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Piranavan, P., Li, Y., Brown, E. et al. (2 more authors) (2018) Immune checkpoint inhibitor-induced hypoparathyroidism associated with calcium-sensing receptor-activating autoantibodies. Journal of Clinical Endocrinology and Metabolism. ISSN 0021-972X

https://doi.org/10.1210/jc.2018-01151

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Immune Checkpoint Inhibitor-Induced Autoimmune Hypoparathyroidism Associated with Calcium-Sensing Receptor-Activating Autoantibodies

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Short Title: Nivolumab therapy and autoimmune hypoparathyroidism

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Disclosure statement: P.P., Y.L., E.H.K., and N.T. have nothing to declare. E.M.B. has a financial interest in the calcimimetic, cinacalcet, through NPS Pharmaceuticals, Inc., Bedminster, NJ, USA.

Grant Support: None

23 **Keywords:** autoantibodies; autoimmunity; calcium-sensing receptor; hypoparathyroidism;
24 immunosuppression

25

26 **Abbreviations:** APS1, autoimmune polyglandular syndrome type 1; CaSR, calcium-sensing
27 receptor; cpm, counts per minute; CTLA-4, cytotoxic T lymphocyte antigen-4; ELISA, enzyme-
28 linked immunosorbent assay; ICI, immune checkpoint inhibitors; IFN, interferon; IL, interleukin;
29 PBS, phosphate-buffered saline; PD-1, programmed cell death protein 1; PD-L1, programmed
30 cell death protein 1 ligand 1; PTH, parathyroid hormone; RLBA, radioligand binding assays;
31 SCLC, small cell lung cancer.

32

33 **Word Count** (excluding abstract, references and figure legends): 3089

34

35 **Abstract Word Count**: 241

36

37 **Precis:** A patient with small cell lung cancer undergoing treatment with nivolumab developed
38 hypocalcemia due to hypoparathyroidism caused by calcium-sensing receptor-activating
39 autoantibodies.

40 **Abstract**

41 **Context:** While therapy with immune checkpoint inhibitors such as nivolumab have substantially
42 improved survival in several types of cancer, increased attention has been given to adverse
43 immune events associated with their use, including the development of endocrine autoimmunity.

44 **Objectives:** First, to describe a patient with a two-year history of metastatic small cell lung
45 cancer who had been treated with nivolumab a few months prior to presentation with the signs
46 and symptoms of severe hypocalcemia and hypoparathyroidism. Second, to investigate the
47 etiology of the patient's hypoparathyroidism, including the presence of activating autoantibodies
48 against the calcium-sensing receptor (CaSR), since humoral and cellular immune responses
49 against the CaSR have been reported in patients with autoimmune hypoparathyroidism.

50 **Case and Results:** A 61-year-old female was admitted with persistent nausea, vomiting,
51 epigastric pain, constipation, and generalized weakness. Laboratory analyses showed low total
52 serum calcium, ionized calcium, and parathyroid hormone (PTH). The patient was diagnosed
53 with severe hypocalcemia as a result of autoimmune hypoparathyroidism after testing positive
54 for CaSR-activating autoantibodies. She was treated with intravenous calcium gluconate
55 infusions followed by a transition to oral calcium carbonate plus calcitriol which normalized her
56 serum calcium. Her serum PTH remained low during her hospitalization and initial outpatient
57 follow-up despite adequate repletion of magnesium.

58 **Conclusions:** This case illustrates autoimmune hypoparathyroidism induced by immune
59 checkpoint inhibitor-blockade. As immune checkpoint inhibitors are now used to treat many
60 cancers, clinicians should be aware of the potential risk for hypocalcemia that may be
61 associated with their use.

62 **Introduction**

63 Immunotherapies targeting the immune checkpoint molecules, such as cytotoxic T lymphocyte
64 antigen-4 (CTLA-4), programmed cell death protein 1 (PD-1), and its ligand PD-L1, are now
65 commonly used in clinical practice to treat malignancies (1). For example, nivolumab is an anti-
66 PD-1 monoclonal antibody that works as an immune checkpoint inhibitor (ICI). It blocks the
67 interaction between PD-1 on the surface of activated T cells and PD-L1 produced by cancer
68 cells, a signalling process that would normally prevent T lymphocytes from attacking tumors.
69 While therapy with ICIs such as nivolumab has substantially improved cancer survival,
70 increased attention has been called to immune-related adverse events associated with their use
71 including endocrine autoimmunity (1, 2). ICI-induced autoimmune endocrinopathies involving
72 the pituitary and thyroid are frequent being reported in up to 10% of patients treated with
73 antibodies against CTLA-4 or PD-1/PD-L1 (1-5). In contrast, primary adrenal insufficiency and
74 type 1 diabetes are uncommon (1-3), while ICI-induced autoimmune involvement of the
75 parathyroid gland is exceedingly rare (4). To date, there have been two reported cases of ICI-
76 induced hypocalcemia, but the etiology of their low calcium levels was not reported (6).

77 A common cause of hypocalcemia is hypoparathyroidism. Normally, low blood calcium
78 concentrations are detected by the parathyroid-expressed calcium-sensing receptor (CaSR),
79 which responds by stimulating parathyroid hormone (PTH) secretion from the parathyroid
80 glands (7). There is a resulting normalization of serum calcium as it is reabsorbed by the
81 kidneys, released by bone, and absorbed by the intestine. However, in circumstances of absent,
82 reduced or ineffective PTH, this regulatory function is lost and hypocalcemia ensues.
83 Hypoparathyroidism can be due to surgical or autoimmune destruction of the parathyroid glands
84 (8). In addition, autoantibodies that stimulate CaSR activity, even when blood calcium levels are
85 lower than normal, can cause the inhibition of PTH secretion from the parathyroid, such that
86 calcium remains below the critical concentration that would normally stimulate PTH secretion

87 (9). Such CaSR-activating autoantibodies have been identified in patients with idiopathic
88 hypoparathyroidism and autoimmune polyendocrine syndrome type 1 (APS1), in which
89 hypoparathyroidism is a prominent manifestation (10, 11).

90 The aims of this study were to describe a patient with a two-year history of metastatic small cell
91 lung cancer (SCLC), who had been treated with nivolumab a few months prior to presentation
92 and developed the signs and symptoms of severe hypocalcemia and hypoparathyroidism, and
93 to investigate a possible autoimmune etiology of the patient's hypoparathyroidism by testing for
94 the presence of CaSR autoantibodies. In addition, NALP5 and cytokine autoantibodies were
95 evaluated since these are indicative of APS1.

Materials and Methods

Case description

A 61-year-old woman with metastatic SCLC was admitted to our hospital with persistent nausea, vomiting, epigastric pain, constipation and generalized weakness in July 2017. In addition to these symptoms, she also reported bilateral distal lower limbs paresthesias but no muscle spasms or cramps. She had three prior admissions at other institutions within the last two months for similar symptoms. Low serum calcium levels were the most notable laboratory finding during these prior hospital admissions. The patient's hypocalcemia was treated during these hospitalizations, and the patient was instructed to take over-the-counter calcium and vitamin D3 supplementation at discharge. She was not taking the recommended calcium and vitamin D supplementation at the time of admission to our hospital.

The patient was diagnosed with SCLC in July 2015, which was initially treated with multiple cycles of platinum-based chemotherapy and radiation therapy. Given the progression of the disease despite the initial treatment, nivolumab therapy was begun in March 2017. The patient received six intravenous infusions of nivolumab, which led to partial remission of her SCLC. The last infusion of nivolumab was two months prior to the current hospitalization.

At our hospital, the patient reported no family history of autoimmune endocrinopathies, neck irradiation or neck surgery. Her physical examination was unremarkable except for mild gait ataxia. Chvostek and Trousseau signs were negative. Laboratory findings upon admission are summarized in Table 1 and showed low total serum calcium, ionized calcium, and PTH levels. A CT scan of her neck, chest and abdomen revealed a primary left lung mass with extensive lymph nodes and bone metastases without any identifiable lesions in the parathyroid glands.

During the current hospitalization, her serum calcium level normalized after she received intravenous calcium gluconate infusions. The patient experienced marked improvement in her

120 symptoms following normalization of serum calcium. Magnesium sulfate was infused to treat
121 hypomagnesaemia. Serum PTH levels remained persistently suppressed despite normalization
122 of serum calcium and magnesium (Fig. 1). Treatment with oral calcium carbonate and calcitriol
123 was started as maintenance therapy for hypocalcemia. At two weeks following discharge from
124 the hospital and continued treatment with calcium and calcitriol, her serum calcium was normal.
125 However, her serum PTH level remained persistently low.

126 Due to the terminal nature of her disease and the inconvenience of additional medical
127 appointments, the patient decided on regularly scheduled follow-ups only with her oncologist at
128 another institution. We called the patient and her oncologist, learning that nivolumab therapy
129 was replaced with a different chemotherapy regimen in March 2018 due to progression of
130 mesenteric nodal metastatic disease. The oncologist reported that the patient's serum calcium
131 was low in March 2018 after the patient stopped taking calcitriol for a few days. Given the nature
132 of her illness, the patient was reluctant to obtain regular laboratory testing.

133 Written consent was obtained from the patient for blood tests and publication.

134 **Measurement of CaSR-binding antibodies**

135 CaSR immunoprecipitation assays for detecting CaSR antibodies were carried out as before
136 (12). The patient's serum and healthy control sera (n = 10) were stored at -80℃. Human
137 embryonic kidney 293 (HEK293) cells were transiently transfected with plasmid pcCaSR-FLAG,
138 and cell extracts were prepared containing expressed CaSR-FLAG protein (11). Aliquots (50-µl)
139 of GammaBind® Sepharose beads (Amersham Biosciences, Little Chalfont, UK) were mixed
140 with patient serum, control sera or positive control anti-CaSR antiserum (Alpha Diagnostic
141 International, San Antonio, TX) in duplicate at 1:100 dilution in 1 ml of immunoprecipitation
142 buffer and incubated for 1 h at 4℃. The bead/IgG complexes were collected by centrifugation
143 and incubated with cell extract containing CaSR-FLAG protein at 4℃ for 16 h. The
144 bead/IgG/protein complexes were collected by centrifugation, washed, denatured, and

145 subjected to SDS-PAGE in 7.5% polyacrylamide gels. The separated proteins were transferred
146 onto Trans-Blot® Transfer Membranes (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK)
147 using standard protocols. Immunoprecipitated CaSR-FLAG protein was detected using anti-
148 FLAG® M2-Peroxidase Conjugate (Sigma-Aldrich, Poole, UK) and an ECL™ Western Blotting
149 Analysis System (Amersham Biosciences) with a final exposure to pre-flashed x-ray film for 5
150 min. Densitometry of the developed films was performed in a Bio-Rad GS 690 Scanning
151 Densitometer with Multi-Analyst Version 1.1 Software (Bio-Rad Laboratories Ltd.), which
152 produced a densitometry value for each individual band. A CaSR antibody index for each serum
153 sample in the immunoprecipitation assay was calculated as the densitometry value of the tested
154 serum/mean densitometry value of 10 control sera. The upper normal limit for the assay was
155 calculated using the mean CaSR antibody index + 3 SD of these control individuals. A CaSR
156 antibody index above the upper normal limit was designated as positive for CaSR antibody
157 reactivity. All assays were run blinded to avoid operator bias.

158 **Measurement of CaSR-activating antibodies**

159 As detailed elsewhere (11), the response of the CaSR to Ca^{2+} was assessed in HEK293 cells
160 stably expressing the receptor (HEK293-CaSR) by measuring intracellular inositol-1-phosphate
161 (IP1) accumulation using an IP-One ELISA Kit (CIS Bio International, Gif-sur-Yvette, France).
162 Monolayer HEK293-CaSR cells were cultured in 24-well plates. The cells were washed with
163 serum-free medium and then Ca^{2+}-free assay buffer containing 10 mM lithium chloride. For
164 investigating antibody effects, cells were pre-incubated for 10 min at 37℃ with duplicate IgG
165 samples (1:100 in assay buffer), which had been prepared from the patient's and control sera (n
166 = 10) by standard methods (11). Assay buffer containing varying concentrations of calcium
167 chloride (0-5 mM final concentration) was added to the cells followed by incubation for 60 min at
168 37℃. HEK293-CaSR cells without pre-incubation with IgG were also included in experiments as
169 controls. Following incubation, cells were lysed for 30 min at 37°C with 50 µl of 2.5% IP-One

170 ELISA Kit Lysis Reagent (CIS Bio International). The accumulation of intracellular IP1 was
171 measured according to an IP-One ELISA Kit, an immunoassay based on competition between
172 free IP1 and IP1-horse-radish peroxidase (HRP) conjugate for binding to anti-IP1 monoclonal
173 antibody. The results for IP1 accumulation were expressed as: percentage inhibition of IP1-HRP
174 binding = [1 - IP1-HRP binding in stimulated cells/IP1-HRP binding in unstimulated cells] x 100.
175 Increasing IP1 accumulation in the HEK293-CaSR cells is reflected by an increase in the
176 percentage inhibition of IP1-HRP binding.

177 **Measurement of NALP5 antibodies**

178 A TnT® T7-Coupled Reticulocyte Lysate System (Promega, Southampton, UK) was used to
179 produce [^{35}S]-labelled NALP5 protein in vitro from the transcription-translation of NALP5 cDNA
180 in plasmid pCMV6-XL5-NALP5 (a gift from Professor Olle Kampe, University Hospital, Uppsala
181 University, Uppsala, Sweden), as detailed in the manufacturer's protocol. Radioligand binding
182 assays (RLBA) were carried out as detailed elsewhere (13). In brief, for each RLBA, an aliquot
183 of NALP5 in vitro transcription-translation reaction containing 100,000 counts per minute (cpm)
184 of trichloroacetic acid-precipitable material, as determined per the manufacturer's instructions,
185 was suspended in 50 μl of IP buffer (20 mM Tris-hydrochloride, pH 8.0, 150 mM sodium
186 chloride, 1% Triton X-100, and 10 mg/ml aprotinin (Bayer, Newbury, UK)). A sample of patient
187 or healthy control serum was then added to a final dilution of 1:100. Anti-NALP5 polyclonal goat
188 antibody (Santa Cruz Biotechnology Inc., Dallas, TX) was used at 1:200 a dilution as a positive
189 control. All serum and antibody samples were tested in duplicate. After overnight incubation at 4
190 C, 50 μl of protein G Sepharose™ 4 Fast Flow (GE Healthcare Life Sciences, Little Chalfont,
191 UK), prepared according to the manufacturer, were added followed by incubation for 1 h at 4°C.
192 Subsequently, the protein G Sepharose-antibody-antigen complexes were collected by
193 centrifugation and washed six times for 15 min in immunoprecipitation buffer at 4℃. The
194 complexes were then transferred to 1 ml of Ultima-Gold® XR scintillation fluid (Packard

195 Bioscience, Groningen, The Netherlands) and immunoprecipitated cpm evaluated in a Beckman
196 LS 6500 Multi-Purpose Scintillation Counter (Beckman Coulter, Inc., Fullerton, CA). A NALP5
197 antibody index for each serum was calculated as: cpm immunoprecipitated by tested
198 serum/mean cpm immunoprecipitated by 10 healthy control sera. Each serum was tested in
199 duplicate in two experiments and the mean NALP5 antibody index calculated. The upper limit of
200 normal for the NALP5 antibody RLBA was calculated using the mean NALP5 antibody index +
201 3SD of 10 healthy controls. Any serum sample with NALP5 antibody index above the upper limit
202 of normal was designated as NALP5 antibody-positive.

203 **Measurement of cytokine antibodies**

204 Antibodies against cytokines were detected in enzyme-linked immunosorbent assays (ELISAs)
205 (14, 15). Interleukin (IL)-22, IL-17A, IL-17F (R and D Systems, Minneapolis, MN), interferon
206 (IFN)-omega, IFN-alpha2A and IFN-lamda1 (IL-29) (Sigma-Aldrich) were prepared according to
207 the manufacturer's instructions. For ELISAs, the required cytokine was diluted in phosphate-
208 buffered saline (PBS) to 0.1 µg/ml and 100-µl samples used to coat the wells of a Corning
209 polystyrene 96-well microtitre plate (Bibby Sterilin Ltd., Bargoed, UK). The plates were then
210 incubated overnight at 4℃. Excess peptide was removed by decanting, and the wells were
211 blocked with blocking buffer (PBS containing 0.1% Tween-20 and 3% bovine serum albumin)
212 for 30 min at 37℃. Plates were washed four times with washing buffer (PBS containing 0.1%
213 Tween-20). Aliquots (100 µl) of serum at a 1:100 dilution in blocking buffer were added to the
214 wells. PBS was applied as a control. The plates were incubated at room temperature for 1 h and
215 then washed four times with washing buffer. Aliquots (100 µl) of anti-human IgG alkaline
216 phosphatase-conjugate (Sigma-Aldrich) diluted to 1:2000 in blocking buffer were added to the
217 wells for 1 h at room temperature. After washing five times with washing buffer, 100 µl of
218 alkaline phosphatase substrate Sigma Fast p-Nitrophenyl Phosphate (Sigma-Aldrich) were
219 applied to each well and plates incubated at room temperature to allow colour development. A

220 LabSystems Integrated EIA Management System (Life Sciences International, Basingstoke,
221 UK) was used to read absorption of the wells at 405 nm. All sera were tested in duplicate and
222 the average OD_{405} value taken. The binding reactivity of each patient and control sera to each
223 cytokine was expressed as an antibody index calculated as: mean OD_{405} of tested serum/mean
224 OD_{405} of 10 healthy control sera. Each serum was tested in two experiments and the mean
225 antibody index calculated. The upper limit of normal for each ELISA was calculated using the
226 mean antibody index + 3SD of 10 healthy control sera. Patient sera with an antibody index
227 greater than the upper limit of normal were regarded as positive for cytokine antibodies.

228 **Results**

229 **Detection of CaSR autoantibodies in the patient's serum**

230 Initially, a CaSR immunoprecipitation assay, as detailed in Materials and Methods, was used to
231 analyse the patient's serum for CaSR autoantibodies. The upper limit of normal for the CaSR
232 immunoprecipitation assay was estimated as a CaSR antibody index of 2.81, calculated from a
233 population of 10 healthy control sera (Fig. 2). The serum sample from the patient tested positive
234 for CaSR autoantibodies with a CaSR antibody index of 40.1 (Fig. 2). The positive control anti-
235 CaSR antiserum had a CaSR antibody index of 20.5 (Fig. 2).

236 Subsequently, the response to Ca^{2+} of the CaSR expressed in HEK293 cells was assessed by
237 measuring intracellular IP1 accumulation as described in Materials and Methods. To determine
238 the effects of IgG from the patient and healthy controls (n = 10) on the response of the CaSR to
239 Ca^{2+}, HEK293-CaSR cells were incubated with IgG samples at a 1:100 dilution prior to no
240 stimulation or stimulation with a range of Ca^{2+} concentrations (0.5, 1.5, 3.0, and 5.0 mM). Each
241 experiment included HEK293-CaSR cells stimulated with Ca^{2+} alone. IP accumulation was
242 measured using the IP-One ELISA Kit as detailed in Materials and Methods and the percentage
243 inhibition of IP1-HRP binding was calculated.

244 Of the 10 control IgG samples analyzed, none had any effect upon the levels of IP1
245 accumulation when compared with stimulation by Ca^{2+} alone (Fig. 3). In contrast, the IgG
246 sample from the patient significantly increased the levels of IP1 accumulation at Ca^{2+}
247 concentrations of 0.5, 1.5 and 3.0 mM when compared with Ca^{2+}-stimulation alone (P values $<$
248 0.001, one-way ANOVA) (Fig. 3). The IgG from the patient had no detectable effect on IP1
249 accumulation, however, when no Ca^{2+} was added to the buffer, indicating that some degree of
250 receptor activation by its natural ligand, Ca^{2+}, was needed to see the functional effect of the IgG.
251 The results indicated the presence of CaSR-stimulating activity in the patient IgG sample.

252 **Detection of NALP5 and cytokine autoantibodies in the patient's serum**

253 The patient's serum sample was evaluated for NALP5 and cytokine antibodies using RBLAs
254 and ELISAs, respectively, as detailed in Materials and Methods. The upper limits of normal for
255 antibody assays against IL-22, IL-17F, IL-17A, IFN-omega, IFN-alpha2A, and IFN-lamda1, and
256 NALP5 were antibody indices of 1.29, 1.25, 1.33, 1.89, 2.03, 1.70, and 1.81, respectively. The
257 patient was negative for autoantibodies against NALP5 and all the cytokines tested; antibody
258 indices were 1.01, 0.98, 0.91, 0.75, 1.02, 1.11, and 1.07 for assays measuring autoantibodies
259 against IL-22, IL-17F, IL-17A, IFN-omega, IFN-alpha2A, and IFN-lamda1, and NALP5,
260 respectively.

261 **Discussion**

262 The biochemical investigations carried out here strongly suggest hypoparathyroidism as the
263 cause of the patient's hypocalcemia. As there was no history of neck radiation, surgery or
264 infiltration of the parathyroids on imaging, an autoimmune etiology of hypoparathyroidism
265 seemed most likely. Furthermore, persistently low PTH despite normalization of serum
266 magnesium excluded the possibility of hypomagnesemia as the cause of hypocalcemia and
267 hypoparathyroidism.

268 Although the parathyroid glands are not the target of most autoimmune diseases, autoimmunity
269 is an important cause of hypoparathyroidism, either as an isolated endocrinopathy or as a
270 component of APS1 (16, 17). Autoimmune hypoparathyroidism can be caused by permanent
271 hypoparathyroidism owing to irreversible, immune-mediated damage to the parathyroid glands
272 or functional hypoparathyroidism due to antibody-induced activation of signaling pathway(s)
273 regulating parathyroid function (18). In APS1, common autoantibody targets include parathyroid-
274 expressed NALP5 and cytokines. However, testing carried out here revealed that the patient
275 was not positive for autoantibodies against NALP5, a marker for parathyroid autoimmunity, or a
276 panel of interferons and interleukins which are diagnostic for APS1 (13-15). As such, the patient
277 was not diagnosed with multiple endocrine autoimmune disease to account for her low
278 parathyroid function. Autoantibodies directed against the parathyroid-expressed CaSR have
279 been recognized to be present in the serum of patients with autoimmune hypoparathyroidism
280 (16), including autoantibodies that can activate the receptor, thereby causing reduced PTH
281 secretion (10, 11).

282 Antibody tests of the patient's serum confirmed our diagnosis of hypoparathyroidism caused by
283 the development of CaSR-activating autoantibodies. Autoantibody-activation of the CaSR was
284 indicated by increased inositol-1-phosphate levels in a cell line expressing the CaSR following
285 treatment with the patient's IgG; the phosphoinositide pathway being a key intracellular mediator

286 of CaSR activation. Thus, by shifting the calcium-PTH curve to the left and decreasing the set-
287 point of the CaSR, PTH is not released at lower serum calcium concentrations resulting in
288 hypocalcemia. The temporal relationship between the initiation of nivolumab infusions suggests
289 that this drug is likely responsible for the development of the CaSR-activating autoantibodies,
290 although was cannot rule out with certainty that the development of anti-CaSR antibodies and
291 hypoparathyroidism were coincidental. Although the patient's hypoparathyroidism appears
292 persistent despite discontinuation of nivolumab, it is possible that improvement could take place
293 with longer follow-up. To the best of our knowledge this is the first report illustrating a case of
294 autoimmune hypoparathyroidism induced by ICI-blockade as the underlying cause.

295 The reasons for differential autoimmune involvement of the endocrine glands between anti-PD1
296 and anti-CTLA-4 therapy are not entirely clear (2). Higher risk of hypophysitis with anti-CTLA-4
297 therapy such as ipilimumab compared to anti-PD1 pembrolizumab may be due to the
298 expression of CTLA-4 in the normal pituitary gland (2). In contrast to the high risk of
299 hypophysitis with anti-CTLA-4 therapy, autoimmune thyroiditis appears to be more common in
300 anti-PD1 therapy. It has been speculated that in addition to effects on T cell immunity, anti-PD1
301 therapy may augment humoral immunity, thus boosting anti-thyroid antibody formation (2).
302 Careful studies of ICIs in vitro and in animal models have also shown that their complex role in
303 T cell immunity and blockade may involve the inhibition of regulatory T cells, which could be a
304 factor in the induction of autoimmunity (19).

305 Given the high risk of autoimmune diseases, including endocrinopathy, during treatment with
306 ICIs, the risk of toxicity should always be balanced against the benefit that may derive from the
307 ICI-blockade. As ICIs are now used to treat many cancers, clinicians should be aware of the
308 potential risk for hypocalcemia associated with their use.

309

310

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376 **Figure Legends**

377 **Figure 1. Time course for changes in biochemical laboratory tests.** The patient's ionized
378 calcium, magnesium, and PTH levels during admission to our hospital are shown. The normal
379 range is indicated by the hatched boxes: ionized calcium, 1.12-1.31 mmol/L; magnesium, 1.62-
380 2.62 mg/dL; and PTH, 12-65 pg/mL.

381 **Figure 2. Detection of patient CaSR autoantibodies.** Serum from the patient, healthy controls
382 (n = 10), and positive control anti-CaSR antiserum were tested in CaSR immunoprecipitation
383 assays at a 1:100 dilution and assigned a CaSR antibody index as detailed in Materials and
384 Methods. The upper limit of normal for the CaSR immunoprecipitation assay was calculated
385 using the mean CaSR antibody index + 3 SD of 10 control sera, which gave a CaSR antibody
386 index value of 2.81. A CaSR antibody index above the upper normal limit was designated as
387 positive for anti-CaSR antibody reactivity. The CaSR antibody index of the patient's sample
388 (40.1) and the positive control anti-CaSR antiserum (20.5) are shown and are positive.

389 **Figure 3. Effect of patient IgG on CaSR activity.** Intracellular IP1 accumulation in HEK293-
390 CaSR cells was measured in response to Ca^{2+}-stimulation (final concentrations of 0-5 mM) after
391 cells were pre-incubated with the patient's IgG sample or healthy control IgG at a 1:100 dilution.
392 Cells without pre-incubation with IgG were also included. The accumulation of intracellular IP1
393 was measured using an IP-One ELISA Kit, an immunoassay based on competition between free
394 IP1 and IP1-horse-radish peroxidase (HRP) conjugate for binding to anti-IP1 monoclonal
395 antibody. The results for IP1 accumulation were expressed as: percentage inhibition of IP1-HRP
396 binding = [1 - IP1-HRP binding in stimulated cells/IP1-HRP binding in unstimulated cells] x 100.
397 Increasing IP1 accumulation in the HEK293-CaSR cells is reflected by an increase in the
398 percentage inhibition of IP1-HRP binding. The results show IP1 accumulation (mean ± SD of six
399 experiments) in Ca^{2+}-stimulated HEK293-CaSR cells that were pre-incubated with either IgG
400 from the patient or IgG from a single control, or that were not pre-incubated with IgG. Only the

401 patient's IgG sample significantly increased the levels of IP1 accumulation when compared with

402 Ca^{2+}-stimulation (at 0.5, 1.5, and 3 mM) of HEK293-CaSR cells not pre-incubated with IgG: *P

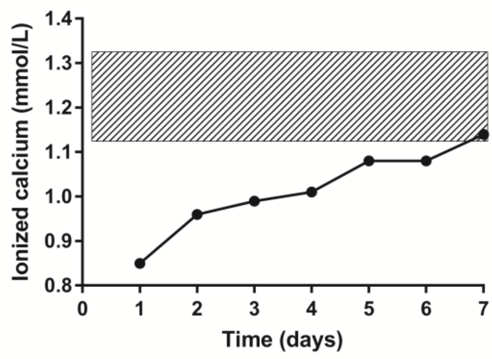
403 values were < 0.001 (One-way ANOVA).

Table 1. Laboratory Test Results Upon Admission to Our Hospital

Test	Results	Normal range
Calcium	5.8 mg/dL (Low)	8.3-10 mg/dL
Albumin	4.2 g/dL (Normal)	3.6-4.8 g/dL
Ionized calcium	0.85 mmol/L (Low)	1.12-1.32 mmol/L
PTH	7.77 pg/mL (Low)	12-65 pg/mL
Magnesium	1.4 mg/dL (Low	1.6-2.6 mg/dL
Potassium	3.4 mEg/L (Low)	3.6-5.6 mEq/L
Phosphate	4.3 mg/dL (Normal)	2.5-4.5 mg/dL
25-hydroxy vitamin D	23.8 ng/mL (Low)	30-200 ng/mL
1,25-dihydroxy vitamin D	10.6 pg/mL (Low)	19.9-79.3 pg/mL
TSH	0.99 mIU/mL (Normal)	0.45-4.5 mIU/mL
EKG-QTc	492 sec (High)	431-470 sec (women)

Figure 1

(a)



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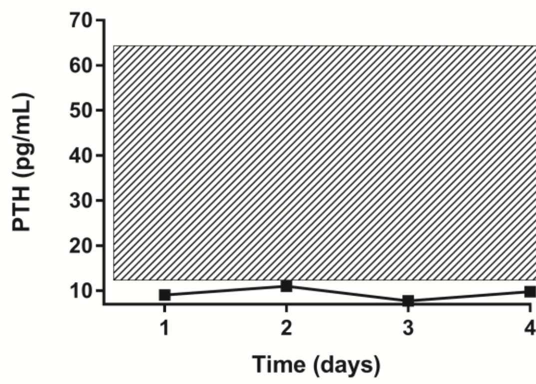

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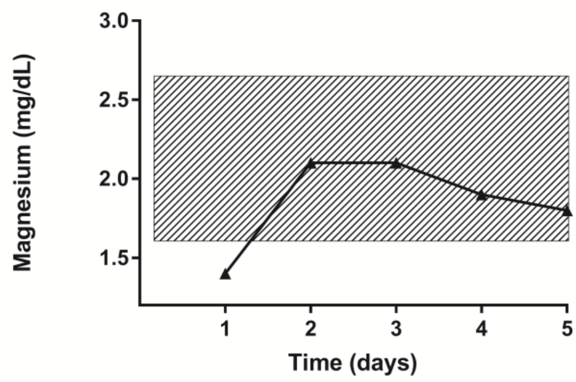

Figure 2

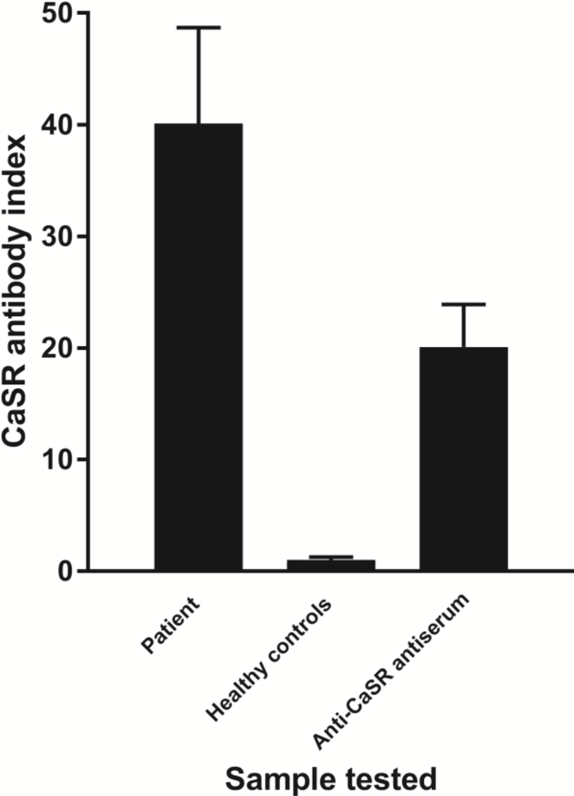

Figure 3