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Activating Antibodies to Calcium-Sensing Receptor In Immunotherapy-Induced Hypoparathyroidism
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Abstract:	Context 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. Objectives 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. Methods Calcium-sensing receptor (CaSR) autoantibodies, their functional activity, Ig subclasses and epitopes involved in the pathogenesis of autoimmune hypoparathyroidism were tested. Results 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. Conclusion

Immunotherapy and Autoimmune Hypoparathyroidism

1 **Activating Antibodies to Calcium-Sensing Receptor In Immunotherapy-**

2 **Induced Hypoparathyroidism**

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11 **Short title:** Immunotherapy and autoimmune hypoparathyroidism

12 **Keywords:** autoimmunity; calcium-sensing receptor; hypoparathyroidism; immune checkpoint

13 inhibitors, epitopes

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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.

18 **Grants**

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 (CLTA-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   cytoxicity or T cell infiltration of the parathyroid as immunological causes of

62   hypoparathyroidism (8-11). With respect to hypoparathyroidism as an irAE, the few

63   reported cases (2,12-14) suggest that the disorder can be due to parathyoid 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 metasteases 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

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   additonal 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**

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

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 IP1accumulation 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

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 cytoxicity

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 hypoparathyrid 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

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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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**

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 Ca^{2+} 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 IP1accumulation 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 IP1were 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 Ca^{2+}; *P values were < 0.05, one-way ANOVA, indicating

473   that they had receptor-stimulating activity.

Table

Immunotherapy and Autoimmune Hypoparathyroidism

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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6 [1]Values outside the reference range are highlighted in bold type-face.
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Table 2 Epitopes, titres, IgG subclass, and functional affinity of the patient's CaSR antibodies

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

[1]CaSR 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.

[2]The 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 cancinoma	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