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Activating Antibodies to Calcium-Sensing Receptor In Immunotherapy-Induced Hypoparathyroidism

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Abstract:	<p>Context</p> <p>Immune checkpoint inhibitors (ICIs), as anti-programmed cell death protein-1 (PD-1), anti-programmed cell death protein-ligand 1 (PD-L1), and anti-cytotoxic T lymphocyte antigen-4 (CTLA-4) monoclonal antibodies, are approved for the treatment of some types of advanced cancer. Their main treatment-related side-effects are immune-related adverse events (irAEs), especially thyroid dysfunction and hypophysitis. Hypoparathyroidism, on the contrary, is an extremely rare irAE.</p> <p>Objectives</p> <p>The aim of the study was to investigate the etiology of autoimmune hypoparathyroidism in a lung cancer patient treated with pembrolizumab, an anti-PD-1.</p> <p>Methods</p> <p>Calcium-sensing receptor (CaSR) autoantibodies, their functional activity, Ig subclasses and epitopes involved in the pathogenesis of autoimmune hypoparathyroidism were tested.</p> <p>Results</p> <p>The patient developed hypocalcemia after 15 cycles of pembrolizumab. Calcium levels normalised with oral calcium carbonate and calcitriol and no remission of hypocalcemia was demonstrated during a nine-month follow-up. The patient was found to be positive for CaSR-stimulating antibodies, of IgG1 and IgG3 subclasses, that were able to recognize functional epitopes on the receptor, thus causing hypocalcemia.</p> <p>Conclusion</p>

Immunotherapy and Autoimmune Hypoparathyroidism

1 **Activating Antibodies to Calcium-Sensing Receptor In Immunotherapy-** 2 **Induced Hypoparathyroidism**

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15 **Disclosure Summary**

16 The authors declare that there is no conflict of interest that could be perceived as prejudicing the
17 impartiality of this study.

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20 or not-for profit sector.

21

22 **ABSTRACT**

23 **Context:** Immune checkpoint inhibitors (ICIs), as anti-programmed cell death protein-1
24 (PD-1), anti-programmed cell death protein-ligand 1 (PD-L1), and anti-cytotoxic T
25 lymphocyte antigen-4 (CTLA-4) monoclonal antibodies, are approved for the treatment of
26 some types of advanced cancer. Their main treatment-related side-effects are immune-
27 related adverse events (irAEs), especially thyroid dysfunction and hypophysitis.
28 Hypoparathyroidism, on the contrary, is an extremely rare irAE.

29 **Objectives:** The aim of the study was to investigate the etiology of autoimmune
30 hypoparathyroidism in a lung cancer patient treated with pembrolizumab, an anti-PD-1.

31 **Methods:** Calcium-sensing receptor (CaSR) autoantibodies, their functional activity, Ig
32 subclasses and epitopes involved in the pathogenesis of autoimmune hypoparathyroidism
33 were tested.

34 **Results:** The patient developed hypocalcemia after 15 cycles of pembrolizumab. Calcium
35 levels normalised with oral calcium carbonate and calcitriol and no remission of
36 hypocalcemia was demonstrated during a nine-month follow-up. The patient was found to
37 be positive for CaSR-stimulating antibodies, of IgG1 and IgG3 subclasses, that were able
38 to recognize functional epitopes on the receptor, thus causing hypocalcemia.

39 **Conclusion:** The finding confirms that ICIs therapy can trigger, amongst other
40 endocrinopathies, hypoparathyroidism which can be caused by pathogenic autoantibodies.

41 **INTRODUCTION**

42 Immune checkpoint inhibitors (ICIs) are monoclonal antibodies directed against T cells
43 surface receptors involved in immune regulation such as cytotoxic T lymphocyte antigen-
44 4 (CTLA-4), programmed cell death protein-1 (PD-1), and programmed cell death protein-
45 ligand 1 (PD-L1) (1-3). ICIs, now used against a variety of solid tumors, are also significant
46 because they induce a broad spectrum of toxicities collectively referred to as immune-
47 related adverse events (irAEs) that can affect any organ or tissue (3). The endocrine glands
48 appear to be preferentially targeted sites resulting in autoimmune endocrinopathies that are
49 akin to the primary form of the autoimmune disease. Examples include thyroid dysfunction,
50 which is common during anti-PD-1 blockade, pituitary dysfunction, which is more often
51 found during anti-CTLA-4 blockade, and type 1 diabetes, which is more prevalent
52 following anti-PD-L1 therapy (3). Other endocrinopathies, such as adrenalitis, central
53 diabetes insipidus or hypoparathyroidism, have rarely been described during ICIs treatment
54 (4,5).

55 In rare cases, primary hypoparathyroidism and hypoparathyroidism in the context of
56 autoimmune polyendocrine syndrome type 1 (APS 1), has been related to the presence of
57 antibodies that activate the calcium-sensing receptor (CaSR), a molecule that controls
58 serum calcium levels via PTH secretion from the parathyroid gland (6,7). Such stimulating
59 antibodies may result in abnormally low PTH levels, even when serum calcium levels are
60 lower than optimal and need to be raised. Other reports implicate antibody-mediated
61 cytotoxicity or T cell infiltration of the parathyroid as immunological causes of
62 hypoparathyroidism (8-11). With respect to hypoparathyroidism as an irAE, the few

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63 reported cases (2,12-14) suggest that the disorder can be due to parathyroid inflammation
64 (12) or CaSR activating antibodies (2).

65 The aims of the current study were to report a lung cancer patient treated with
66 pembrolizumab, an anti-PD-1, who had developed hypoparathyroidism with symptomatic
67 hypocalcemia, and to investigate the aetiology of the endocrine disorder including the
68 presence of activating antibodies to the CaSR, their epitopes and IgG subclasses.

69 **PATIENT AND METHODS**

70 **Case presentation**

71 A 52-year-old man was treated with pembrolizumab (anti-PD-1) at 2 mg/kg every three
72 weeks, starting from September 2017, for his metastatic lung adenocarcinoma. ICIs therapy
73 was continued for 21 cycles (14 months) and interrupted in November 2018 due to diarrhea
74 which ceased after a few days. In February 2019, the patient was admitted to ER for recent
75 appearance of confusion, drowsiness, muscle weakness, and cramps. He had suffered from
76 epilepsy and depression for 15 years and was treated with topiramate, gabapentin, and
77 citalopram. Blood tests revealed hyponatremia and hypocalcemia, thus the patient was
78 referred to our Endocrine Unit for further evaluation.

79 The patient did not have history of autoimmune diseases, neck surgery or neck irradiation.
80 No evidence for metastases in the neck region were apparent at the latest CT scan. Family
81 history was also negative for hypocalcemic disorders and autoimmune diseases.

82 At physical examination, the patient was euvolemic, his blood pressure was in the normal
83 range (125/80 mmHg), and Chvostek and Trousseau signs were both positive. To rule out
84 endocrine causes of hyponatremia, blood tests for basal pituitary hormones, thyroid
85 function, electrolytes, urine analysis with urinary sodium excretion, urine osmolality,
86 plasma osmolality, were performed. To evaluate adrenal reserve, a cosyntropin test (250
87 µg intramuscular injection) was carried out. The results of analytes and antibody tests are
88 given in Table 1. These showed a normal adrenal and thyroid function. Severe
89 hyponatremia was confirmed and accompanied by elevated sodium excretion and urine
90 osmolality. Hypocalcemia was associated with inappropriately normal levels of PTH, low
91 levels of 25-hydroxy vitamin D and normal magnesium values. A revision of the clinical

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92 records of the patient demonstrated that, two months before starting ICIs, calcium and
93 PTH levels were in the normal range (25 pg/mL and 9.2 mg/dL, respectively) and that a
94 mild hypocalcemia (7.8 mg/dL) had appeared after the 15th dose (at 11 months) of
95 pembrolizumab and had worsened over time, even after ICIs withdrawal (Figure 1A). PTH
96 levels decreased overtime remaining detectable (Figure 1B). The patient was treated with
97 intravenous calcium gluconate infusions and switched to oral calcium carbonate (1 g per
98 day) and calcitriol (1 µg per day) which normalized his serum calcium concentration within
99 a few days.

100 It also became apparent that mild hyponatremia (132 mEq/L) had developed after three
101 cycles of pembrolizumab, worsened over time and became severe (120 mEq/L) after ICIs
102 withdrawal (Figure 1C). Adrenal insufficiency and hypothyroidism were ruled out thus
103 pointing to a syndrome of inappropriate antidiuretic hormone secretion (SIADH) as the
104 cause of hyponatremia. SIADH was likely due to a multifactorial origin including the
105 oncologic disease and concomitant therapy with citalopram and other drugs for epilepsy.
106 After fluid restriction and citalopram withdrawal an improvement of hyponatremia was
107 observed (Figure 1C).

108 The patient was regularly followed at our Endocrine Unit after ICIs withdrawal for an
109 additional nine months. However, despite treatment with oral calcium carbonate plus
110 calcitriol, no remission of hypocalcemia was demonstrated during this period (Figure 1A).

111 Informed consent for biochemical, *in vitro* studies and publication was obtained from the
112 patient.

113 **CaSR immunoprecipitation assays**

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114 CaSR immunoprecipitation assays used to detect CaSR antibodies were undertaken as
115 detailed elsewhere (15). Briefly, human embryonic kidney 293 (HEK293) cells were
116 transiently transfected with pcCaSR-FLAG. Cell extract containing expressed CaSR-
117 FLAG protein was then prepared and stored at -80°C. GammaBind® Sepharose beads (50-
118 µl samples) (GE Healthcare, Little Chalfont, UK) were mixed with sera at a 1:100 dilution
119 in immunoprecipitation buffer, and incubated at 4°C for 1 h. The beads and IgG complexes
120 were collected and incubated with cell extract containing CaSR-FLAG protein for 16 h at
121 4°C. The bead-IgG-CaSR-FLAG protein complexes were collected and subjected to SDS-
122 PAGE and immunoblotting using anti-FLAG® M2-Peroxidase Conjugate (Sigma-Aldrich,
123 Poole, UK) and an ECL™ Western Blotting Analysis System (GE Healthcare). The
124 densitometry of bands on developed films resulting from immunoprecipitated CaSR-FLAG
125 protein was performed in a Bio-Rad GS 690 Scanning Densitometer with Multi-Analyst
126 Software (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK). A CaSR antibody index for
127 each serum sample was calculated as the densitometry value of the tested serum/mean
128 densitometry value of 12 control sera. The upper normal limit for the assay was calculated
129 using the mean CaSR antibody index + 3 SD of 12 controls.

130 **CaSR peptide ELISAs**

131 CaSR peptide ELISAs to identify CaSR antibody binding sites were done as detailed
132 previously (16). The peptides (Cambridge Peptides, Birmingham, UK) used represented
133 amino acid residues 41-69, 114-126, 171-195, 344-358, and 374-391 of the CaSR
134 sequence. In brief, 20 ng of the required peptide were applied to the wells of a 96-well
135 microtiter plate overnight at 4°C. Plate wells were blocked with blocking buffer (PBS
136 containing 0.1% Tween 20 and 3% BSA) for 30 min at 37°C, and washed with PBS

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137 containing 0.1% Tween 20. Patient and control sera were added to wells at a 1:100 dilution
138 and incubated at room temperature for 1 h before washing. Antibody binding was detected
139 using anti-human IgG conjugated to alkaline phosphatase (Sigma-Aldrich, Poole, U.K.)
140 and alkaline phosphatase substrate SIGMAFAST p-Nitrophenyl phosphate (Sigma-
141 Aldrich) with OD values read at 405 nm. A CaSR antibody index for each serum sample
142 was calculated as the OD₄₀₅ of the tested serum/mean OD₄₀₅ value of 20 control sera.
143 The upper normal limits for the ELISAs were calculated using the mean CaSR antibody
144 index + 3 SD of 20 controls.

145 To estimate CaSR antibody titres, the patient's serum was analysed at dilutions of 1:100 to
146 1:10,000. Titres were defined as the serum dilution at which antibody binding was detected
147 above the upper limit of normal for the CaSR peptide ELISA.

148 **CaSR antibody purification**

149 Initially, IgG was isolated from sera using protein G Sepharose 4 Fast Flow (GE
150 Healthcare) affinity chromatography, according to the manufacturer's instructions.
151 Antibodies against a specific CaSR peptide (41–69, 114–126, 171–195, 344–358 and 374-
152 391) were isolated by affinity chromatography using a CarboxyLink Immobilization Kit
153 (Thermo Fisher Scientific, Waltham, MA, USA). All purified antibodies were dialysed,
154 concentrated, and stored at –20°C at 10 mg/ml.

155 **CaSR antibody IgG subclass, functional affinity and specificity**

156 To determine the IgG subclass of purified CaSR antibodies, anti-human IgG1, IgG2, IgG3,
157 and IgG4 alkaline phosphatase conjugates (SouthernBiotech, Birmingham, AL) were
158 applied as the secondary antibody in CaSR peptide ELISAs.

159 To determine functional affinities, purified CaSR antibodies were incubated at non-
160 saturating dilutions with a range of concentrations (0–1000 nM) of the required CaSR
161 peptide for 30 min before analysis in the appropriate CaSR peptide ELISA. Functional
162 affinity was expressed as the concentration of peptide that blocked 50% of antibody
163 binding in CaSR peptide ELISAs.

164 To evaluate CaSR antibody specificity, purified CaSR antibodies were preabsorbed at a
165 non-saturating dilution with a 200X M excess of the required CaSR peptide before analysis
166 in CaSR peptide ELISAs. Antibody binding following preabsorption was expressed as a
167 percentage of antibody binding without preabsorption.

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

169 As described elsewhere (16), the stimulatory effects of Ca^{2+} on HEK293 cells expressing
170 the CaSR (HEK293-CaSR) were measured by assessing intracellular inositol-1-phosphate
171 (IP1) accumulation. Monolayer HEK293-CaSR cells were cultured in 24-well plates before
172 washing with serum-free medium and Ca^{2+} -free assay buffer containing 10 mM lithium
173 chloride. For investigating CaSR antibody effects, cells were preincubated for 10 min at
174 37°C with patient CaSR antibodies or control IgG at a 1:100 dilution, and then stimulated
175 with 1.5 mM calcium chloride for 60 min at 37°C. HEK293-CaSR cells without
176 preincubation with IgG were also included as controls. Subsequently, cells were lysed for
177 30 min at 37°C with 50 μl of 2.5% IP-One ELISA Kit Lysis Reagent (CIS Bio International,
178 Gif-sur-Yvette, France). The accumulation of intracellular IP1 was assessed using an IP1
179 ELISA Kit (CIS Bio International), according to the manufacturer's protocol.

180 **RESULTS**

181 **Detection of patient's CaSR autoantibodies**

182 Immunoprecipitation assays detected CaSR antibodies in the patient's serum with a CaSR
183 antibody index of 61.3 (Figure 2) compared with the upper limit of normal for the assay of
184 2.73 (Figure 2). To identify the binding sites of the patient's CaSR antibodies, previously
185 characterised epitopes (6,16) at CaSR amino acid residues 41–69, 114–126, 171–195, 344-
186 358 and 374-391 were tested in ELISAs against the patient's serum. Antibody reactivity
187 was detected against epitopes 41-69, 114-126, 171-195 but not against CaSR peptides 344-
188 358 and 374-391 (Figure 3). Antibody titres against each relevant epitope were investigated
189 in ELISAs. Titres were 1:1000 for antibodies against epitope 41–69 and 114-126, and
190 1:5000 against epitope 171–195 (Table 2).

191 **Determination of CaSR antibody IgG subclass, functional affinity, and specificity**

192 Following purification of the patient's CaSR antibodies, ELISAs were used to analyse their
193 IgG subclass. The results indicated that antibodies against CaSR epitopes 41–69 and 171–
194 195 were of IgG1 subclass, and that antibodies against 114–126 were subtype IgG3 (Table
195 2). The functional affinities of the patient's CaSR antibodies were analysed in ELISAs.
196 The results showed that functional affinities ranged from 10^{-8} to 10^{-7} M (Table 2). The
197 specificity of the patient's CaSR autoantibodies was analysed by preabsorption of the
198 purified antibodies with CaSR peptides representing identified epitopes. Any effects upon
199 CaSR antibody binding were then assessed in ELISAs. The results showed that binding
200 was only significantly reduced by preabsorption with the CaSR peptide recognised as the
201 antibody epitope; no detectable cross-reactivity between different CaSR antibodies was
202 evident (Table 2).

203 **Analysis of CaSR antibody functional effects**

204 The effect of the patient's CaSR antibodies on receptor activity was analysed by
205 preincubation of HEK293-CaSR cells with CaSR antibody samples prior to stimulating
206 with Ca^{2+} . Intracellular IP1 accumulation was then measured as the indicator of CaSR-
207 stimulation. Autoantibody-stimulation of the CaSR would be expected to increase
208 intracellular IP1 levels shifting the inositol-1-phosphate-calcium curve leftwards and
209 decreasing the set-point that is normal for the receptor.

210 The results showed that preincubation of HEK293-CaSR cells with antibodies against
211 CaSR epitopes 114–126 and 171-195 gave a statistically significant increase in IP1
212 accumulation upon Ca^{2+} stimulation at 0.5, 1.5, and 3.0 mM, compared with Ca^{2+}
213 stimulation alone; **P* values were < 0.05, one-way ANOVA (Figure 4).

214 Both antibody types were therefore considered as having CaSR-activating activity. In
215 contrast, no effect on IP1 accumulation was evident from pre-treatment with antibodies
216 against the CaSR epitope 41-69 (Figure 4).

217 **Previous cases of ICI-induced hypoparathyroidism**

218 The previously reported cases of ICI-induced hypoparathyroidism are summarised in Table
219 3. In two cases, the pathomechanism of hypoparathyroidism was not determined (13,14)
220 Inflammation of the parathyroid was the cause in one case (12), and the presence of CaSR-
221 activating antibodies in another (2).

222 **DISCUSSION**

223 In recent years, therapeutic antibodies have been introduced into clinical practice in order
224 to target key regulators of peripheral immune-tolerance, namely anti-CTLA-4, anti-PD-1,
225 and anti-PD-L1, with the goal of activating the immune system against cancer cells (1). An

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226 undesirable, but somewhat expected, effect of immunotherapy is the triggering of
227 autoimmune diseases, referred to as irAEs (3,17). Some irAEs are very common, such as
228 thyroid dysfunction (accounting for about 10% during anti-PD-1 therapy) and hypophysitis
229 (accounting for up to 12% during anti-CTLA-4 therapy), but others such as
230 hypoparathyroidism, adrenalitis and diabetes insipidus are extremely rare (2-4,12-14,17).

231 ICIs induce a general inflammatory response that can facilitate the development of irAEs
232 through several mechanisms. These mechanisms, which are not mutually exclusive,
233 include an increase in T cell activity against antigens shared by tumor and healthy tissues,
234 and an elevation of pre-existing autoantibody levels or the development of novel antibody
235 responses. In addition, the production of pro-inflammatory cytokines such as IL-17 may
236 occur. Finally, another possible mechanism is when a direct binding of an anti-CTLA-4
237 antibody to CTLA-4 - when expressed ectopically on cells other than T lymphocytes-
238 occur causing a complement-mediated inflammation as described in ICI-induced
239 hypophysitis (1,18).

240 To date, only four cases of hypoparathyroidism following cancer immunotherapy have
241 been described and are summarised in Table 3 (2,12-14). However, the disease mechanism
242 although suspected in terms of an autoimmune response, has not been determined in all
243 four patients. In one case, a mechanism of parathyroid inflammation was suggested (12)
244 and in a second, the presence of CaSR activating antibodies was confirmed (2).

245 An autoimmune aetiology of hypoparathyroidism is rare (19), although it is a major
246 manifestation of autoimmune polyendocrine syndrome type 1 (APS1) (16).

247 In autoimmune cases of hypoparathyroidism, T cells are the most likely mediators causing
248 a mononuclear infiltration found in the parathyroid (8). Indeed, CaSR specific cytotoxic T

249 cells have been reported in 82.2% of idiopathic hypoparathyroid patients (10,11). However,
250 an equivalent destruction of the gland could be achieved by antibody-mediated cytotoxicity
251 which has also been proposed as a pathomechanism (9). In addition, CaSR activating
252 antibodies that are able to reduce the secretion of PTH from the parathyroids - thus keeping
253 serum calcium levels artificially low - have been reported in patients with
254 hypoparathyroidism (7,16)

255 In accordance with the results reported by Piranavan and colleagues (2), the serum of the
256 patient here described, was markedly positive for CaSR antibodies. In addition, we found
257 that these CaSR antibodies recognised the epitopes 41–69, 114–126, and 171–195 on the
258 receptor (13). Interestingly, the recognition of these epitopes was previously reported in
259 100%, 31%, and 38% of 16 APS1 patients, respectively (16). So they appear to be common
260 and major binding sites for autoantibodies against the CaSR, but different from the most
261 frequent CaSR T cell epitopes that were discovered in hypoparathyroid patients by Mahtab
262 and co-workers (11).

263 The finding that the patient's CaSR antibodies were of IgG1 and IgG3 subclasses is in
264 agreement with the previous findings (16). They have, therefore, the potential to bind
265 complement and be involved in antibody-mediated cytotoxicity (9,20), but these immune
266 mechanisms were not evaluated here.

267 The increase of IP1 accumulation in a cell line expressing the CaSR upon treatment with
268 the patient's purified CaSR antibodies, demonstrated the activating role of the antibodies
269 against CaSR epitopes 114-126 and 171-195. Activation of the CaSR caused a leftward
270 shift of the inositol-1-phosphate-calcium curve and a decrease of the normal set-point of
271 the CaSR. This would cause a reduction of PTH secretion even at lower than optimal serum

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272 calcium levels, thus causing hypocalcemia. With regard to the function of the CaSR,
273 epitope 114-126 forms part of the molecule in which point mutations or deletions cause
274 autosomal dominant hypoparathyroidism by increasing sensitivity to calcium (21,22).
275 Antibody binding to this epitope could favor the active conformation of the receptor
276 leading to lowering of PTH secretion even when calcium levels are below optimum. This
277 action resembles that observed with stimulating antibodies to the TSH receptor in Graves'
278 disease (23).

279 Site-directed mutagenesis and molecular models have shown that epitope 171-195 is
280 crucial for Ca^{2+} binding (21,24,25) thus it is easy to conceive of how antibody binding to
281 this part of the CaSR could adversely affect the interaction of calcium ions with this binding
282 site.

283 Antibodies against the CaSR epitope at 41-69, did not affect the function of the receptor.
284 Such neutral binding autoantibodies that do not have any detectable functional effects have
285 also been reported against the TSH receptor in patients with autoimmune thyroid disease
286 (26), although they may be capable of exacerbating the autoimmune response via other
287 antigen-driven mechanisms.

288 In conclusion, the findings in this study suggest that CaSR-activating antibodies are able
289 to contribute to ICI-induced hypoparathyroidism by reducing the secretion of PTH from
290 the parathyroid thus keeping serum calcium levels artificially low.

291 Other immune mechanisms that could occur simultaneously and result in destruction of the
292 parathyroid, including T cells and cytotoxic antibodies, were not investigated in this study.

293 Overall, it is important that clinicians are aware of the potential risk for hypocalcemia

294 associated with the use of immunotherapies as, in general, irAEs are unpredictable in
295 presentation and timing (27).

296 **Author contribution statement**

297 IL and AB followed the patient and designed the study. E H K designed and performed the
298 laboratory experiments and analysed the results. All authors contributed to the writing of
299 the manuscript.

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303

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440 **LEGENDS TO FIGURES**

441 **Figure 1**

442 Biochemical and hormonal test results during immunotherapy and after ICI-withdrawal.
443 (A) Total serum calcium; (B) PTH; and (C) sodium. The normal ranges are shaded.

444 **Figure 2**

445 Detection of CaSR antibodies in the patient's serum. Sera from the patient and healthy
446 controls (n = 12) were tested for binding to the CaSR using a CaSR immunoprecipitation
447 assay. The results are shown for sera tested in duplicate in three experiments. The upper
448 limit of normal for the CaSR immunoprecipitation assay (mean CaSR antibody index + 3
449 SD of 12 control sera) was a CaSR antibody index of 2.73. The CaSR antibody index of
450 the patient's serum sample was 61.3, indicating positivity for CaSR antibodies.

451 **Figure 3**

452 Identification of CaSR antibody binding sites. Sera from the patient and 20 healthy controls
453 were evaluated in ELISAs for antibodies against CaSR peptides 41–69, 114–126, 171–195,
454 344-358, and 374-391. The results are shown for sera tested in duplicate in three
455 experiments. The upper limits of normal (mean CaSR antibody index + 3 SD of 20 control
456 sera) were 1.84, 1.93, 2.83, 1.75, and 2.02 for the 41-69, 114-126, 171-195, 344-358, and
457 374-391 peptide ELISAs, respectively. The patient's serum had antibody indices of 30.1,
458 10.6, 15.4, 1.10, and 1.23 for the 41-69, 114-126, 171-195, 344-358, and 374-391 peptide
459 ELISAs, respectively, indicating binding sites at three different epitopes.

460 **Figure 4**

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461 Detection of CaSR-stimulating activity of the patient's CaSR antibodies. Intracellular
462 IP1(IP1) accumulation in HEK293-CaSR cells was measured in response to stimulation by
463 0.5-5 mM Ca²⁺ after they were preincubated with the patient's CaSR antibody samples or
464 healthy control antibody (n = 12). Cells without preincubation with antibody were included
465 in experiments. Intracellular IP1 accumulation was measured using an IP-One ELISA, and
466 the results expressed as: percentage inhibition of IP1-HRP binding = [1 - IP1-HRP binding
467 in stimulated cells/IP1-HRP binding in unstimulated cells] x 100. Increasing intracellular
468 levels of IP1 were reflected by an increase in the percentage inhibition of IP1-HRP binding
469 in the IP-One ELISA. The results are shown for the patient's CaSR antibodies tested in
470 four experiments. Preincubation with antibodies against epitopes 114-126 and 171-195
471 increased the levels of IP1 accumulation significantly in HEK293-CaSR cells at
472 concentrations of 0.5-3.0 mM Ca²⁺; **P* values were < 0.05, one-way ANOVA, indicating
473 that they had receptor-stimulating activity.

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1 **Table 1** Biochemical and hormonal features at first evaluation, three months after anti-
 2 PD-1 immunotherapy withdrawal

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Test	Value ¹	Reference range
Sodium	120	135-145 mEq/L
Potassium	3.85	3.5-5.1 mEq/L
Calcium	6.2	8.6-10.2 mg/dL
Ionised calcium	0.8	1.13-1.32 mEq/L
Phosphorus	3.7	2.5-4.5 mg/dL
Magnesium	2.2	1.7-2.5 mg/dL
Albumin	4.6	3.5-5 g/dL
Creatinin	0.7	0.7-1.2 mg/dL
Urea	14	10-50 mg/dL
Uric acid	2.6	3.5-7 mg/dL
Glucose	76	74-109 mg/dL
Cosyntropin test		
Cortisol (Time 0')	9.8	
Cortisol (Time 30')	29.3	
Cortisol (Time 60')	38.3	
ACTH	12	<50 ng/L
PTH	18	8-40 pg/mL
25-hydroxyvitamin D	12	> 30 µg/L
FSH	1.4	1.3-19.5 mIU/mL
LH	4.8	1.4-12.7 mIU/mL
Testosterone	3.52	1.75-7.8 µg/L
PRL	10.9	2-13 ng/mL male
IGF-1	99	57-202 µg/L
FT4	1.14	0.7-1.7 ng/dL
TSH	1.4	0.4-4 mcIU/mL
TgAb	Negative	<30 IU/mL
TPOAb	Negative	<10 IU/mL
Plasma osmolality	255	280-295 mOsm/Kg
Urinary osmolality	250	300-800 mOsm/kg
Urinary sodium excretion	70	54-190 mEq/L

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¹Values outside the reference range are highlighted in bold type-face.

Table 2 Epitopes, titres, IgG subclass, and functional affinity of the patient's CaSR antibodies

CaSR antibody epitope	Titre¹	IgG subclass	Functional affinity (M)²	Specificity
41-69	1:1000	IgG1	3×10^{-7}	No cross reactivity with antibodies against epitopes 114-126 and 171-195.
114-126	1:1000	IgG3	4×10^{-7}	No cross reactivity with antibodies against epitopes 41-69 and 171-195.
171-195	1:5000	IgG1	7×10^{-8}	No cross reactivity with antibodies against epitopes 41-69 and 114-126.

¹CaSR antibody titres were defined as the dilution of the patient's serum at which antibody binding could still be detected above the upper limits of normal for the CaSR peptide ELISAs.

²The functional affinity of purified CaSR antibodies was calculated as the concentration of the relevant CaSR peptide that blocked 50% of CaSR antibody binding in CaSR peptide ELISAs.

Immunotherapy and Autoimmune Hypoparathyroidism

1 **Table 3** Reported cases of hypoparathyroidism following ICI therapy
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Study	Patient	Immunotherapy	PTH level	Endocrine and other disorders
Win et al (14)	73-year-old male with metastatic melanoma	Nivolumab (anti-PD-1); ipilimumab (anti-CTLA-4)	< 1 pg/mL (reference range, 9 to 80 pg/mL)	Hypoparathyroidism (autoimmune pathomechanism suspected but not determined); autoimmune thyroiditis.
Umeguchi et al (13)	64-year-old male with stage IVB non-small cell lung carcinoma	Pembrolizumab (anti-PD-1)	8 pg/mL (reference range, 10 to 65 pg/mL)	Hypoparathyroidism (autoimmune pathomechanism suspected but not determined).
Trinh et al (12)	53-year-old male with stage IV melanoma	Nivolumab (anti-PD-1); ipilimumab (anti-CTLA-4)	7 pg/mL (reference range, 15 to 65 pg/mL)	Autoimmune hypoparathyroidism due to inflammation of parathyroid; immune-mediated colitis; inflammatory oligoarthritis.
Piranavan et al (2)	61-year-old female with small cell lung cancer	Nivolumab (anti-PD-1)	8 pg/mL (reference range, 12 to 65 pg/mL)	Autoimmune hypoparathyroidism due to CaSR-activating antibodies.

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Fig. 1







