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AUTHORS: Geddes, R F; Jepson, R E; Forcada, Y; Elliott, J; Syme, H M

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Original Article

Associations between single nucleotide polymorphisms in the calcium sensing receptor and chronic kidney disease-mineral and bone disorder in cats

R.F. Geddes [a,*], R.E.Jepson [a], Y. Forcada[a], J. Elliott [b] and H.M. Syme [a]

[a]*Department of Clinical Sciences and Services, Royal Veterinary College, Hawkshead Lane, North Mymms, Hertfordshire, AL9 7TA, UK*
[b]*Department of Comparative Biomedical Sciences, Royal Veterinary College, 4 Royal College Street, London, NW1 0TU, UK*

*Corresponding author. Tel: +44 1707 666 366
E-mail address: rgeddes@rvc.ac.uk (R.F. Geddes)

Highlights:

- Twelve polymorphisms have now been identified in the feline calcium sensing receptor.
- One polymorphism was associated with parathyroid hormone concentration at diagnosis of CKD in non-pedigree cats.
- No associations yet identified between identified polymorphisms and ionised calcium or phosphate in non-pedigree cats.

Abstract

Feline chronic kidney disease (CKD) is associated with high variability in severity of CKD-mineral and bone disorder (CKD-MBD). The calcium sensing receptor (CaSR) regulates circulating parathyroid hormone (PTH) and calcium concentrations. Single nucleotide polymorphisms (SNPs) in the CaSR are associated with severity of secondary renal hyperparathyroidism and total calcium concentrations in human patients receiving haemodialysis. The objective of this study was to explore associations between polymorphisms in the feline CaSR (fCaSR) and biochemical changes observed in CKD-MBD.

Client owned cats (≥ 9 years) were retrospectively included. SNP discovery was performed in 20 cats with azotaemic CKD and normal or dysregulated calcium concentrations. Non-pedigree cats (n=192) (125 with azotaemic CKD and 66 healthy), Persians (n=40) and Burmese (n=25) were genotyped for all identified SNPs using KASP. Biochemical parameters from the date of CKD diagnosis or from first visit to the clinic (healthy cats) were used. Associations between genotype and ionized calcium, total calcium, phosphate, PTH and FGF-23 were performed for non-pedigree cats using logistic regression.

Sequence alignment against the fCaSR sequence revealed eight novel exonic SNPs. KASP genotyping had high accuracy (99.6%) and a low failure rate (<6%) for all SNPs. Allele frequencies varied between breeds. In non-pedigree cats, one synonymous SNP CaSR:c.1269G>A was associated with logPTH concentration (adjusted for plasma creatinine concentration), with a recessive model having the best fit (G/G vs A/A-G/A, P=0.031).

Genetic variation in the fCaSR is unlikely to explain the majority of the variability in presence and severity of CKD-MBD in cats.

Keywords

CaSR, CKD-MBD, phosphate, PTH, FGF-23, KASP, Feline

Introduction

Decreased glomerular filtration rate in chronic kidney disease (CKD) leads to phosphate retention and disruption to calcium-phosphate homeostasis, which can ultimately lead to renal osteodystrophy. The term CKD-mineral and bone disorder (CKD-MBD) is now used to describe these abnormalities in humans (Moe et al., 2006). The biochemical changes involved in CKD-MBD have been documented in azotaemic cats, including hyperphosphataemia, hypocalcaemia, hyperparathyroidism (Barber and Elliott, 1998) and elevations in the phosphatonin fibroblast growth factor 23 (FGF-23) (Geddes et al., 2013). Additionally, pre-azotaemic cats have increased PTH and FGF-23 compared to cats that remain non-azotaemic (Finch, 2009; Finch et al., 2013). However, for as yet undetermined reasons, cats with similar degrees of CKD show marked individual variation in the presence and severity of CKD-MBD (Barber and Elliott, 1998; Geddes et al., 2013).

The calcium sensing receptor (CaSR), a G protein-coupled transmembrane receptor, is intricately involved in calcium homeostasis. The human CaSR has an extracellular domain of 612 amino acids, a 250 amino acid domain of 7 transmembrane helices and an intracellular carboxy-terminal tail of 216 amino acids. When activated by extracellular calcium the receptor couples with several G-proteins and regulates multiple signalling pathways. This receptor is expressed in a number of organs, but most abundantly in the parathyroid gland and the kidney. In the parathyroid gland, stimulation of the CaSR reduces PTH secretion, with a sigmoidal relationship between extracellular calcium concentration and PTH secretion; the calcium 'set-point' is defined as the concentration of calcium that results in suppression of PTH secretion to 50% of maximum (Brown, 1983). The CaSR also regulates PTH synthesis and cellular proliferation (Ho et al., 1995). In the kidney, the CaSR regulates calcium ion reabsorption by inhibiting both PTH-stimulated transcellular calcium reabsorption (Motoyama and Friedman,

2002) and paracellular calcium reabsorption that is independent of PTH (Loupy et al., 2012; Toka et al., 2012).

Single nucleotide polymorphisms (SNPs) in the human CaSR have been associated with serum ionized (He et al., 2012) and total calcium (He et al., 2012; Kapur et al., 2010; O'Seaghdha et al., 2010) concentrations in healthy people and associated with PTH (Eren et al., 2009; Yano et al., 2000) and total calcium (Eren et al., 2009) concentrations in CKD patients on haemodialysis. The feline CaSR (fCaSR) protein (RefSeq. NM_001164654.1) is 1081 amino acids, 3 amino acids longer than the human receptor (UniProtKB P41180.3), with the 3 additional amino acids occurring consecutively in the intracellular tail of the receptor. Four SNPs have previously been identified (Gal, 2010), however associations of these SNPs and variables involved in calcium-phosphate homeostasis have not been previously explored.

We hypothesised that SNPs in the fCaSR would be associated with severity of CKD-MBD and explored this hypothesis by developing a 95% reference interval for ionised serum calcium in older cats, performing further SNP discovery and subsequently genotyping cats with and without azotaemia to explore associations between genotype and the variables ionised calcium, total calcium, PTH, phosphate and FGF-23.

Materials and methods

Determination of a 95% reference interval for ionised serum calcium concentrations in older cats

Jugular venepuncture was performed during routine health screening of domestic shorthaired (DSH) and domestic longhaired (DLH) cats ≥9 years of age. Whole blood ionised calcium measurements were performed using a portable analyser (iSTAT, Abbott Laboratories). Healthy cats that were non-azotaemic (plasma creatinine concentration ≤177 µmol/L) and remained non-azotaemic for at least 6 months post sampling were used to establish the reference interval for ionised calcium. The distribution of the measurements was determined to be Gaussian by visual inspection of a histogram and the Shapiro-Wilk test. The results were assessed for outliers using the Dixon method (Dixon, 1953), and the reference interval was calculated parametrically (mean ± 2*SD).

Case selection

Cases were identified from records of two clinics for geriatric cats (all cats ≥9 years of age), the People's Dispensary for Sick Animals, Bow and the Beaumont Sainsbury Animal Hospital, Camden (from 1st January 2000 to 15th July 2013). Written consent was obtained from the cat's owner for residual blood samples to be used in research studies at the time of their first appointment at the geriatric cat clinics. The study was approved by the Royal Veterinary College Ethics and Welfare Committee (URN 2013 1258, 2nd December 2013). Cats with a sample of buffy coat-enriched packed cells previously stored at -80 °C were included. For SNP discovery, cats of any breed with azotaemic CKD (defined as plasma creatinine concentration >177 µmol/L and concurrent urine specific gravity <1.035 on the day of diagnosis) and subsequently started on a renal diet (Veterinary diet Renal, Royal Canin SAS, France) were identified. Records for each cat were searched for an ionised calcium measurement taken post-

CKD diagnosis. Ten cats with ionised hypercalcaemia and ten cats with low/normal ionised calcium concentrations were selected.

For the larger genotyping study, four groups of cats were selected: non-pedigree cats diagnosed with azotaemic CKD and with an ionised calcium measurement from the day of CKD diagnosis (cats being fed 'renal' diets were excluded unless they were positive control samples), healthy non-pedigree cats, Persian cats and Burmese cats. We included Persian and Burmese cats specifically following identification of possible breed-specific SNPs in the initial genotyping phase of the study.

SNP discovery

Genomic DNA was extracted from buffy coat enriched packed cells stored at -80°C using a commercially available kit (GenElute blood genomic DNA extraction kit, Sigma-Aldrich). A proteinase K digestion was performed for samples that had been stored without the addition of phosphate-buffered-saline and EDTA. DNA concentration was determined using spectrophotometry (Nanodrop 1000 Spectrophotometer, Thermo Scientific). PCR was performed with the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) using previously published primers (Gelain et al., 2006) to assess DNA quality. The coding sequence of fCASR was available on EnsEMBL genome browser (release 63) (ENSFCAT00000008718, GenBank Assembly ID GCA_000181335.2). Missing intronic sequence flanking exon 3 was identified using basic local alignment search tool (BLAST) and confirmed with sequencing. Previously published primers (Gal, 2010) and primers designed using Primer 3[1] were used to sequence all six exons in full (see Table 1). All primers were obtained from Sigma-Aldrich, UK.

[1] See: Primer3 v 0.4.0, available at http://bioinfo.ut.ee/primer3-0.4.0/primer3/

PCR was performed in 25 µL volumes: 13 µL of water, 5 µL of 5xHi-Spec Additive (Bioline), 2.5 µL of Immobuffer (Bioline), 1.25 µL of 50 mM $MgCl_2$, 0.25 µL of 25 mM dNTP (dNTP Mix, Bioline), 2 µL of primer mix (at 20 pmol/µL), 0.1 µL of Immolase DNA polymerase (Bioline) with 1 µL of genomic DNA (template). Positive and negative control PCRs used primers for feline GAPDH (Gelain et al., 2006) and 1 µL genomic DNA or water respectively as the template. Annealing temperature was determined as the lowest primer melting temperature minus 5°C. Using a PCR cycler (DNA Engine Tetrad 2, Bio-Rad), reactions were as follows: 95°C for 10 minutes, 35 cycles of 94°C for 40 seconds, 57-59°C for 30 seconds and 72°C for 1 minute, then 72°C for 10 minutes. PCR products were separated by horizontal gel electrophoresis using a 2% agarose gel containing a nucleic acid stain (Safeview Nucleic Acid Stain, NBS Biologicals) and analysed under 302 nm UV light (Chemi-Doc, Bio-Rad). DNA bands were extracted using a gel extraction kit (GenElute Gel Extraction Kit, Sigma-Aldrich), according to the manufacturer's instructions and submitted for Sanger sequencing (GATC Biotech, London or Source Bioscience, Nottingham or DNA Sequencing and Services, Dundee). Sequences were analysed using CLC Main Workbench 6 (CLC Bio). The functional effect of non-synonymous SNPs was predicted online using PolyPhen-2.[2]

Association of genotype with variables involved in CKD-MBD

Determination of SNP genotype was performed using Kompetitive Allele Specific PCR (KASP, LCG Genomics). Genomic DNA was extracted (as above), diluted and pipetted into 384-well plates (7.5 ng per well). Plates were dried at 65°C for a minimum of 1.5 hours. Positive and negative control wells were included on all plates by using previously sequenced genomic DNA or empty wells respectively. Primers were designed using Primerpicker (LGC

[2] See: Polyphen-2 available at http://genetics.bwh.harvard.edu/pph2/

Genomics) (see Table 2). Assay mix was prepared using 12 µL of each allele specific primer, 30 µL of the common reverse primer (all primers at a concentration of 100 µM) and 46 µL of nucleic free water. Buffer mix was prepared using KASP Reaction Mix aliquots and $MgCl_2$ (50 mM) to give a final concentration of 1.8 mM. Master mix (made up of 2 µL assay mix and 126 µL buffer mix) was added to each well (2.5 µL). Using a PCR cycler (PTC 225, Bio-Rad) reactions were as follows: 94°C for 15 minutes, 10 cycles of 94°C for 20 seconds with touch down over 65-70°C for 60 seconds (reducing by 0.8°C per cycle), 26 cycles of 94°C for 20 seconds and 57°C for 60 seconds. PCR plates were read on a Taqman 7900HT system (Applied Biosystems, Life Technologies) and analysed using SDS 2.3 (Qiagen). Small clusters that did not contain a positive control sample were confirmed using PCR and sequencing as described previously. SNPs present in only one cat were also confirmed by repeat PCR and sequencing.

Routine biochemical variables were measured at an external laboratory (Idexx, UK). Plasma FGF-23 and PTH concentrations were determined for all cases from EDTA plasma samples previously stored at -80°C, using an ELISA (FGF-23 ELISA kit, Kainos) and an immunoradiometric assay (Total intact PTH immunoradiometric assay – coated bead version, Scantibodies), both previously validated for use in cats (Geddes et al., 2013; Williams et al., 2012). PTH concentrations below the limit of detection, < 0.55 pmol/L (5.2 pg/mL), were assigned a value of 0.28 pmol/L (2.6 pg/mL).

Statistical analysis

Associations between quantitative or binary response variables and SNPs were analysed by logistic regression using SNPSTATS (Sole et al., 2006). In azotaemic CKD, associations were adjusted for plasma creatinine concentration by including creatinine as a co-variate. Quantitative response variables were assessed for normality by inspection of

histograms and Shapiro-Wilk tests and highly skewed variables were logarithmically transformed. We used SNPSTATS to examine allele frequency, calculate the Hardy-Weinberg equilibrium (using the exact Hardy-Weinburg test) and assess linkage disequilibrium. Akaike information criteria (AIC) were used to determine the inheritance model with the best fit (Akaike, 1974). Statistical significance was set at $P < 0.05$. Unless otherwise stated, variables are reported as median [25^{th}, 75^{th} percentiles].

Results

Fifty-two cats had measurements for ionised serum calcium performed and remained non-azotaemic for a minimum of 6 months. The Dixon method identified two results to be outliers and these were removed. The data was normally distributed (P = 0.715) and the 95% reference interval for geriatric feline whole blood ionised serum calcium was determined to be 1.19-1.37 mmol/L.

SNP discovery

The 10 cats with azotaemic CKD and ionised hypercalcaemia included 5 DSH, 1 DLH, 2 Burmese, 1 Persian and 1 Russian blue cross. Median ionised serum calcium concentration was 1.56 mmol/L (range 1.42 - 1.85 mmol/L). The ten cats with azotaemic CKD and low (n=1) or normal (n=9) ionised serum calcium concentrations included 7 DSH, 2 Burmese and one British shorthaired. Median ionised serum calcium concentration was 1.24 mmol/L (range 1.14 -1.30 mmol/L).

Sequence alignment from all 20 cats against the fCaSR reference sequence confirmed the presence of four previously published SNPs (Gal, 2010) and eight novel SNPs in the coding region (see Table 5 and Figure 1). Five SNPs were breed specific: CaSR:c.981C>T was only present with a minor allele frequency >10% in Burmese cats and CaSR:c.1992C>T, CaSR:c.2061C>T, CaSR:c.2780C>T and CaSR:c.3088G>A were only present with a minor allele frequency >10% in Persian cats (see Tables 4a-c).

A prediction of the functional effect of the three non-synonymous SNPs using PolyPhen-2 (Adzhubei et al., 2010) suggested that p.(Pro927Leu) might be possibly damaging (score 0.874 (sensitivity 0.83, specificity 0.93) but that the other two SNPs were benign

[p.(Ala1030Thr) score 0.018 (sensitivity 0.95, specificity 0.80), p.(Arg1044Pro) score 0.415 (sensitivity 0.89, specificity 0.90)].

Genotyping studies using KASP

We found KASP to be a rapid and straightforward method for genotyping cats. Nineteen of the cats from the SNP discovery study were used as positive controls (we had insufficient sample remaining from the twentieth cat) and KASP genotyping matched the genotyping by sequencing in 227/228 (99.6%) reactions. Rates of failed samples were <6% for each individual SNP, with 80/3084 (2.6%) samples failing in total, due to lack of amplification or failure to adequately cluster on the allelic discrimination plot making assignment of genotype unreliable. It was not possible to repeat KASP for the small number of failed samples as each KASP reaction needs a minimum number of samples (n=22) to obtain clusters of each genotype on the allelic discrimination plot, making this cost and time prohibitive.

Two-hundred and fifty-seven cats were genotyped for all 12 SNPs in the fCaSR using KASP, including 192 non-pedigree (161 DSH and 31 DLH), 40 Persian and 25 Burmese cats. Allele frequencies for individual SNPs varied greatly between breeds (see Tables 4 a-c), but were very similar for DSH and DLH cats so all non-pedigree cats were analysed together. Due to the small numbers of Persian and Burmese cats, and due to not having ionised serum calcium measurements or samples available from some cases to measure PTH and FGF-23, we could not perform further analyses of the SNPs for these breeds.

The 192 non-pedigree cats included 116 cats at diagnosis of azotaemic CKD, 9 cats previously diagnosed with CKD (used as positive controls) and 67 healthy older cats. Five

SNPs had a minor allele frequency of >10% in the non-pedigree population (see Table 4) and were analysed for associations with plasma concentrations of PTH, ionised serum calcium concentration, total serum calcium concentration, FGF-23 and serum phosphate concentration. All five SNPs were in Hardy-Weinberg equilibrium ($P > 0.05$) indicating independence between the two alleles and that genetic variation between the different alleles is remaining constant across generations. No SNPs were found to be in complete linkage (inherited together in all cases due to being closely located on a chromosome); therefore all five SNPs were analysed separately.

The 66 non-pedigree healthy older cats included 56 DSH and 10 DLH, of which 34 cats were neutered males and 32 were neutered females. Median age was 12.2 [10.9, 13.8] years, with the following results at the first screening visit: ionised calcium concentration – 1.29 [1.25, 1.31] mmol/L; plasma total calcium concentration – 2.46 [2.35, 2.54] mmol/L; plasma PTH concentration – 0.76 [0.28, 1.19] pmol/L (7.2 [2.6, 11.2] pg/mL); plasma FGF-23 concentration – 5.0 [3.5, 7.4] pmol/L (140.1[98.7, 207.6] pg/mL); and plasma phosphate concentration – 1.16 [1.02, 1.29] mmol/L. We found no associations between any SNPs and plasma/whole blood concentrations of logPTH, ionised and total calcium, logFGF-23 or phosphate in healthy cats.

The 116 non-pedigree cats at diagnosis of azotaemic CKD included 95 DSH and 21 DLH, of which 55 were males (2 entire, 53 neutered) and 61 were females (1 entire, 60 neutered). Most cats were in International Renal Interest Society (IRIS) stage 2 (plasma creatinine concentration 140-249 µmol/L, n = 84), with smaller numbers in stages 3 (plasma creatinine concentration 250-440 µmol/L, n = 25) and 4 (plasma creatinine concentration >440 µmol/L, n = 7). The cats had a median age of 14.5 years [12.0, 16.5], with the following results at CKD diagnosis (no cat was eating a 'renal' diet at this time): ionised calcium concentration – 1.29

[1.25, 1.33] mmol/L; plasma total calcium concentration – 2.54 [2.42, 2.64] mmol/L; plasma PTH concentration – 1.69 [0.76, 4.21] pmol/L (15.9 [7.2, 39.7] pg/mL); plasma FGF-23 concentration – 20.9 [11.6, 69.6] pmol/L (583.5 [324.1, 1946.9]) pg/mL; and plasma phosphate concentration – 1.29 [1.10, 1.66] mmol/L.

Concentrations of whole blood ionised calcium, plasma phosphate and PTH by genotype are shown in Figure 2. We found one association: CaSR:c.1269G>A was associated with logPTH concentration (adjusted for plasma creatinine concentration), with a recessive model having the best fit (G/G vs A/A-G/A, $P = 0.031$) (see Table 5).

Discussion

Our study revealed eight novel SNPs in the feline calcium sensing receptor, in addition to four previously identified SNPs. Three (including two novel SNPs) were non-synonymous (p.(Pro927Leu), p.(Ala1030Thr) and p.(Arg1044Pro)) (den Dunnen et al., 2016), resulting in a change in the amino acid sequence of the fCaSR protein and therefore potentially affecting receptor function. All three were in the intracellular tail of the fCaSR, in a nearby (but not identical) location to three non-synonymous SNPs identified in humans: p.Ala986Ser, p.Arg990Gly, p.Gln1011Glu (Heath et al., 1996). In healthy human subjects, the haplotype (combination of all three) of these SNPs accounts for 49.1 % of variation in ionised serum calcium concentrations (Scillitani et al., 2004) and a meta-analysis of 11 studies examining p.Ala986Ser found a strong association between this SNP and total and ionised calcium concentrations but no association with PTH concentration (He et al., 2012). In human CKD patients on haemodialysis, investigators found associations between p.Arg990Gly genotype and serum calcium and phosphate concentrations, (Eren et al., 2009) and with PTH concentrations (Eren et al., 2009; Yano et al., 2000). Additionally investigators have found associations between p.Gln1011Glu and with calcium, phosphate and PTH concentrations (Eren et al., 2009). In our study, the minor allele frequency of p.(Pro927Leu), p.(Ala1030Thr) and p.(Arg1044Pro) varied greatly amongst different cat breeds. In non-pedigree breeds, we only examined associations of p.(Arg1044Pro) and plasma/whole blood markers of calcium homeostasis, because it had a minor allele frequency >10 %. The substitution of proline for arginine is usually disfavoured (Betts and Russell, 2007) and could therefore affect receptor function; however we found no associations between this SNP and ionised calcium, total calcium, phosphate, PTH or FGF-23 concentrations in healthy cats or in cats at diagnosis of azotaemic CKD.

A number of SNPs had a minor allele frequency of >10% in Persian cats but not in Burmese or non-pedigree cats, including two non-synonymous SNPs p.(Pro927Leu) and p.(Ala1030Thr). Although 40 Persian cats were genotyped in this study a large number of them did not have ionised calcium measurements available or did not have sample available for measurement of PTH, therefore association studies were not performed for these cases. The potential significance of these SNPs in the Persian breed is currently unknown. The substitution of leucine for proline is usually disfavoured (Betts and Russell, 2007), and is predicted to be potentially damaging (score 0.874); therefore additional studies exploring the significance of p.(Pro927Leu) in the Persian cats are warranted. Furthermore, because Persians expressed all three of the non-synonymous SNPs identified in the fCaSR, the haplotype of these SNPs could also be examined in this breed.

The small number of Burmese cats genotyped in our study demonstrated marked differences in the presence of fCaSR SNPs when compared to non-pedigree and Persian cats. No Burmese cats demonstrated polymorphism at CaSR:c.732C>T, CaSR:c.1992C>T, CaSR:c.2061C>T, CaSR:c.2780C>T or CaSR:c.3088G>A, but they demonstrated a SNP at nucleotide 981 which was not present in non-pedigree breeds and only present at a low minor allele frequency in Persians. This highlights the need to segregate different cat breeds when performing genetic association studies in this species.

We found KASP genotyping to be accurate and efficient. We examined all SNPs with a minor allele frequency >10% in healthy and azotaemic non-pedigree cats for associations with ionised and total calcium, phosphate, FGF-23 and PTH concentrations. We found no associations between SNPs and any of these variables in the healthy cats. In samples collected

from cats with azotaemic CKD, we found one synonymous SNP (CaSR:c.1269G>A) was associated with plasma PTH concentration, but with no other variables. Because prevalence of secondary renal hyperparathyroidism varies with disease severity (Barber and Elliott, 1998), we adjusted the statistical model for creatinine to account for this. The model of best fit was recessive, with the genotype G/G being associated with higher plasma PTH concentrations when compared to the other genotypes, however G/G was not associated with any difference in ionised calcium, total calcium, phosphate or FGF-23 concentrations. This result is similar to a study of 122 Japanese haemodialysis patients that found associations between two SNPs and PTH concentration without any difference in corrected serum calcium or phosphate concentration (Yano et al., 2000).

CaSR:c.1269G>A is a synonymous SNP and therefore does not change the amino acid sequence of the fCaSR protein, making it more difficult to predict what the effect of this polymorphism could be. It might have no functional significance, or might be in linkage with a SNP elsewhere (and therefore inherited with another SNP) that is the actual cause of a functional change in the receptor and, therefore, the phenotypic variation. However, in some cases synonymous SNPs have been linked directly to disease pathogenesis (Bartoszewski et al., 2010; Macaya et al., 2009; O'Driscoll et al., 2003; Ramser et al., 2008). Synonymous SNPs are thought to be able to modify mRNA splicing, change mRNA stability and therefore affect protein expression, and alter function due to changes in protein conformation (Sauna and Kimchi-Sarfaty, 2011). The mechanism by which CaSR:c.1269G>A could affect PTH secretion in azotaemic cats is unknown and further studies are required to investigate if it could have any effect on the function or expression of the fCaSR and therefore increase PTH secretion.

In humans, mutations that inactivate the CaSR result in familial hypocalciuric hypercalcaemia (FHH) in heterozygotes and neonatal severe hyperparathyroidism (NSHPT) in homozygotes. In both cases, a reduction in CaSR activity increases the set-point for calcium (the concentration of calcium that reduces PTH secretion by 50%) resulting in hypercalcaemia and inappropriately normal or elevated PTH concentrations (D'Souza-Li et al., 2002). Conversely, activating mutations result in autosomal dominant hypocalcaemia (ADH) resulting in hypocalcaemia and hypoparathyroidism. These conditions are not recognised in the cat, but feline idiopathic hypercalcaemia is an increasingly recognised problem (Cook, 2009). However, the hypercalcaemia in this circumstance is generally PTH-independent, with low PTH concentrations (McClain et al., 1999; Midkiff, 2000), therefore, if the CaSR is involved in this condition, it would have to be via PTH-independent regulation of calcium.

A limitation of the present study is that we examined only the fCaSR exons for SNPs. Intronic mutations associated with PTH concentrations in Japanese haemodialysis patients (Yano et al., 2000) and with development of FHH (D'Souza-Li et al., 2001) have been identified. Therefore, it is possible that there could have been additional unidentified SNPs in introns, the promoter, or 3'- or 5'- untranslated regions and genotyping of these regions should be considered now that coverage of the intronic regions is available.

In conclusion, 12 SNPs have been identified to date in the fCaSR. The frequency of these polymorphisms varies markedly between cat breeds, which should be taken into account when planning future association studies of fCaSR genotype to phenotype. The KASP method was fast and accurate in genotyping cats. We found a significant association between a synonymous SNP and plasma PTH concentrations in azotaemic cats at diagnosis. Further

investigations are required to explore additional reasons for the marked variability in the presence and severity of feline CKD-MBD.

Conflict of interest statement

The Renal Research Clinic at the Royal Veterinary College acknowledges the support of Royal Canin for its research on feline hyperphosphataemia and chronic kidney disease. R.Geddes was in receipt of an Everts Luff Trust Research Training Fellowship. None of the authors has any other financial or personal relationships that could inappropriately influence or bias the content of the paper.

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Table 1: Primers for sequencing the six exons of the feline calcium sensing receptor and for feline GAPDH

Name	Forward primer (5'-3')	Tm[c] °C	Reverse primer (5'-3')	Tm[c] °C
Exon 1[a]	AGGGAGTGAACTGCACCAAG	64.3	GCTTTTCTCCAACCACCAGA	64.1
Exon 2[a]	CCATGATTCAAACCCAGCTT	63.8	CGCATTGCCCCATATAAGAA	64.4
Exon 3i	GAATGCTCAAAAAGCCAACC	63.4	ACTCCTTGGCAAAACCATTG	63.8
Exon 3ii	TAGTGGCCCAGACCTTGAAC	64.0	AGGCAATTTGAACCCAAAGC	65.0
Exon 4	CATATGTGGTAGCCCTGTGG	62.8	CCTAAGGCCCTTCCAAAGTC	63.7
Exon 5 [a]	CTCTTCCGTGCTTGTGTCAA	64.1	CAGGAGGGCATGTTCCTTTA	63.9
Exon 6i	TGACCGCATCCAATAATCTG	63.4	ACCTCCTCGATGGTGTTACG	63.9
Exon 6ii[a]	ACCTTCAGCATGCTCATCTTC	64.2	GTCTGAGGCGATTCCTCATC	63.6
GAPDH[b]	TCTTCCAGGAGCGAGATCC	64.5	AGGGATGATGTTCTGGCAGC	66.5

i, first part of exon; ii, second part of exon

[a] from (Gal, 2010)

[b] from (Gelain et al., 2006)

[c] Melting Temperature

Table 2: Primers for KASP for all 12 SNPs identified in the feline calcium sensing receptor

Exon, mRNA position and nucleotide change of SNP	**Allele 1 (FAM), allele 2 (VIC) and common reverse primer (C) (5'-3')** **The allele specific nucleotide for the forwards primers are underlined.**
Exon3_732 C>T	Allele 1: GAAGGTGACCAAGTTCATGCTATCGACTTCAGTGAACTCATCTCC Allele 2: GAAGGTCGGAGTCAACGGATTCATCGACTTCAGTGAACTCATCTCT C: TTGCTGGATCTCTTCTTCATCAGAATACT
Exon3_981 C>T	Allele 1: GAAGGTGACCAAGTTCATGCTCTGAAGGCTGGACAGATC Allele 2: GAAGGTCGGAGTCAACGGATTCGCTCTGAAGGCTGGACAGATT C: GACTTTCTGCAGGAATTCCCGGAA
Exon3_987 T>C	Allele 1: GAAGGTGACCAAGTTCATGCTACTTTCTGCAGGAATTCCCGGAAA Allele 2: GAAGGTCGGAGTCAACGGATTCTTTCTGCAGGAATTCCCGGAAG C: ATTCGCTCTGAAGGCTGGACAGAT
Exon3_1065 C>A	Allele 1: GAAGGTGACCAAGTTCATGCTTCTTGGAGGTGGCAGTTAAAG Allele 2: GAAGGTCGGAGTCAACGGATTACCTTCTTGGAGGTGGCAGTTAAAT C: GGTTTTGCCAAGGAGTTTTGGGAAG
Exon3_1269 G>A	Allele 1: GAAGGTGACCAAGTTCATGCTGGGCATGAGCAATGGAATAGACC Allele 2: GAAGGTCGGAGTCAACGGATTAGGGCATGAGCAATGGAATAGACT C: TTACGGATATCCTACAATGTCTACTTAGC
Exon6_1863 G>C	Allele 1: GAAGGTGACCAAGTTCATGCTATTGCACTCACTCTCTTTGCTGTG Allele 2: GAAGGTCGGAGTCAACGGATTGCACTCACTCTCTTTGCTGTC C: CCAGCACGAAGGCTGTCAGGAA
Exon6_1992 C>T	Allele 1: GAAGGTGACCAAGTTCATGCTTGCTCTGCTGCTTCTCCAGC Allele 2: GAAGGTCGGAGTCAACGGATTCTTGCTCTGCTGCTTCTCCAGT C: GGGCTCACCAATGAAGAACAGGG
Exon6_2061 C>T	Allele 1: GAAGGTGACCAAGTTCATGCTATGCATGATATGCAGAGCACGAAG Allele 2: GAAGGTCGGAGTCAACGGATTGATGCATGATATGCAGAGCACGAAA C: CCAGCCAGCCTTTGGCATCAG
Exon6_2109 C>T	Allele 1: GAAGGTGACCAAGTTCATGCTGATCTTGGCCTCAAACACCAGG Allele 2: GAAGGTCGGAGTCAACGGATTGGATCTTGGCCTCAAACACCAGA C: TCCTAGTGAAAACCAACCGTGTCCT
Exon6_2780 C>T	Allele 1: GAAGGTGACCAAGTTCATGCTGCTGCTTTTGCCTCTCGG Allele 2: GAAGGTCGGAGTCAACGGATTGCTGCTGCTTTTGCCTCTCGA C: AGTGAAGACCCCTTCCCACAGC
Exon6_3088 G>A	Allele 1: GAAGGTGACCAAGTTCATGCTGGAGACAGACTCAGAACTGAGTG Allele 2: GAAGGTCGGAGTCAACGGATTGGAGACAGACTCAGAACTGAGTA C: CTACAGGCCCTTGAAGACCTCTC
Exon6_3131 G>C	Allele 1: GAAGGTGACCAAGTTCATGCTCAGGGTCCTCCATCTCTGGTC Allele 2: GAAGGTCGGAGTCAACGGATTCAGGGTCCTCCATCTCTGGTG C: AGGGCCTGTAGATGGGGACTTC

Table 3: A summary of all 12 SNPs identified in the feline calcium sensing receptor

SNP	E	D	S or NS	Hypercalcaemic cats (20 alleles)				Normocalcaemic cats (20 alleles)			
				Major allele		Minor allele		Major allele		Minor allele	
CaSR:c.732C>T	3	ECD	S	C	18 (90.0%)	T	2 (10.0%)	C	17 (85.0%)	T	3 (15.0%)
CaSR:c.981C>T	3	ECD	S	C	17 (85.0%)	T	3 (15.0%)	C	17 (85.0%)	T	3 (15.0%)
CaSR:c.987T>C	3	ECD	S	T	14 (70.0%)	C	6 (30.0%)	T	13 (65.0%)	C	7 (35.0%)
CaSR:c.1065C>A	3	ECD	S	C	16 (80.0%)	A	4 (20.0%)	C	13 (65.0%)	A	7 (35.0%)
CaSR:c.1269G>A	3	ECD	S	A	12 (60.0%)	G	8 (40.0%)	A	10 (50.0%)	G	10 (50.0%)
CaSR:c.1863G>C	6	TM1	S	G	19 (95.0%)	C	1 (5.0%)	G	20 (100%)	C	0 (0%)
CaSR:c.1992C>T	6	TM2	S	C	18 (90.0%)	T	2 (10.0%)	C	20 (100%)	T	0 (0%)
CaSR:c.2061C>T	6	TM3	S	C	18 (90.0%)	T	2 (10.0%)	C	20 (100%)	T	0 (0%)
CaSR:c.2109C>T	6	ICL2	S	C	19 (95.0%)	T	1 (5.0%)	C	20 (100%)	T	0 (0%)
CaSR:c.2780C>T	6	ICD	NS p.(Pro927Leu)	C	18 (90.0%)	T	2 (10.0%)	C	20 (100%)	T	0 (0%)
CaSR:c.3088G>A	6	ICD	NS p.(Ala1030Thr)	G	18 (90.0%)	A	2 (10.0%)	G	20 (100%)	A	0 (0%)
CaSR:c.3131G>C	6	ICD	NS p.(Arg1044Pro)	G	11 (55.0%)	C	9 (45.0%)	G	11 (55.0%)	C	9 (45.0%)

Previously identified SNPs underlined (Gal, 2010).

E, exon; ECD, extracellular domain; TM, transmembrane domain; ICL2, intracellular loop; ICD, intracellular domain; NS, non-synonymous; S, synonymous

Tables 4 (a – c): Allele frequencies of all 12 SNPs in the fCaSR for 257 cats by breed

(a) Allele frequencies for non-pedigree cats (DSH and DLH) (n = 192)

SNP	Number of cats	Major allele			Minor allele		
		Nucleotide	n	Proportion	Nucleotide	n	Proportion
CaSR:c.732C>T	**188**	**C**	**328**	**87 %**	**T**	**48**	**13 %**
CaSR:c.981C>T	181	C	361	100 %	T	1	0 %
CaSR:c.987T>C	**183**	**T**	**238**	**65 %**	**C**	**128**	**35 %**
CaSR:c.1065C>A	**191**	**C**	**253**	**66 %**	**A**	**129**	**34 %**
CaSR:c.1269G>A	**188**	**A**	**207**	**55 %**	**G**	**169**	**45 %**
CaSR:c.1863G>C	187	G	371	99 %	C	3	1 %
CaSR:c.1992C>T	187	C	368	98 %	T	6	2 %
CaSR:c.2061C>T	183	C	359	98 %	T	7	2 %
CaSR:c.2109C>T	188	C	371	99 %	T	5	1 %
CaSR:c.2780C>T	188	C	371	99 %	T	5	1 %
CaSR:c.3088G>A	188	G	364	97 %	A	12	3 %
CaSR:c.3131G>C	**192**	**G**	**275**	**72 %**	**C**	**109**	**28 %**

SNPs with a minor allele frequency of >10 % are highlighted in bold.

(b) Allele frequencies for Persian cats (n = 40)

SNP	Number of cats	Major allele			Minor allele		
		Nucleotide	n	Proportion	Nucleotide	n	Proportion
CaSR:c.732C>T	40	C	78	97.5 %	T	2	2.5 %
CaSR:c.981C>T	37	C	71	95.9 %	T	3	4.0 %
CaSR:c.987T>C	**37**	**T**	**50**	**67.6 %**	**C**	**24**	**31.0 %**
CaSR:c.1065C>A	**40**	**C**	**66**	**82.5 %**	**A**	**14**	**17.0 %**
CaSR:c.1269G>A	**39**	**A**	**49**	**62.8 %**	**G**	**29**	**39.0 %**
CaSR:c.1863G>C	39	G	78	100.0 %	C	0	0.0 %
CaSR:c.1992C>T	**37**	**C**	**61**	**82.4 %**	**T**	**13**	**17.0 %**
CaSR:c.2061C>T	**38**	**C**	**61**	**80.3 %**	**T**	**15**	**19.0 %**
CaSR:c.2109C>T	40	C	80	100.0 %	T	0	0.0 %
CaSR:c.2780C>T	**39**	**C**	**65**	**83.3 %**	**T**	**13**	**16.0 %**
CaSR:c.3088G>A	**38**	**G**	**64**	**84.2 %**	**A**	**12**	**15.0 %**
CaSR:c.3131G>C	**40**	**G**	**42**	**52.5 %**	**C**	**38**	**46.0 %**

SNPs with a minor allele frequency of >10 % are highlighted in bold.

(c) Allele frequencies for Burmese cats (n = 25)

SNP	Number of cats	Major allele			Minor allele		
		Nucleotide	n	Proportion	Nucleotide	n	Proportion
CaSR:c.732C>T	24	C	48	100 %	T	0	0 %
CaSR:c.981C>T	**24**	**C**	**26**	**54 %**	**T**	**22**	**46 %**
CaSR:c.987T>C	25	T	46	96 %	C	2	4 %
CaSR:c.1065C>A	25	C	48	96 %	A	2	4 %
CaSR:c.1269G>A	**25**	**A**	**26**	**52 %**	**G**	**24**	**48 %**
CaSR:c.1863G>C	25	G	50	100 %	C	0	0 %
CaSR:c.1992C>T	25	C	50	100 %	T	0	0 %
CaSR:c.2061C>T	25	C	50	100 %	T	0	0 %
CaSR:c.2109C>T	25	C	50	100 %	T	0	0 %
CaSR:c.2780C>T	25	C	50	100 %	T	0	0 %
CaSR:c.3088G>A	24	G	48	100 %	A	0	0 %
CaSR:c.3131G>C	**25**	**G**	**4**	**8 %**	**C**	**46**	**92 %**

SNPs with a minor allele frequency of >10 % are highlighted in bold.

Table 5: Feline synonymous SNP CaSR:c.1269G>A association with plasma logPTH concentration at diagnosis of azotaemic CKD

SNP 1269 association with logPTH, adjusted by plasma creatinine concentration						
Model	**Genotype**	**n**	**Response mean (SE)**	**Difference (95 % CI)**	***P* value**	**AIC**
Co-dominant	A/A	29	1.23 (0.09)	0.00	0.075	156
	G/A	45	1.11 (0.08)	-0.09 (-0.31-0.14)		
	G/G	26	1.41 (0.11)	0.19 (-0.07-0.44)		
Dominant	A/A	29	1.23 (0.09)	0.00	0.9	156.8
	G/A-G/G	71	1.22 (0.07)	0.01 (-0.20-0.23)		
Recessive	**A/A-G/A**	**74**	**1.16 (0.06)**	**0.00**	**0.031**	**152**
	G/G	**26**	**1.41 (0.11)**	**0.24 (0.02-0.45)**		
Over-dominant	A/A-G/G	55	1.31 (0.07)	0.00	0.075	153.5
	G/A	45	1.11 (0.08)	-0.18 (-0.37-0.02)		
Log-additive	---	---	---	0.09 (-0.04-0.22)	0.18	155

Frequency and significance of CaSR:c.1269G>A genotype association with plasma logPTH concentration in non-pedigree cat breeds at diagnosis of azotaemic CKD (n = 100 as three cats failed genotyping of this SNP and 10 cats had no PTH measurement available). The model highlighted in bold was the best fit and indicated a significant association between homozygote G/G cats and increased plasma logPTH concentration.

AIC, Akaike information criteria; CI, confidence interval.

Figure Legends

Figure 1: Eight novel SNPs identified in the feline calcium sensing receptor.

Sequences obtained from cats used in the SNP discovery compared to reference sequences obtained from EnsEMBL. The breed of cat from which the sequence was obtained is shown in each case.

Y, heterozygous C/T; S, heterozygous G/C.

Figure 2: Column bar graphs showing phosphate, ionised calcium and PTH concentrations by CaSR:c.1269G>A genotype for cats at diagnosis of azotaemic CKD.

Column bar graphs of plasma phosphate, whole blood ionised calcium and plasma PTH concentrations by CaSR:c.1269G>A genotype measured in 113 non-pedigree cats at diagnosis of azotaemic CKD. Columns represent the median and error bars represent the interquartile range.

Genotype was found to be significantly associated with logPTH concentration; logPTH concentration was significantly higher for G/G when compared to A/A-A/G ($P = 0.037$). No significant association was found with phosphate or ionised calcium concentrations ($P \geq 0.14$).