the *CASR* in those on treatment with AHD differs from the effects of the variants in those not on antihypertensive treatment. However, the directions of the observed differences between men and women were the same in the subgroups not receiving AHD. Genetic analyses were not performed in the control group. In addition to the variants causing FHH, a number of polymorphisms in the carboxyl terminus have also been identified which, in general, are considered to be nonpathologic. However, some of the polymorphism have been associated with slightly increased calcium levels (43, 44). Our study was not designed to study whether such non-FHH polymorphisms are of importance to cardiovascular health. Further studies in larger cohorts are needed to test such hypothesis.

Conclusion

In conclusion, our results do not suggest major overall adverse effects on cardiovascular health of FHH. No major differences were found in central blood pressure

or measures of arterial stiffness. However, our data suggest differential effects on volume and BP regulation between men and women with an inactivating variant in the *CASR*. The minor decrease in diastolic BP FHH-female could protect against cardiovascular diseases. Accordingly, individuals with an inactivating variant in the gene encoding the *CASR* do not seem to be at any major increased risk of cardiovascular diseases.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/EJE-16-0369>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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References

- 1 Brown EM, Gamba G, Riccardi D, Lombardi M, Butters R, Kifor O, Sun A, Hediger MA, Lytton J & Hebert SC. Cloning and characterization of an extracellular Ca²⁺-sensing receptor from bovine parathyroid. *Nature* 1993 **366** 575–580. (doi:10.1038/366575a0)
- 2 Ward BK, Magno AL, Walsh JP & Ratajczak T. The role of the calcium-sensing receptor in human disease. *Clinical Biochemistry* 2012 **45** 943–953. (doi:10.1016/j.clinbiochem.2012.03.034)
- 3 Brown EM & MacLeod RJ. Extracellular calcium sensing and extracellular calcium signaling. *Physiological Reviews* 2001 **81** 239–297.
- 4 Nissen PH, Christensen SE, Heickendorff L, Brixen K & Mosekilde L. Molecular genetic analysis of the calcium sensing receptor gene in patients clinically suspected to have familial hypocalciuric hypercalcemia: phenotypic variation and mutation spectrum in a Danish population. *Journal of Clinical Endocrinology & Metabolism* 2007 **92** 4373–4379. (doi:10.1210/jc.2007-0322)
- 5 Marx SJ, Attie MF, Stock JL, Spiegel AM & Levine MA. Maximal urine-concentrating ability: familial hypocalciuric hypercalcemia versus typical primary hyperparathyroidism. *Journal of Clinical Endocrinology & Metabolism* 1981 **52** 736–740. (doi:10.1210/jcem-52-4-736)
- 6 Christensen SE, Nissen PH, Vestergaard P & Mosekilde L. Familial hypocalciuric hypercalcaemia: a review. *Current Opinion in Endocrinology & Diabetes and Obesity* 2011 **18** 359–370. (doi:10.1097/MED.0b013e32834c3c7c)
- 7 Tyler Miller R. Control of renal calcium, phosphate, electrolyte, and water excretion by the calcium-sensing receptor. *Best Practice & Research Clinical Endocrinology & Metabolism* 2013 **27** 345–358. (doi:10.1016/j.beem.2013.04.009)

- 8 Luft FC. Putative mechanism of blood pressure reduction induced by increases in dietary calcium intake. *American Journal of Hypertension* 1990 **3** 156S–160S. (doi:10.1093/ajh/3.8.156)
- 9 McCarron DA & Reusser ME. Finding consensus in the dietary calcium-blood pressure debate. *Journal of the American College of Nutrition* 1999 **18** 398S–405S. (doi:10.1080/07315724.1999.10718904)
- 10 Odenwald T, Nakagawa K, Hadtstein C, Roesch F, Gohlke P, Ritz E, Schaefer F & Schmitt CP. Acute blood pressure effects and chronic hypotensive action of calcimimetics in uremic rats. *Journal of the American Society of Nephrology* 2006 **17** 655–662. (doi:10.1681/ASN.2005090914)
- 11 Rybczynska A, Boblewski K, Lehmann A, Orlewska C, Foks H, Drewnowska K & Hoppe A. Calcimimetic NPS R-568 induces hypotensive effect in spontaneously hypertensive rats. *American Journal of Hypertension* 2005 **18** 364–371. (doi:10.1016/j.amjhyper.2004.10.019)
- 12 Zitt E, Woess E, Mayer G & Lhotta K. Effect of cinacalcet on renal electrolyte handling and systemic arterial blood pressure in kidney transplant patients with persistent hyperparathyroidism. *Transplantation* 2011 **92** 883–889. (doi:10.1097/TP.0b013e31822d87e8)
- 13 Ziegelstein RC, Xiong Y, He C & Hu Q. Expression of a functional extracellular calcium-sensing receptor in human aortic endothelial cells. *Biochemical and Biophysical Research Communications* 2006 **342** 153–163. (doi:10.1016/j.bbrc.2006.01.135)
- 14 Smajilovic S, Hansen JL, Christoffersen TEH, Lewin E, Sheikh SP, Terwilliger EF, Brown EM, Haunso S & Tfelt-Hansen J. Extracellular calcium sensing in rat aortic vascular smooth muscle cells. *Biochemical and Biophysical Research Communications* 2006 **348** 1215–1223. (doi:10.1016/j.bbrc.2006.07.192)
- 15 Alam MU, Kirton JP, Wilkinson FL, Towers E, Sinha S, Rouhi M, Vizard TN, Sage AP, Martin D, Ward DT *et al*. Calcification is associated with loss of functional calcium-sensing receptor in vascular smooth muscle cells. *Cardiovascular Research* 2009 **81** 260–268. (doi:10.1093/cvr/cvn279)
- 16 März W, Seelhorst U, Wellnitz B, Tiran B, Obermayer-Pietsch B, Renner W, Boehm BO, Ritz E & Hoffmann MM. Alanine to serine polymorphism at position 986 of the calcium-sensing receptor associated with coronary heart disease, myocardial infarction, all-cause, and cardiovascular mortality. *Journal of Clinical Endocrinology & Metabolism* 2007 **92** 2363–2369. (doi:10.1210/jc.2006-0071)
- 17 Babinsky VN, Hannan FM, Youhanna SC, Maréchal C, Jadoul M, Devuyt O & Thakker RV. Association studies of calcium-sensing receptor (CaSR) polymorphisms with serum concentrations of glucose and phosphate, and vascular calcification in renal transplant recipients. *PLoS ONE* 2015 **10** e0119459. (doi:10.1371/journal.pone.0119459)
- 18 Smajilovic S, Yano S, Jabbari R & Tfelt-Hansen J. The calcium-sensing receptor and calcimimetics in blood pressure modulation. *British Journal of Pharmacology* 2011 **164** 884–893. (doi:10.1111/bph.2011.164.issue-3)
- 19 Smajilovic S & Tfelt-Hansen J. Novel role of the calcium-sensing receptor in blood pressure modulation. *Hypertension* 2008 **52** 994–1000. (doi:10.1161/HYPERTENSIONAHA.108.117689)
- 20 Schillaci G, Pucci G, Pirro M, Monacelli M, Scarponi AM, Manfredelli MR, Rondelli F, Avenia N & Mannarino E. Large-artery stiffness: a reversible marker of cardiovascular risk in primary hyperparathyroidism. *Atherosclerosis* 2011 **218** 96–101. (doi:10.1016/j.atherosclerosis.2011.05.010)
- 21 Walker MD, Rubin M & Silverberg SJ. Non-traditional manifestations of primary hyperparathyroidism. *Journal of Clinical Densitometry* 2013 **16** 40–47. (doi:10.1016/j.jocd.2012.11.008)
- 22 Jakobsen NF, Rolighed L, Nissen PH, Mosekilde L & Rejnmark L. Muscle function and quality of life is not impaired in familial hypocalciuric hypercalcemia (FHH): a cross-sectional study on physiological effects of inactivating variants in the calcium sensing receptor gene (CaSR). *European Journal of Endocrinology* 2013 **169** 349–357. (doi:10.1530/EJE-13-0224)
- 23 Hermann AP, Thomsen J, Vestergaard P, Mosekilde L & Charles P. Assessment of calcium intake. A quick method compared to a 7 days food diary. *Calcified Tissue International* 1999 **64** (Supplement 1) S82.
- 24 Rejnmark L, Vestergaard P, Heickendorff L & Mosekilde L. Determinants of plasma PTH and their implication for defining a reference interval. *Clinical Endocrinology* 2011 **74** 37–43. (doi:10.1111/cen.2010.74.issue-1)
- 25 Hojskov CS, Heickendorff L & Møller HJ. High-throughput liquid-liquid extraction and LCMSMS assay for determination of circulating 25(OH) vitamin D3 and D2 in the routine clinical laboratory. *Clinica Chimica Acta* 2010 **411** 114–116. (doi:10.1016/j.cca.2009.10.010)
- 26 Pedersen EB, Danielsen H & Spencer ES. Effect of indapamide on renal plasma flow, glomerular filtration rate and arginine vasopressin in plasma in essential hypertension. *European Journal of Clinical Pharmacology* 1984 **26** 543–547. (doi:10.1007/BF00543482)
- 27 Pedersen EB, Eiskjær H, Madsen B, Danielsen H, Egeblad M & Nielsen CB. Effect of captopril on renal extraction of renin, angiotensin II, atrial natriuretic peptide and vasopressin, and renal vein renin ratio in patients with arterial hypertension and unilateral renal artery disease. *Nephrology Dialysis Transplantation* 1993 **8** 1064–1070.
- 28 Pedersen RS, Bentzen H, Bech JN & Pedersen EB. Effect of water deprivation and hypertonic saline infusion on urinary AQP2 excretion in healthy humans. *American Journal of Physiology: Renal Physiology* 2001 **280** F860–F867.
- 29 Graffe CC, Bech JN & Pedersen EB. Effect of high and low sodium intake on urinary aquaporin-2 excretion in healthy humans. *American Journal of Physiology: Renal Physiology* 2012 **302** F264–F275. (doi:10.1152/ajprenal.00442.2010)
- 30 Mathiesen SK, Larsen T, Lauridsen TG, Vase H, Gjørup PH, Nykjaer KM, Nielsen S & Pedersen EB. Effect of amiloride and spironolactone on renal tubular function, ambulatory blood pressure, and pulse wave velocity in healthy participants in a double-blinded, randomized, placebo-controlled, crossover trial. *Clinical and Experimental Hypertension* 2012 **34** 588–600. (doi:10.3109/10641963.2012.681730)
- 31 Hager H, Kwon TH, Vinnikova AK, Masilamani S, Brooks HL, Frøkjær J, Knepper MA & Nielsen S. Immunocytochemical and immunoelectron microscopic localization of alpha-, beta-, and gamma-ENaC in rat kidney. *American Journal of Physiology: Renal Physiology* 2001 **280** F1093–F1106.
- 32 Nissen PH, Christensen SE, Ladefoged SA, Brixen K, Heickendorff L & Mosekilde L. Identification of rare and frequent variants of the CASR gene by high-resolution melting. *Clinica Chimica Acta* 2012 **413** 605–611. (doi:10.1016/j.cca.2011.12.004)
- 33 Van Bortel LM, Laurent S, Boutouyrie P, Chowienczyk P, Cruickshank JK, De BT, Filipovsky J, Huybrechts S, Mattace-Raso FU, Protogerou AD *et al*. Expert consensus document on the measurement of aortic stiffness in daily practice using carotid-femoral pulse wave velocity. *Journal of Hypertension* 2012 **30** 445–448. (doi:10.1097/hjh.0b013e32834fa8b0)
- 34 Vlachopoulos C, Aznaouridis K & Stefanadis C. Prediction of cardiovascular events and all-cause mortality with arterial stiffness: a systematic review and meta-analysis. *Journal of the American College of Cardiology* 2010 **55** 1318–1327.
- 35 Lauridsen TG, Vase H, Bech JN, Nielsen S & Pedersen EB. Direct effect of methylprednisolone on renal sodium and water transport via the principal cells in the kidney. *European Journal of Endocrinology* 2010 **162** 961–969. (doi:10.1530/eje-10-0030)
- 36 Sands JM, Naruse M, Baum M, Jo I, Hebert SC, Brown EM & Harris HW. Apical extracellular calcium/polyvalent cation-sensing receptor regulates vasopressin-elicited water permeability in rat kidney inner medullary collecting duct. *Journal of Clinical Investigation* 1997 **99** 1399–1405. (doi:10.1172/jci119299)

- 37 Procino G, Mastrofrancesco L, Tamma G, Lasorsa DR, Ranieri M, Stringini G, Emma F, Svelto M & Valenti G. Calcium-sensing receptor and aquaporin 2 interplay in hypercalciuria-associated renal concentrating defect in humans. An in vivo and in vitro study. *PLoS ONE* 2012 **7** e33145. (doi:10.1371/journal.pone.0033145)
- 38 Riccardi D & Brown EM. Physiology and pathophysiology of the calcium-sensing receptor in the kidney. *American Journal of Physiology: Renal Physiology* 2010 **298** F485–F499. (doi:10.1152/ajprenal.00608.2009)
- 39 Peterson LN. Vitamin D-induced chronic hypercalcemia inhibits thick ascending limb NaCl reabsorption in vivo. *American Journal of Physiology* 1990 **259** F122–F129.
- 40 Mironova E, Chen Y, Pao AC, Roos KP, Kohan DE, Bugaj V & Stockand JD. Activation of ENaC by AVP contributes to the urinary concentrating mechanism and dilution of plasma. *American Journal of Physiology: Renal Physiology* 2015 **308** F237–F243. (doi:10.1152/ajprenal.00246.2014)
- 41 Jakobsen NF, Rolighed L, Moser E, Nissen PH, Mosekilde L & Rejnmark L. Increased trabecular volumetric bone mass density in familial hypocalciuric hypercalcemia (FHH) type 1: a cross-sectional study. *Calcified Tissue International* 2014 **95** 141–152. (doi:10.1007/s00223-014-9877-0)
- 42 Hannan FM, Nesbit MA, Zhang C, Cranston T, Curley AJ, Harding B, Fratter C, Rust N, Christie PT, Turner JJO *et al.* Identification of 70 calcium-sensing receptor mutations in hyper- and hypo-calcaemic patients: evidence for clustering of extracellular domain mutations at calcium-binding sites. *Human Molecular Genetics* 2012 **21** 2768–2778. (doi:10.1093/hmg/dds105)
- 43 Scillitani A, Guarnieri V, De Geronimo S, Muscarella LA, Battista C, D'Agruma L, Bertoldo F, Florio C, Minisola S, Hendy GN *et al.* Blood ionized calcium is associated with clustered polymorphisms in the carboxyl-terminal tail of the calcium-sensing receptor. *Journal of Clinical Endocrinology & Metabolism* 2004 **89** 5634–5638. (doi:10.1210/jc.2004-0129)
- 44 Hannan FM & Thakker RV. Calcium-sensing receptor (CaSR) mutations and disorders of calcium, electrolyte and water metabolism. *Best Practice & Research Clinical Endocrinology & Metabolism* 2013 **27** 359–371. (doi:10.1016/j.beem.2013.04.007)

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