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Minambres, I., Corcoy, R., Weetman, A.P. et al. (1 more author) (2020) Autoimmune hypercalcemia due to autoantibodies against the calcium-sensing receptor. Journal of Clinical Endocrinology and Metabolism, 105 (7). pp. 2229-2236. ISSN 0021-972X

https://doi.org/10.1210/clinem/dgaa219

This is a pre-copyedited, author-produced version of an article accepted for publication in The Journal of Clinical Endocrinology & Metabolism following peer review. The version of record [Inka Miñambres, Rosa Corcoy, Anthony P Weetman, E Helen Kemp, Autoimmune hypercalcemia due to autoantibodies against the calcium-sensing receptor, The Journal of Clinical Endocrinology & Metabolism, , dgaa219] is available online at: https://doi.org/10.1210/clinem/dgaa219

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1 **Autoimmune hypercalcemia due to autoantibodies against the calcium-**

2 **sensing receptor**

3

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5

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14 **Short title:** Autoimmune hypocalciuric hypercalcemia

15 **Keywords:** autoimmunity; calcium-sensing receptor; hyperparathyroidism; epitopes

16 **Word count:** 3103 (not including abstract, figures legends or references)

17 **Abstract word count:** 240

18 **Disclosure Summary:** EHK, IM, and RC have nothing to disclose. APW has received

19 lecture fees from Merck & Co., Inc., Kenilworth, NJ, USA.

20 **Grants:** This research did not receive any specific grant from any funding agency in the
21 public, commercial or not-for profit sector.

22

23 **Abstract**

24 **Context:** Autoimmune hypocalciuric hypercalcemia (AHH) is an acquired disorder
25 caused by the presence of blocking autoantibodies against the calcium-sensing receptor
26 (CaSR). Few cases of this condition have been described to date in the literature.

27 **Objective:** The objectives of this study were to describe two patients in whom the
28 presence of AHH was suspected and to assess the patients for the presence of CaSR
29 antibodies.

30 **Methods:** CaSR antibodies were detected and characterised by immunoprecipitation
31 assays, CaSR peptide ELISAs, and functional assays based on the calcium-stimulated
32 accumulation of inositol-1-phosphate in a mammalian cell line expressing the CaSR.

33 **Results:** Both patients presented with an acquired form of hypocalciuric hypercalcemia.
34 Mutational analyses of *CASR*, *GNA11* and *AP2S1* for familial hypocalciuric
35 hypercalcemia, were negative. According to the presence of Hashimoto's disease in one
36 patient and latent autoimmune diabetes of adulthood and thyroid autoimmunity in the
37 other, AHH was suspected. Immunoprecipitation assays detected CaSR antibodies in both
38 patients. Analysis of the antibody binding sites revealed two main epitopes at amino acids
39 41-69 and 114-126. Preincubation with purified CaSR antibodies against epitope 114-126
40 resulted in a significant decrease in inositol-1-phophate accumulation upon calcium-
41 stimulation of mammalian cells expressing the CaSR, suggesting that the antibodies had
42 receptor-blocking activity.

43 **Conclusions:** AHH is to be suspected in patients with an acquired biochemical pattern of
44 PTH-dependant hypocalciuric hypercalcemia, especially in those with other concomitant

45 autoimmune diseases. Diagnosis by means of detecting CaSR antibodies may help to
46 better characterise this probably under-reported condition.
47

48 **Introduction**

49 The calcium-sensing receptor (CaSR) is a G protein–coupled receptor that regulates
50 parathyroid hormone (PTH) secretion from the parathyroid glands by responding to
51 extracellular levels of calcium (1-3). Increases in extracellular calcium levels activate the
52 CaSR to inhibit PTH secretion, while decreases inhibit the receptor such that PTH release
53 is elevated (1-3). Functionally, PTH upregulates calcium resorption from the bone and
54 downregulates phosphate excretion from the kidneys thereby increasing and decreasing
55 serum calcium and phosphate, respectively (1-3). The CaSR is also expressed in the
56 kidneys where it enhances urinary calcium excretion in response to high levels of the
57 analyte independently of concomitant receptor-mediated changes in the level of
58 circulating PTH (2, 3). Thus, regulation of blood calcium is tightly controlled so that its
59 concentration is held within strict limits.

60 Homozygous mutations that inactivate the CaSR prevent feedback inhibition of the
61 parathyroid by extracellular calcium causing markedly elevated PTH levels and
62 hypercalcemia, a disorder known as neonatal severe hyperparathyroidism (4, 5).
63 Heterozygous mutations of the receptor lead the condition referred to as familial
64 hypocalciuric hypercalcemia (FHH) (4, 5). Such mutations decrease the CaSR's
65 sensitivity to calcium, resulting in reduced receptor stimulation at normal blood calcium
66 levels. As a result, inhibition of PTH secretion does not occur until higher serum calcium
67 levels are reached and reabsorption of renal calcium also continues. Thus, individuals
68 with FHH have PTH-dependent hypercalcemia with a normal or modestly elevated PTH
69 level as well as inappropriately normal or frankly low urinary calcium excretion, the
70 hypocalciuria principally distinguishing FHH from primary hyperparathyroidism (6-8). In

71 addition to the *CASR*, mutations in the *AP2S1* (encodes adaptor protein-2 σ subunit) and
72 *GNA11* (encodes G-protein subunit α11) genes can also results in FHH, but these are less
73 common causes (9-11).

74 An acquired form of hypercalcemia accompanied by hypocalciuria, caused by antibodies
75 that blocked the CaSR thus preventing it from reacting to elevated calcium, was first
76 described by Kifor and colleagues (12). Referred to as autoimmune hypocalciuric
77 hypercalcemia (AHH), the condition mimicked the biochemical pattern observed in
78 patients with FHH, but was also in the setting of various other autoimmune disorders
79 (12). Since this first report of AHH, only a few new cases have been highlighted (13-18).
80 Herein, we present two patients with hypercalcemia and low urinary calcium:creatinine
81 clearance ratios (UCCR), where the presence of AHH was suspected as both individuals
82 displayed autoimmune manifestations. In order to clarify the aetiology, the patients were
83 assessed for CaSR antibodies that might inhibit the receptor causing elevated levels of
84 PTH even in the presence of raised calcium.

85

86 **Patients and methods**

87 **Case presentations**

88 Patient 1 was a 66-year-old male that came to our clinic in 2012 for adjustment of his
89 levothyroxine treatment. He had Hasimoto's thyroiditis, but no other autoimmune
90 diseases. During follow-up, levothyroxine was adjusted in order to maintain a normal
91 thyroid-stimulating hormone (TSH) level, but an assymptomatic hypercalcemia was
92 detected where previously the patient had had normal blood calcium concentrations.
93 Since then, all tests performed showed persistent hypercalcemia with albumin-adjusted
94 serum calcium levels of 2.68-2.70 mmol/L (normal range, 2.15-2.55 mmol/L) (Figure 1),
95 constant hypocalciuria with a low UCCR of < 0.01 (normal range, 0.01-0.02) (Figure 1),
96 and decreased serum phosphate levels of 0.73-0.87 mmol/L (normal range, 0.87-1.45
97 mmol/L). Magnesium levels were normal at 0.93-1.0 mmol/L (normal range, 0.65-1.05
98 mmol/L). PTH levels were elevated and ranged from 18-36 pmol/L (normal range, 1.3-
99 6.8 pmol/L) and glomerular filtration rate (GFR) was normal at >60 mL/min/1.73m^2.

100 The patient had no history of personal or familial hypercalcaemia and mutational
101 analyses of *CASR*, *GNA11* and *AP2S1* were negative. Sestamibi scan showed no evidence
102 of hyperfunctioning parathyroid glands. Autoimmunity studies performed in January
103 2015 showed positivity for thyroid peroxidase (TPO) antibodies at 462 IU/mL (normal
104 range, <100 IU/mL), perinuclear anti-neutrophil cytoplasmic antibodies, anti-
105 mitochondrial antibodies at a 1:160 titre (normal range, titre of < 1:80), and anti-
106 mitochondrial M2 antibody. A bone densitometry analysis perfomed in 2015 revealed
107 osteoporosis of the hip, so alendronate treatment was initiated with minimal impact on

108 calcium levels. At his last visit the patient was treated with levothyroxine, cholecalciferol,
109 alendronate, amlodipine, and telmisartan as he was diagnosed with hypertension during
110 follow-up.

111 Patient 2 was a 51-year-old male with hypercalcemia since 2008, but who previous to that
112 had normal calcium levels. Maximal albumin-adjusted calcium levels were high at 2.67
113 mmol/L (Figure 1), UCCR oscilated between 0.0022 and 0.130 (Figure 1), and phosphate
114 concentrations were normal at 1.39-1.45 mmol/L. Magnesium levels were normal 0.74
115 mmol/L. PTH was repeatedly normal at 2.1-3.0 pmol/L and GFR was normal at >60
116 mL/min/1.73m^2.

117 Sestamibi scan showed no evidence of hyperfunctioning parathyroid glands. The patient
118 had no family or personal history of calcium disorders and mutational analyses of the
119 *CASR*, *GNA11* and *AP2S1* genes were negative. He had latent autoimmune diabetes of
120 adulthood (LADA) treated with insulin glargine and aspart, and thyroid autoimmunity
121 with normal thyroid function. Additional diagnoses were benign monoclonal
122 gammopathy and hypertension that had been treated until 2011 with hydrochlorothiazide.
123 In June 2013, he had abdominal discomfort and his pancreatic enzyme levels were 50%
124 above their reference values. However, an abdominal magnetic resonance imaging scan
125 was normal. The symptoms disappeared and his pancreatic enzyme levels decreased, but
126 did not completely normalise. Given the persistence of the abnormality the advice of a
127 gastroenterologist was sought and the possibility of autoimmune pancreatitis was
128 considered. At the end of follow-up, the patient was treated with insulin glargine and
129 aspart, enalapril, amlodipine, pravastatin, and cholecalciferol.

130 In both patients, AHH was suspected and analyses for the presence of CaSR antibodies
131 were performed. The study was approved by the Ethical Committee of the Hospital de la
132 Santa Creu I Sant Pau, Barcelona, Spain. Each patient had given written informed
133 consent.

134 **CaSR immunoprecipitation assays**

135 CaSR immunoprecipitation assays used to detect CaSR antibodies were undertaken as
136 detailed elsewhere (19). Briefly, human embryonic kidney 293 (HEK293) cells were
137 transiently transfected with pcCaSR-FLAG (19). Cell extract containing expressed CaSR-
138 FLAG protein was then prepared and stored at -80°C. GammaBind® Sepharose beads
139 (GE Healthcare, Little Chalfont, UK) were mixed with patient or control sera or with
140 anti-CaSR antibody (Alpha Diagnostic International, San Antonio, TX, USA) at a 1:100
141 dilution in immunoprecipitation buffer, and incubated at 4°C for 1 h. Subsequently, the
142 beads and antibody complexes were collected and incubated with cell extract containing
143 CaSR-FLAG protein for 16 h at 4°C. The bead-antibody-CaSR-FLAG protein complexes
144 were then collected and subjected to SDS-PAGE and immunoblotting using anti-FLAG®
145 M2-Peroxidase Conjugate (Sigma-Aldrich, Poole, UK) and an ECL™ Western Blotting
146 Analysis System (GE Healthcare). The densitometry of bands on developed films
147 resulting from immunoprecipitated CaSR-FLAG protein was performed in a Bio-Rad GS
148 690 Scanning Densitometer with Multi-Analyst Software (Bio-Rad Laboratories Ltd.,
149 Hemel Hempstead, UK).

150 A CaSR antibody index for each serum sample was calculated as the densitometry value
151 of the tested serum/mean densitometry value of 10 control sera. Each serum was tested in

152 duplicate in three separate experiments and its mean CaSR antibody index calculated.
153 The upper normal limit for the assay was calculated using the CaSR antibody index +
154 3SD of 10 controls. Any serum with a CaSR antibody index above the upper limit of
155 normal was designated as positive for CaSR antibodies.

156 **Antibody purification**

157 IgG was isolated from sera using protein G Sepharose 4 Fast Flow (GE Healthcare)
158 affinity chromatography, according to the manufacturer's instructions (20). Antibodies
159 against a specific CaSR peptide were isolated by affinity chromatography using a
160 CarboxyLink Immobilization Kit (Thermo Fisher Scientific, Waltham, MA, USA) (20).
161 All purified antibodies were dialysed, concentrated, and stored at –20°C at 10 mg/ml.

162 **CaSR peptide ELISAs**

163 CaSR peptide ELISAs to identify CaSR antibody binding sites were carried out as
164 detailed previously (20). The peptides (Cambridge Peptides, Birmingham, UK) used
165 represented amino acid residues 41-69, 114-126, 171-195, 344-358, and 374-391 of the
166 CaSR sequence. In brief, 20 ng of the required peptide were applied to the wells of a 96-
167 well microtiter plate overnight at 4°C. Plate wells were blocked with blocking buffer
168 (PBS containing 0.1% Tween 20 and 3% BSA) for 30 min at 37°C, and washed with PBS
169 containing 0.1% Tween 20. Patient and control sera were added to wells at a 1:100
170 dilution and incubated at room temperature for 1 h before washing. Antibody binding was
171 detected using anti-human IgG conjugated to alkaline phosphatase (Sigma-Aldrich) and
172 alkaline phosphatase substrate SIGMAFAST *p*-Nitrophenyl phosphate (Sigma-Aldrich)
173 with OD values read at 405 nm.

174 A CaSR antibody index for each serum sample was calculated as the OD405 of the tested
175 serum/mean OD405 value of 20 control sera. For each ELISA, sera were tested in
176 triplicate in three separate experiments and their mean CaSR peptide antibody indices
177 calculated. The upper limit of normal for each CaSR peptide ELISA was calculated using
178 the mean CaSR peptide antibody index + 3SD of 20 healthy control sera. Any serum with
179 a CaSR peptide antibody index above the upper limit of normal was designated as
180 positive for antibodies against the CaSR peptide tested.

181 To estimate CaSR antibody titres, the patients' sera were analysed at dilutions of 1:100
182 upto 1:10,000. Titres were defined as the serum dilution at which antibody binding was
183 detected above the upper limit of normal for the ELISA. To determine CaSR antibody
184 IgG subtype, anti-human IgG1, IgG2, IgG3, and IgG4 alkaline phosphatase conjugates
185 (SouthernBiotech, Birmingham, AL, USA) were applied as the secondary antibodies.

186 **Intracellular inositol-1-phosphate accumulation assay**

187 The response of mammalian HEK293 cells stably expressing the CaSR (HEK293-CaSR)
188 to calcium was assessed by measuring intracellular inositol-1-phosphate (IP1)
189 accumulation, as described elsewhere (20). For investigating the functional effects of
190 CaSR antibodies, monolayer HEK293-CaSR cells were preincubated for 10 min at 37°C
191 with the patients' purified CaSR antibodies or control IgG at a 1:100 dilution in calcium-
192 free assay buffer containing 10 mM lithium chloride. The cells were then stimulated with
193 0.5-5 mM calcium chloride for 60 min at 37°C. HEK293-CaSR cells without
194 preincubation with IgG were also included as controls. Subsequently, cells were lysed for

30 min at 37°C with 50 µl of 2.5% IP-One ELISA Kit Lysis Reagent (CIS Bio International, Gif-sur-Yvette, France).

The accumulation of IP1 in the recovered cell lysates was assessed using an IP-One ELISA Kit (CIS Bio International), according to the manufacturer's protocol. The IP-One ELISA was based on competition between free IP1 and an IP1-horseradish peroxidase (HRP) conjugate for binding to an anti-IP1 monoclonal antibody. Therefore, any increase in IP1 in the HEK293-CaSR cells following calcium-stimulation was reflected by a decrease in IP1-HRP binding. The results for IP1 accumulation were expressed as: percentage inhibition of IP1-HRP binding = [1 - IP1-HRP binding in stimulated cells/IP1-HRP binding in unstimulated cells] x 100. Each CaSR antibody was tested in six separate experiments and the mean percentage inhibition of IP1-HRP binding calculated. At each calcium concentration (0.5-5 mM), the accumulation of IP1 was compared between HEK293-CaSR cells preincubated with CaSR antibody or control IgG and those not, using one-way ANOVA. P values (two-tailed) < 0.05 were considered significant.

210 **Results**

211 **Detection of CaSR antibodies in the patients' serum**

212 Immunoprecipitation assays were used to detect CaSR antibodies in the patients' sera.
213 The upper limit of normal for the immunoprecipitation assay (mean CaSR antibody index
214 + 3SD of 10 control sera) was a CaSR antibody index of 2.72. Both patients had a CaSR
215 antibody index (mean ± SD) above the upper normal limit at 25.6 ± 5.8 and 43.2 ± 7.2 for
216 Patient 1 and 2, respectively, and were therefore considered antibody-positive (Figure 2).

217 **Identification of CaSR antibody epitopes**

218 Peptides representing previously identified CaSR epitopes at amino residues 41–69, 114–
219 126, and 171–195, 344-358 and 374-391 (12, 13, 21) were used in ELISAs to identify the
220 binding sites of the patients' receptor antibodies. Antibody reactivity against epitope 41-
221 69 was detected in both patients (Table 1). An antibody response against epitope 114-126
222 was apparent in patient 1 and 2 (Table 1). No antibody response was detected against
223 CaSR peptides 171-195, 344-358 or 374-391 (Table 1).

224 **CaSR antibody titres and subclass**

225 Antibody titres against each relevant epitope were again investigated in CaSR peptide
226 ELISAs. Titres were 1:1000 for antibodies against epitope 41–69 (Patient 1 and 2),
227 1:5000 against 114-126 (Patient 1), and 1:10,000 against 114-126 (Patient 2). Following
228 purification of the patients' CaSR antibodies using affinity chromatography, ELISAs
229 were used to determine their IgG subclass. The results indicated that antibodies against

230 CaSR epitope 41–69 were of the IgG1 subclass, and that antibodies against 114–126 were
231 of subclass IgG1 or IgG3 (Table 2).

232 **Functional effects of CaSR antibodies**

233 The effects of the patients' CaSR antibodies on calcium-stimulation of the receptor were
234 analysed by preincubation of HEK293-CaSR cells with CaSR antibody samples prior to
235 stimulating with calcium. The accumulation of intracellular IP1 was assessed as the
236 indicator of CaSR-stimulation. The results indicated that preincubation of HEK293-CaSR
237 cells with antibodies against CaSR epitope 41-69 (Patient 1 and 2) did not affect IP1
238 accumulation (data not shown). Preincubation with antibodies against epitope 114–126
239 (Patient 1 and 2) resulted in a statistically significant decrease in IP1 accumulation upon
240 calcium-stimulation compared with calcium-stimulation alone at calcium concentrations
241 of 0.5, 1.5 and 3.0 mM; P values were < 0.05, one-way ANOVA (Figure 3).

242

Discussion

Autoimmune hypocalciuric hypercalcemia mimics the biochemical pattern observed in patients with FHH, but is due to the presence of antibodies that inhibit the CaSR from reacting appropriately to elevated blood calcium levels (12-18). Here, two patients are presented with suspected AHH based on the presence of other autoimmune disorders, Hashimoto's thyroiditis in one patient and LADA and thyroid autoimmunity in the other, along with the appearance of an acquired hypercalcemia with persistent hypocalciuria. In addition, neither patient had a personal or familial history of hypercalcemia, genetic mutations associated with FHH (9-11), nor hyperfunctioning parathyroid glands. Analysis of the patients' sera using immunoprecipitation assays detected CaSR antibodies in both individuals supporting a diagnosis of AHH.

Analysis of the CaSR antibody binding sites revealed one epitope at amino acids 41-69, which had been previously identified in patients with autoimmune hypoparathyroidism and one patient with AHH (20, 21). Interestingly, the epitope overlaps the CaSR loop1 domain (amino acids 50-59) which, if deleted, reduces receptor activation (22, 23). However, functional analysis of the antibodies against the extracellular 41-69 epitope were found to have no detectable effect upon CaSR activity. This finding is in accordance with previous reports of CaSR antibodies that bind to this site, albeit they were from patients with autoimmune hypoparathyroidism (20).

The epitope at amino acids 114-126 overlaps the CaSR loop 2 domain (amino acids 117-136). Deletion of the loop 2 domain or point mutations present within this region can increase the sensitivity of the CaSR to calcium and can cause autosomal dominant hypoparathyroidism (22, 23). Of note, antibodies against epitope 114-126 that activate

266 the receptor have been identified in patients with autoimmune hypoparathyroidism (20).
267 In the present study, the patients' antibodies against the CaSR 114-126 epitope
268 demonstrated receptor-blocking activity such that even at raised blood calcium, PTH
269 would still be secreted from the parathyroid due to a right-shift of the CaSR's set point. In
270 view of the contrasting results, antibody binding to the epitope could either activate or
271 inhibit the receptor, depending on whether binding of the specific antibody favoured the
272 active or inactive conformation(s), respectively.

273 Antibodies against epitopes at amino acids 214-236, 374-391, and 344-358 of the CaSR,
274 which have previously been reported in patients with AHH (12, 13), were not identified
275 in the present study. Similar results to those here have been reported for an AHH patient
276 with CaSR antibodies in that no antibodies against CaSR peptides 214-236, 374-391 and
277 344-358 were detected in this individual (14).

278 Previously, 11 patients with AHH have been described (12-18, 21). Two of these were
279 unusual in that they were identified by family screening (12); the remaining cases appear
280 to be sporadic. All 11 cases presented with usually mild and asymptomatic
281 hypercalcemia, normal or decreased blood phosphate levels, and normal or elevated PTH
282 values. Hypocalciuria was confirmed in some patients, but not always checked. In six
283 patients, the serum PTH levels were at least 1.5 times above the reference range. This
284 appears to be in contrast to FHH in which around 80% of patients have a serum PTH
285 level within the reference range; the ratio of measured serum PTH level to the upper level
286 of the reference range was 0.7 in a recent series of 65 FHH patients (24). In this series,
287 the median age at diagnosis of FHH was 49 years and 68% of the patients were female.
288 Of the 11 originally presenting patients, seven were female and the median age at

289 diagnosis was 66 years (age range, 18-82 years). Six patients had at least one associated
290 autoimmune disorder: autoimmune thyroiditis occurred in three, with single cases of
291 LADA, celiac disease, bullous pemphigoid, autoimmune hypophysitis, and rheumatoid
292 arthritis. Three patients had anti-nuclear antibodies or perinuclear anti-neutrophil
293 cytoplasmic antibodies. Our patients presented with similar characteristics to the
294 previously described cases having mild and asymptomatic hypercalcemia, hypocalciuria,
295 and the presence of other autoimmune diseases; one of the two patients had a greatly
296 elevated PTH. Both patients were male, so that in the total of 13 cases now reported, the
297 male:female sex ratio is 0.86.

298 Although the frequency of AHH may be rare, it could be that cases of this disorder are
299 misdiagnosed as having primary hyperparathyroidism or FHH (6-8). Determinant traits
300 for the differentiation between these two conditions and AHH are the presence of new-
301 onset hypercalcemia, which is only possible if previously normal serum calcium levels
302 are available, and hypocalciuria (6-8). Unlike FHH, most cases of AHH are sporadic and
303 have an associated autoimmune disorder, but like FHH, AHH patients often have few or
304 no symptoms. In contrast to FHH, the serum PTH level in AHH patients is often raised
305 significantly.

306 Neither of the two patients described in this study received treatment for their
307 hypercalcemia. Parathyroidectomy was excluded in both cases in light of the suspicion of
308 AHH and the futility of surgery in these cases as well as in other types of autoimmune
309 hypercalcaemia (13, 18, 25). Treatment with glucocorticoids has been assessed in three
310 AHH patients, with a therapeutic benefit in two (13, 16, 18). Calcimimetic activators of

311 CaSR could be a promising treatment of severe hypercalcemia as they act as stimulators
312 of the CaSR, thus antagonising the effects of the receptor-blocking antibodies (15).

313 In conclusion, AHH is to be suspected in patients with an acquired biochemical pattern of
314 PTH-dependant hypocalciuric hypercalcemia, especially in those with other concomitant
315 autoimmune diseases. Its suspicion may preclude from parathyroidectomy, and treatment
316 with the calcimimetic drug cinacalcet should be considered in symptomatic cases.
317 Diagnosis by means of detection of CaSR antibodies is not routinely implemented, but
318 may help to better characterise this probably under-reported condition.

319

320 **Author contribution statement**

321 IM, RC, and EHK designed the study. IM and RC carried out the patient assessments.
322 EHK undertook the laboratory work. All authors analysed and interpreted the study data.
323 IM and EHK wrote the initial draft of the manuscript. All authors approved the final
324 version of the manuscript.

325 **Acknowledgements**

326 We thank Dr Edward M. Brown (Division of Endocrinology, Diabetes, and Hypertension,
327 Department of Medicine, Brigham and Women's Hospital and Harvard Medical School,
328 Boston, MA 02115, USA) for his critical appraisal of the manuscript. We acknowledge
329 the patients' agreement to be included in this study.

330

331

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423 **Figure Legends**

424 **Figure 1**

425 (A) Total serum calcium concentration and (B) urinary calcium:creatinine clearance ratio
426 (UCCR) of Patient 1 and Patient 2 during the entire follow-up period. The shaded area
427 shows in (A) the normal range for total serum calcium at 2.15-2.55 mmol/L and in (B)
428 the normal range for UCCR at 0.01-0.02.

429 **Figure 2**

430 Assessment of patients' sera for CaSR antibodies. Sera from Patient 1 and 2, and 10
431 healthy controls were tested for CaSR-binding activity using an immunoprecipitation
432 assay. (A) The upper limit of normal for the immunoprecipitation assay (mean CaSR
433 antibody index + 3SD of 10 control sera) was a CaSR antibody index of 2.72. The results
434 (mean CaSR antibody index ± SD) are shown for each patient serum sample tested in
435 three separate experiments. The CaSR antibody index was 25.6 ± 5.8 and 43.2 ± 7.2 for
436 Patient 1 and 2, respectively. (B) Immunoblots from the immunoprecipitation assay are
437 shown for anti-CaSR antibody (Lane 1); Patient 1 serum (Lane 2); Patient 2 serum (Lane
438 3); and a control serum (Lane 4).

439 **Figure 3**

440 Effect of patients' CaSR antibodies on CaSR function. Intracellular IP1 accumulation in
441 HEK293-CaSR cells was measured in response to stimulation by 0.5-5.0 mM calcium
442 after they were preincubated with the patients' CaSR antibody samples. Cells without
443 preincubation with antibody and also preincubated with control IgG were also included.
444 Intracellular IP1 accumulation was measured using an IP-One ELISA and was expressed

445 as: percentage inhibition of IP1-HRP binding = [1-IP1-HRP binding in stimulated
446 cells/IP1-HRP binding in unstimulated cells] x 100. The results are shown for the
447 patients' CaSR antibodies tested in six separate experiments. When compared with
448 HEK293-CaSR cells not preincubated with CaSR antibody, preincubation with the
449 patients' antibodies against epitope 114-126 decreased the levels of IP1 accumulation
450 significantly in HEK293-CaSR cells at concentrations of 0.5, 1.5, and 3.0 mM calcium; P
451 values were < 0.05, one-way ANOVA.

452

453 **Table 1:** Epitope identification for patient CaSR antibodies

454

CaSR peptide used in the ELISA[1]	Upper limit of normal for the ELISA	Patient 1 CaSR antibody index[3]	Patient 2 CaSR antibody index[3]
CaSR 41-69	2.23	**10.9**	**14.3**
CaSR 114-126	1.94	**8.4**	**12.1**
CaSR 171-195	2.09	1.12	1.09
CaSR 344-35	1.82	1.03	0.98
CaSR 374-391	1.73	0.95	1.08

455
456

457 [1]CaSR peptide ELISAs were used to identify CaSR antibody binding sites.

458 [2]The upper limit of normal for each ELISA was calculated using the mean CaSR
459 antibody index + 3SD of 20 controls.

460 [3]The CaSR antibody index for each serum sample was calculated as the OD405 of the
461 tested serum/mean OD405 value of 20 control sera. A CaSR antibody index (mean of
462 three separate experiments) above the upper limit of normal for the ELISA indicated
463 positivity for CaSR antibodies, and these are indicated in bold type.

464

465 **Table 2:** Subclass identification of antibodies against CaSR epitopes 41-69 and 114-126
466
467
468
469

CaSR peptide used in the ELISA/IgG subclass secondary antibody[1]	Upper limit of normal for the ELISA[2]	Patient 1 CaSR antibody index[3]	Patient 2 CaSR antibody index[3]
CaSR 41-69/IgG1	1.84	**9.1**	**15.3**
CaSR 41-69/IgG2	1.89	1.06	1.10
CaSR 41-69/IgG3	1.78	1.23	0.98
CaSR 41-69/IgG4	1.83	1.01	0.96
CaSR 114-126/IgG1	1.72	**10.6**	1.03
CaSR 114-126/IgG2	1.75	1.02	1.16
CaSR 114-126/IgG3	1.91	1.12	**16.8**
CaSR 114-126/IgG4	1.07	1.07	0.97

470
471
472

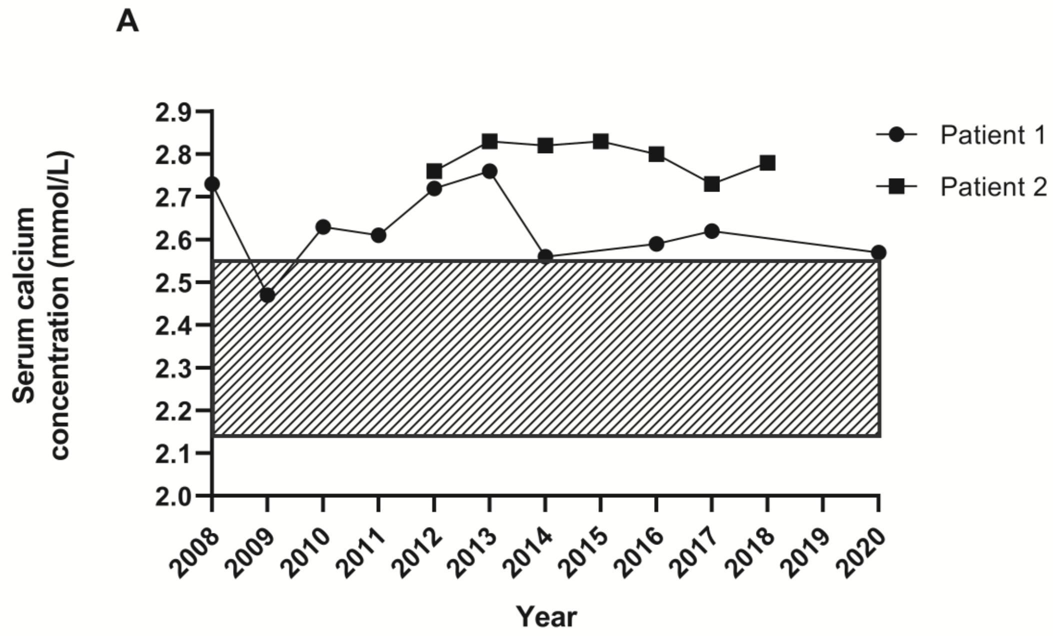
473 [1]To determine CaSR antibody IgG subclass, CaSR peptide ELISAs were used with anti-
474 human IgG1, IgG2, IgG3 or IgG4 alkaline phosphatase conjugates as the secondary
475 antibody.

476 [2]The upper limit of normal for each ELISA was calculated using the mean CaSR
477 antibody index + 3SD of 20 controls.

478 [3]The CaSR antibody index for each serum sample was calculated as the OD405 of the
479 tested serum/mean OD405 value of 20 control sera. A CaSR antibody index (mean of
480 three separate experiments) above the upper limit of normal for the ELISA indicated
481 positivity for CaSR antibodies, and these are indicated in bold type.

482

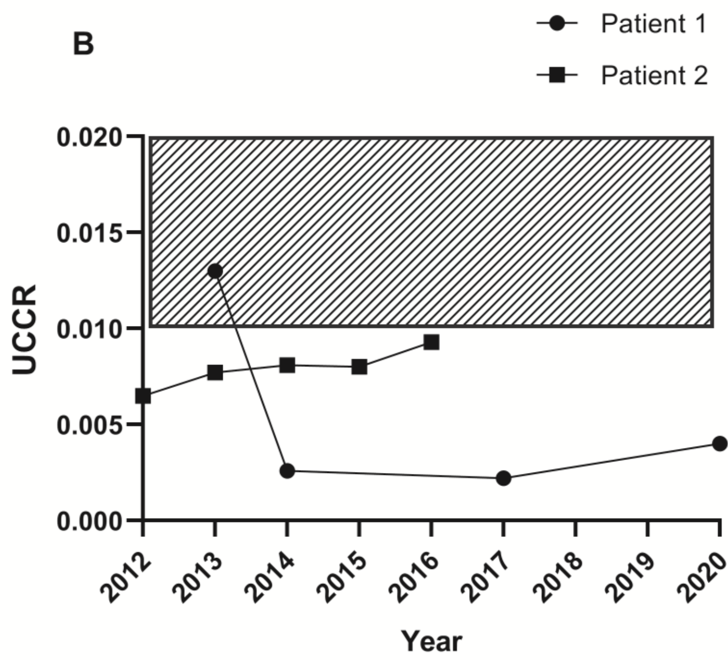
483 **Figure 1A**



484

485

486 **Figure 1B**



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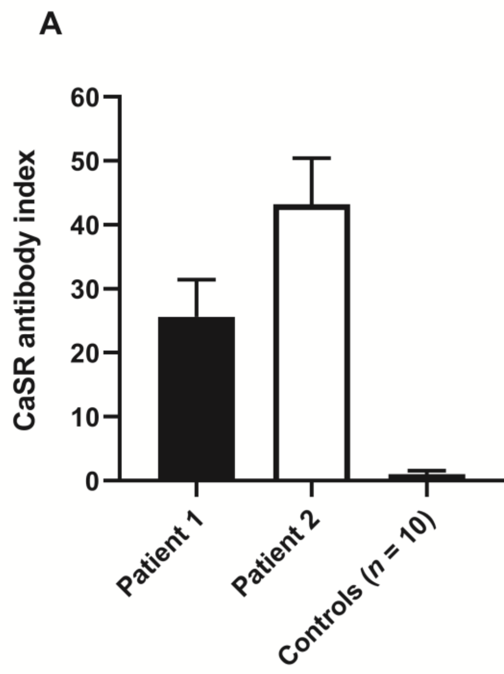
489 **Figure 2A**



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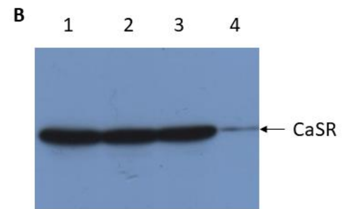
491

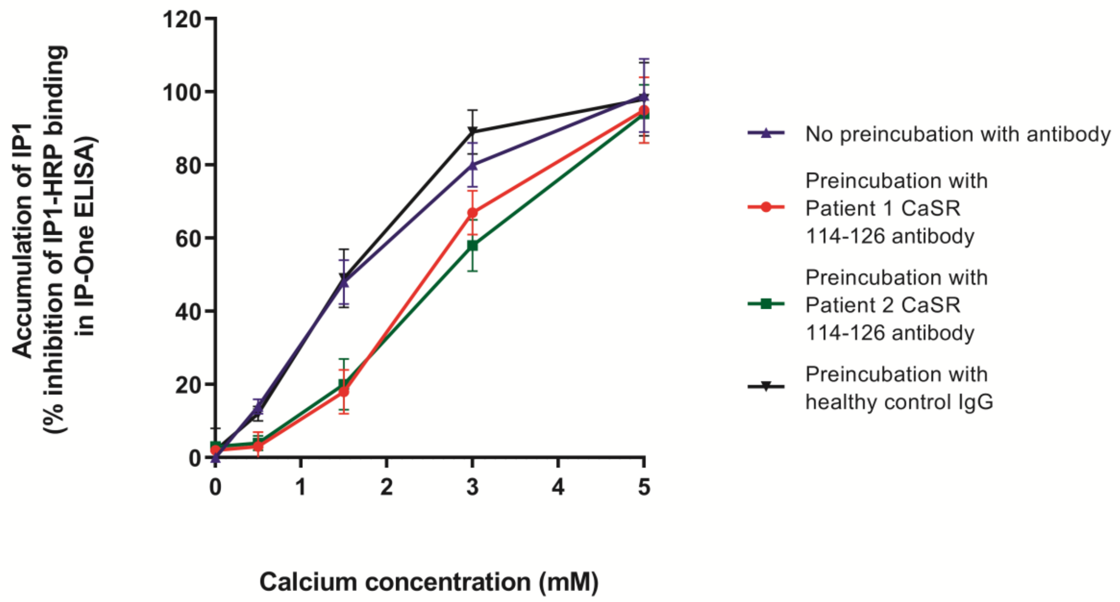
492 **Figure 2B**



493

494

495 **Figure 3**



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