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Immunosuppressive therapy of autoimmune hypoparathyroidism in a patient with activating autoantibodies against the calcium-sensing receptor

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Short Title: Immunosuppression of autoimmune hypoparathyroidism

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CONFLICT OF INTEREST STATEMENT

All authors declare there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

KEYWORDS

Autoantibody; autoimmunity; azathioprine; calcium-sensing receptor; hypoparathyroidism; immunosuppression; prednisone

ABBREVIATIONS

AHH, autoimmune hypocalciuric hypercalcemia; AKI, acute kidney injury; CaSR, calcium-sensing receptor; ER, emergency room; ERK1/2, extracellular signal-regulated kinase 1 and 2; FHH, familial hypocalciuric hypercalcemia; IP, inositol phosphate

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Summary

Context: Activating antibodies directed at the extracellular calcium-sensing receptor (CaSR) have been described in autoimmune hypoparathyroidism in the setting of isolated hypoparathyroidism or autoimmune polyglandular syndrome type 1.

Materials and methods: A 34-year-old female presented with hypocalcemia (6.0 mg/dL) and hypomagnesemia (1.1 mg/dL) accompanied by low serum PTH (2.4 pg/mL) as well as urinary calcium and magnesium wasting. She was diagnosed with hypoparathyroidism, which was refractory to standard therapy. She was started on 60 mg prednisone and 150 mg azathioprine treatment daily on suspicion of an autoimmune aetiology. The patient was tested for CaSR antibodies.

Results: The patient was positive for CaSR antibodies of the IgG1 subtype, which stimulated phosphorylation of extracellular signal-regulated kinases 1 and 2 (ERK1/2) and inositol phosphate (IP) accumulation. Post-treatment with prednisone and azathioprine, her serum calcium and magnesium normalised, as did her CaSR antibody titre and antibody-mediated stimulation of ERK1/2 phosphorylation and IP accumulation.

Conclusion: This is the first demonstration of CaSR antibody-mediated hypoparathyroidism responsive to immunosuppressive therapy, adding to the evidence that autoimmune hypoparathyroidism can be, in some cases, reversible and not the result of autoimmune parathyroid destruction.

INTRODUCTION

Blizzard and coworkers were the first to demonstrate anti-parathyroid antibodies in patients with idiopathic hypoparathyroidism, although the parathyroid antigen(s)'s identity was uncertain.[1] Subsequently, the extracellular calcium-sensing receptor (CaSR) was identified as a target of anti-parathyroid antibodies.[2-4] Later, inactivating antibodies against the receptor were demonstrated in four patients with PTH-dependent hypercalcemia, three of whom also had hypocalciuria.[5] This clinical presentation is similar to that of familial hypocalciuric hypercalcemia (FHH), which is, in most cases, caused by inactivating CaSR mutations.[6] CaSR antibody-mediated inhibition of CaSR activity in two of these cases was demonstrated by showing antibody-mediated inhibition of Ca^{2+}-elicited activation of inositol phosphate (IP) accumulation and extracellular-regulated kinases 1 and 2 (ERK1/2) activity.[5]

The same group then demonstrated activating anti-CaSR antibodies in two patients with autoimmune hypoparathyroidism.[7] These patients' clinical presentation resembled that of autosomal dominant hypoparathyroidism caused by activating CaSR mutations.[6] While very uncommon, additional examples of inactivating or activating CaSR antibodies have been reported in PTH-dependent hypercalcemia or hypocalcemia, respectively.[8-11] In one case with inactivating antibodies, the hypercalcemia was steroid responsive, while in a second it was not.[8,9]

Here, we report a unique case of severe hypoparathyroidism caused by activating antibodies to the CaSR, which was responsive to immunosuppressive therapy.

2 MATERIALS AND METHODS

2.1 Patient clinical history

A 34-year-old Caucasian female with a past medical history of hypertension, arthritis, and obesity presented to our institution with perioral and bilateral hand numbness, tingling and muscle cramps. Four months prior, she was seen at an outside hospital emergency room (ER) for abdominal pain. A CT scan of the abdomen and pelvis did not reveal any intra-abdominal pathology. A basic metabolic panel was normal, including total serum calcium of 9.0 mg/dL. She was discharged with a diagnosis of viral gastroenteritis. Eight weeks later, she presented back to the same ER with bilateral hand and perioral numbness and tingling for four days. Physical exam revealed a positive Chvostek's sign. Total serum calcium was 7.5 mg/dL (normal range, 8.3-10.6 mg/dL), PTH was inappropriately normal at 48 pg/mL (normal range, 14-72 pg/mL), magnesium was 1.5 mg/dL (normal range, 1.8-2.4 mg/dL), and ionised calcium was 3.2 mg/dL (normal range, 4.6-5.4 mg/dL). Intravenous calcium gluconate and magnesium sulphate was administered, and she was discharged on high dose calcium carbonate (1500 mg three times daily) and magnesium oxide (400 mg daily). The following day she presented to the same ER with similar symptoms. Total serum calcium was 7.5 mg/dL and magnesium was 1.6 mg/dL. She received intravenous magnesium and was discharged with nephrology follow-up.

After nephrology evaluation, she was initiated on calcitriol (0.25 µg twice daily) and ergocalciferol weekly; calcium carbonate and magnesium oxide were continued. Outpatient laboratory studies two days after the patient's visit showed total

serum calcium of 6.4 mg/dL, potassium 3.5 mg/dL (normal range, 3.5-5.1 mg/dL), and magnesium of 1.5 mg/dL. PTH was 30.5 pg/mL, and 25-hydroxyvitamin D was 22.5 ng/mL (normal, > 30 ng/mL). One week later, she presented to the ER again with symptomatic hypocalcemia. She was given intravenous magnesium and calcium and discharged home. She returned to the ER the same evening with persistent symptomatic hypocalcemia. Total serum calcium was noted to be 6.3 mg/dL, ionised calcium 3.2 mg/dL, and magnesium 1.4 mg/dL. She was discharged after receiving intravenous calcium gluconate and magnesium sulphate. The following day, she presented back to the ER with worsening perioral and bilateral hands numbness and tingling as well as blurry vision and diplopia. An electrocardiogram showed prolonged QTc, and physical exam revealed positive Chvostek's sign. She was transferred to a university hospital for persistent symptomatic hypocalcemia and hypomagnesemia.

At the university hospital, additional work-up included serum cortisol level which was 14.2 μ/dL (normal range, 6.7-22.6 μ/dL). Repeat PTH was 4.0 pg/ml and phosphate 6.3 mg/dL (normal range, 2.5-4.5 mg/dL). Renal ultrasound showed right and left kidneys measured 12.2 cm and 10.9 cm, respectively, with normal echogenicity, no masses, calculi or nephrocalcinosis. Thyroid ultrasound showed the right and left lobes measured 4.8 cm and 5.5 cm, respectively. No discrete thyroid nodules or cysts were visualized. The parathyroid glands were not appreciated. She continued to receive intravenous calcium and magnesium and was discharged after two and a half weeks on oral amlodipine for hypertension, calcitriol, magnesium and calcium supplements, and ergocalciferol. She presented back to the same hospital within 48 h with recurrent symptomatic hypocalcemia. Total serum calcium was 6.5 mg/dL, phosphate was 5.9

mg/dL, and magnesium was 1.5 mg/dL. She received intravenous calcium and magnesium supplementation daily and was discharged after five days on calcitriol, calcium and magnesium.

The following day, the patient was presented to our institution for persistent symptomatic hypocalcemia, this being her third hospitalisation. Physical examination was notable for a positive Chvostek's sign. Total serum calcium was 6.0 mg/dL, magnesium 1.1 mg/dL, and PTH 2.4 pg/ml. Fractional excretion of calcium and magnesium were 6.45% and 23%, respectively. She was diagnosed with severe hypocalcemia and hypomagnesemia due to acquired hypoparathyroidism. She received daily intravenous calcium and magnesium to maintain serum levels close to or within the respective normal ranges. Her subsequent treatment with immunosuppressive therapy is detailed in the results section.

2.2 CaSR immunoprecipitation assays

CaSR immunoprecipitation assays for detecting CaSR antibodies were carried out as before.[12] The patient's pre- and post-immunosuppressive treatment serum samples (n = 2), and previously studied healthy control sera (n = 10),[12] were stored at -80°C. Human embryonic kidney 293 (HEK293) cells were transiently transfected with plasmid pcCaSR-FLAG. Cell extracts containing expressed CaSR-FLAG protein were prepared. Aliquots (50-µl) of GammaBind® Sepharose beads (Amersham Biosciences, Little Chalfont, UK) were mixed with sera (1:100 dilution) in duplicate in 1 ml of immunoprecipitation buffer and incubated for 1 h at 4°C. The bead/IgG complexes were collected by centrifugation and incubated with cell extract containing CaSR-FLAG

protein at 4°C for 16 h. The bead/IgG/protein complexes were collected by centrifugation, washed, denatured, and subjected to SDS-PAGE in 7.5% polyacrylamide gels. The separated proteins were transferred onto Trans-Blot® Transfer Membranes (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK) using standard protocols. Immunoprecipitated CaSR-FLAG protein was detected using anti-FLAG® M2-Peroxidase Conjugate (Sigma-Aldrich, Poole, UK) and an ECL™ Western Blotting Analysis System (Amersham Biosciences) with a final exposure to pre-flashed x-ray film for 5 min. Densitometry was performed in a Bio-Rad GS 690 Scanning Densitometer with Multi-Analyst Version 1.1 Software (Bio-Rad Laboratories Ltd.).

A CaSR antibody index for each serum in the immunoprecipitation assay was calculated as the densitometry value of the tested serum/mean densitometry value of 10 control sera. The upper normal limit for the assay was calculated using the mean CaSR antibody index + 3 SD of these control individuals. A CaSR antibody index above the upper normal limit was designated as positive for CaSR antibody reactivity. All assays were run blinded to avoid operator bias.

2.3 Measurement of CaSR-stimulated inositol-1-phosphate accumulation

The CaSR's response to Ca^{2+} was assessed in HEK293 cells stably expressing the receptor (HEK293-CaSR) by measuring intracellular inositol-1-phosphate (IP1) accumulation using a specific IP-1 ELISA (CIS Bio International, Gif-sur-Yvette, France).[11] The IP-1 ELISA is highly specific, with no cross-reactivity to myo-inositol, IP2, IP3 or IP4. Results obtained using this assay are comparable to those measuring inositol phosphate by tritium-labeling.[13]

Monolayer HEK293-CaSR cells were cultured as in 24-well plates.[11] The cells were washed with serum-free medium and then Ca^{2+}-free assay buffer containing 10 mM lithium chloride.[11] Cells were pre-incubated for 10 min at 37°C with duplicate IgG samples (1:100 in assay buffer) prepared from the patient's and control sera.[11] Assay buffer containing varying concentrations of calcium chloride (0-5 mM final concentration) was added to the cells followed by incubation for 60 min at 37°C. HEK293-CaSR cells without pre-incubation with IgG were included as experimental controls. Following incubation, cells were lysed for 30 min at 37°C with 50 µl of 2.5% IP-One ELISA Kit Lysis Reagent (CIS Bio International, Gif-sur-Yvette, France).

The accumulation of intracellular IP1 was measured (IP-One ELISA Kit, CIS Bio International), an immunoassay based on competition between free IP1 and IP1-horseradish peroxidase (HRP) conjugate for binding to anti-IP1 monoclonal antibody. IP1 accumulation is expressed as: percentage inhibition of IP1-HRP binding = [1 - IP1-HRP binding in stimulated cells/IP1-HRP binding in unstimulated cells] x 100. Increased IP1 accumulation in the HEK293-CaSR cells produces an increase in the percentage inhibition of IP1-HRP binding.

2.4 Measurement of CaSR-stimulated ERK1/2 phosphorylation

The CaSR's response to Ca^{2+} was also assessed by measuring phosphorylation of extracellular signal-regulated kinases 1 and 2 (ERK1/2).[11] HEK293-CaSR cell monolayers grown in 96-well plates were incubated with IgG samples and Ca^{2+} exactly as above. HEK293-CaSR cells without pre-incubation with IgG were included as controls. Following incubation, cells were fixed for 20 min at room temperature with 100

µl of 4% Cell Fixing Buffer (Cellular Activation of Signaling ELISA (CASE™) Kit, SuperArray Bioscience Corporation, Frederick, MD, USA). ERK1/2 phosphorylation was measured using the CASE™ Kit (SuperArray Bioscience Corporation), and results are expressed as the ratio of phosphorylated ERK1/2 to total ERK1/2.[11]

2.5 CaSR peptide ELISAs

Peptide ELISAs were used to verify the binding of CaSR antibodies to identified CaSR epitopes. Lyophilised peptides were solubilised and stored according to the manufacturer's instructions (Cambridge Peptides Ltd., Birmingham, UK). For ELISAs, the required peptide was diluted in PBS to 200 ng/ml, and 100-µl aliquots were used to coat the wells of a 96-well microtiter plates. The plates were then incubated overnight at 4°C. Excess peptide was removed by decanting, and the wells were blocked with blocking buffer (PBS containing 0.1% (w/v) Tween-20 and 3% (w/v) bovine serum albumin) for 30 min at 37°C. Plates were washed four times with washing buffer (PBS containing 0.1% (v/v) Tween-20). Duplicate 100-µl samples of sera at a 1:200 dilution in blocking buffer were added to the wells. PBS was applied as a control to measure any non-specific binding of ELISA reagents in the absence of sera. The plates were incubated at room temperature for 1 h and then washed four times with washing buffer. 100-µl of goat anti-human IgG conjugated to alkaline phosphatase (Sigma-Aldrich), diluted to 1:2000 in blocking buffer, was added to each well for 1 h at room temperature. After washing five times with washing buffer, 100 µl of alkaline phosphatase substrate Sigma Fast *p*-nitrophenyl phosphate (Sigma-Aldrich) were applied to each well and plates incubated at room temperature to allow color development. A LabSystems

Integrated EIA Management System spectrophotometer (Life Sciences International, Basingstoke, UK) was used to read absorption of the wells at 405 nm.

All sera were tested in duplicate, and the average OD_{405} value taken. The CaSR peptide-binding reactivity of each patient and control serum was expressed as an antibody index calculated as: mean OD_{405} of tested serum/mean OD_{405} of a population of 16 healthy control sera. Each serum was tested in three experiments, and the mean antibody index calculated. For each ELISA, the upper limit of normal was calculated as mean antibody index + 3SD of 16 healthy individuals. Sera with an antibody index above the upper limit of normal were designated as positive for CaSR antibodies.

For estimating antibody titres, sera were analysed at dilutions ranging from 1:200 to 1:10,000. Titers were defined as the serum dilution at which antibody binding could still be detected above the upper limits of normal for the CaSR peptide ELISAs, as detailed above. To determine the IgG subclass of purified CaSR antibodies, anti-human IgG1, IgG2, IgG3 and IgG4 alkaline phosphatase-conjugates (Southern Biotech, Birmingham, AL, USA) were applied as the secondary antibody at a 1:2000 dilution, and antibody positivity was defined as above.

2.6 IgG and CaSR antibody purification

IgG was isolated from sera using protein G-Sepharose 4 Fast Flow (Amersham Biosciences AB, Uppsala, Sweden) affinity chromatography, according to the manufacturer's instructions. IgG was eluted using 0.2 M glycine hydrochloride (pH 3.0), and the collected 1-ml fractions neutralized with 50 µl of 1 M Tris base (pH 9.0).

Fractions containing IgG, as determined by photometry at 280 nm, were extensively dialysed against PBS, and concentrated using an Amicon Concentrator (Amicon Inc., Beverly, MA, USA). IgG samples were sterilised with a Millex Filter Unit (Millipore Corp., Bedford, MA, USA) and stored at 10 mg/ml at –20°C. Antibodies against specific CaSR epitopes were purified using peptide affinity chromatography. The required CaSR peptides (2 mg) were coupled to CarboxyLink™ Columns according to a CarboxyLink™ Immobilization Kit (ThermoFisher Scientific, Altrincham, UK). IgG samples were applied to the peptide affinity columns in PBS, eluted in IgG Elution Buffer (ThermoFisher Scientific), and then dialysed, concentrated, and stored as detailed above.

Statistical analyses

Statistical analyses were performed using Student's unpaired *t* tests. *P* values < 0.05 (two-tailed) were regarded as statistically significant.

3 Results

3.1 Response of severe hypocalcemia and acquired hypoparathyroidism to immunosuppressive therapy

The patient had no previous personal or family history of abnormalities of calcium or magnesium metabolism, hence ruling out a genetic cause of hypoparathyroidism; therefore, an autoimmune basis was considered. Antibodies to PTH were assayed, but were not detected. The possibility that CaSR-activating antibodies were causing hypocalcemia and low PTH was investigated since such autoantibodies have been reported previously in individuals with autoimmune hypoparathyroidism.[7-11] While testing for antibodies was underway, she was started on immunosuppressive treatment with prednisone 60 mg and azathioprine 150 mg daily, given the severity of her clinical presentation and clinical course. In addition, she continued to receive calcitriol and oral calcium and magnesium supplements. This approach was based on the assumption that reducing the titre of such antibodies might be beneficial in this severe case of hypoparathyroidism unresponsive to aggressive therapy of calcium and magnesium supplementation. Moreover, in a previously described case of inactivating CaSR antibodies as the cause of PTH-dependent, hypocalciuric hypercalcemia, the patient's hypercalcemia remitted during glucocorticoid therapy.[8] Furthermore, given the morbidities associated with long-term steroid use, including, but not limited to, diabetes mellitus, and avascular necrosis of the hip, azathioprine was added as a steroid-sparing agent, since it was anticipated that the patient would require long-term immunosuppression based on the earlier report.[8] Azathioprine is one of the oldest

immunosuppressive drugs in continuous use. It blocks the de novo pathway of purine synthesis, and its relative specificity for lymphocytes results from their lack of a salvage pathway.[14] Azathioprine has been used as a steroid-sparing agent for the long-term maintenance therapy for autoimmune diseases, such as lupus nephritis and pauci-immune glomerulonephritis.[15-16]

The patient's total and ionised calcium, magnesium, and phosphate levels prior to and subsequent to treatment prednisone and azathioprine are shown in Figure 1. Forty-eight hours after starting immunosuppressive therapy, she no longer required intravenous calcium supplementation and within four days she did not require intravenous magnesium. All oral supplementation was continued. In addition, her PTH level was 2.4 pg/mL and 2.0 pg/mL on hospital day 1 and day 4, respectively, while receiving calcitriol, and increased to 7.6 pg/mL after treatment with immunosuppressants.

The patient was discharged home 36 days after the initial presentation at our institution on prednisone 60 mg daily and azathioprine 150 mg daily, and calcitriol 1.5 µg daily, as well as calcium and magnesium supplements. The detailed time course of prednisone dose and serum calcium concentration after this discharge is shown in Table 1. Initially, prednisone was tapered down to 20 mg daily as an outpatient. Thirteen days later, the patient was re-hospitalised for symptomatic hypocalcemia. Total serum calcium was 6.8 mg/dL, magnesium 1.3 mg/dL, phosphate 4.3 mg/dL, and PTH 2.2 pg/ml. The dose of prednisone was increased back to 60 mg and calcitrol to 2.0 µg daily. Within three days, total serum calcium levels trended up and peaked at 13.5 mg/dL six days after the dose of prednisone was increased. She developed acute kidney injury (AKI) due to

hypercalcemia, which were resolved with intravenous fluid and withholding calcitriol and calcium supplements. She was restarted and discharged on a lower dose of calcitriol (0.5 µg daily).

Following the next taper to 40 mg prednisone, the patient relapsed again with total serum calcium at 6.9 mg/dL (Table 1). Serum calcium levels normalised when the dose of prednisone was increased back to 60 mg. Calcitrol was increased to 0.5 µg twice daily. Again, the patient became overtly hypercalcemic and developed AKI due to concurrent increases in calcitriol and calcium supplements with the relapse. The hypercalcemia resolved after calcitriol and calcium supplements were withheld briefly. Given the severity of the patient's hypocalcemia, her multiple ER visits and hospitalisations, and the need for daily intravenous calcium infusion to maintain serum calcium within the desired range, an attempt was not made to taper off calcitriol after immunosuppression was initiated. In our view, the benefit of continuing calcitriol was felt to outweigh any risk of ongoing treatment. Since the patient developed hypocalcemia when the prednisone dose was lowered despite the use of azathioprine as a potential steroid-spring agent, azathioprine was tapered off as it did not seem to have an effect on serum calcium levels.

During the next taper in prednisone dose from 60 mg at four months after initial discharge to 0 mg at seven months after initial discharge, serum calcium concentrations remained in the range of 8-9 mg/dL (Table 1). In this period, the patient had recurrence of AKI so calcitrol was lowered to 0.25 µg twice daily. Over 15 months after initial discharge, calcitrol was also tapered off gradually and the patient remains off calcitrol.

At the patient's most recent follow-up, 1.5 years after being admitted to our institution, her serum calcium level was slightly below normal at 8.0 mg/dL and PTH was within normal at 16.1 pg/ml. Her medications now include calcium carbonate (750 mg daily), without any need for immunosuppressants or calcitrol.

3.2 Detection of CaSR antibodies

CaSR immunoprecipitation assays for CaSR antibodies showed that the pre-treatment serum sample from the patient tested positive: the CaSR antibody index of 18.9 for the serum sample was greater than the upper limit of normal (a CaSR antibody index of 2.75) calculated from a population of 10 healthy control sera (Figure 2A). The patient's post-treatment sample had a CaSR antibody index of 1.25, a value within the range of normal, suggesting that immunosuppressive treatment reduced CaSR antibody levels. Analysis of CaSR antibody reactivity in CaSR peptide ELISAs indicated that the recognised binding site was between amino acids 114-126**,** a known location for activating mutations of the CaSR (http://www.casrdb.mcgill.ca/) (Figure 2B), and that the antibody titre against this epitope was 1:2000. The CaSR antibodies against the 114-126 epitope were of the IgG1 subclass (Figure 2C).

3.3 CaSR-modulating effects of the patient's IgG

To determine the effects of the CaSR antibodies on CaSR function, HEK293-CaSR cells were incubated with IgG (1:100 dilution) prior to measurement of Ca^{2+}-induced IP1 accumulation and ERK1/2 phosphorylation. The results indicated that only the patient's pre-treatment IgG sample significantly increased the levels of IP1 accumulation (Figure

2D) and ERK1/2 phosphorylation (Figure 2E) when compared with Ca^{2+}-stimulation of HEK293-CaSR cells that were not pre-incubated with IgG.

4 Discussion

CaSR antibodies occur in a substantial number of patients with idiopathic hypoparathyroidism or hypoparathyroidism as part of autoimmune polyglandular syndromes.[2,7,11,12,17,18] In most cases, the biological activity of the CaSR antibodies has not been tested or was negative. Occasional patients have had biologically active caSR antibodies.[7,11] Here, we describe the fifth case of autoimmune hypoparathyroidism, who harbors IgG1 CaSR antibodies activating IP accumulation and ERK1/2 and directed at an epitope known to contain naturally-occurring activating mutations (http://www.casrdb.mcgill.ca/) and to participate in receptor activation. The associated inhibition of PTH secretion *in vivo* may result from activation of one or both signaling pathways or perhaps others not evaluated here. Indeed, the CaSR is known to activate multiple signaling pathways, including activation and inhibition of adenylate cyclase, stimulation of phospholipase A2, and activation of protein kinase C and various mitogen-activated protein kinases.[19] Of these, activation of protein kinase C and the ERK1/2 pathway have been shown to modulate PTH secretion,[20,21] although the evidence is incomplete and disparate in some cases. Nevertheless, our data show that two prominent PTH signalling pathways regulated by the CaSR are modulated by the CaSR antibodies in our patient.

As in two earlier cases,[7] residual parathyroid function in our case was documented despite the CaSR antibodies. In the first such report,[7] the hypoparathyroidism remitted spontaneously in one case and normal appearing parathyroid tissue was documented in the second during incidental thyroid surgery.[7] In the present case, the hypocalcemia

improved dramatically with immunosuppressive therapy and relapsed when the dose of prednisone was decreased. Though antibody titres were not measured during the relapse, the presumption is antibody titres were reduced with high dose steroids as hypocalcemia resolved within 48 h after dose increased similar to her prior hospitalisation. The patient had been refractory to calcitriol and high dose calcium supplements prior to prednisone initiation. The disappearance of antibodies combined with high dose calcitriol and calcium supplements during relapse caused the patient to become hypercalcemic. These cases are proof-of-principle that autoimmune hypoparathyroidism is occasionally not the result of irreversible destruction of the parathyroid glands and is at least theoretically treatable by lowering the level of circulating CaSR antibodies and/or blocking their action. In the case described here, the CaSR antibody titre decreased rapidly and markedly during immunosuppressive therapy, concomitant with restoration of normocalcemia, further supporting the pathogenic, functional role of the CaSR antibodies in this case (i.e., autoimmune hypoparathyroidism without parathyroid destruction). Indeed, at her most recent clinical follow-up, the patient was maintained normocalcemic with oral calcium supplements alone, without any need for calcitrol or immunosuppressants. This documents that her antibody-mediated hypoparathyroidism was in near total remission.

An alternative therapeutic approach in patients such as ours would be to block the activating action of CaSR antibodies with a CaSR antagonist, e.g., a calcilytic.[22] Although there is no assurance that the drug would effectively block the action of the antibody, it, at least theoretically, could promote an inactive conformation of the receptor, thereby blocking or blunting the antibody's action and, as a consequence,

enhancing PTH secretion and renal tubular calcium reabsorption. A brief therapeutic trial could test parathyroid secretory capacity and hence the potential therapeutic utility of this approach.

It is of interest to compare the stimulatory actions of CaSR antibodies, along with their implications for the pathogenesis and treatment of the associated hypoparathyroidism, to autoimmune hypocalciuric hypercalcemia (AHH) resulting from inactivating CaSR antibodies.[5,8-10] Any patient having PTH-dependent hypercalcemia caused by inactivating antibodies must, by definition, have viable parathyroid tissue. This contrasts with most cases of autoimmune hypoparathyroidism associated with anti-CaSR antibodies (and/or antibodies to other parathyroid epitopes), in which there appears to be irreversible, immunologic destruction.[1,17,23] It is currently unknown whether prolonged exposure to inactivating antibodies in AHH eventually damages parathyroid tissue and causes hypoparathyroidism. As noted above, hypoparathyroidism caused by activating CaSR antibodies could potentially be tested for residual parathyroid function using a calcilytic. In contrast to a CaSR antagonist in this setting, a CaSR activator is a potential therapy in AHH, by counteracting the blocking effect of the CaSR antibody, thereby restoring normocalcemia. In fact, the calcimimetic, cinacalcet, ameliorated the hypercalcemia in a case of AHH.[24] It will be interesting to determine the effects of cinacalcet in additional cases of AHH. Notably, cinacalcet has also been shown to improve the hypercalcemia in several cases of FHH caused by inactivating CaSR mutations.[6]

In summary, this is the first demonstration of CaSR antibody-mediated hypoparathyroidism responsive to immunosuppressive therapy, adding to the evidence of the role of activating CaSR antibodies in hypoparathyroidism.

References

1. Blizzard RM, Chee D, Davis W. The incidence of parathyroid and other antibodies in the sera of patients with idiopathic hypoparathyroidism. *Clin Exp Immunol*. 1966;1:119-128.

2. Li Y, Song YH, Rais N, et al. Autoantibodies to the extracellular domain of the calcium-sensing receptor in patients with acquired hypoparathyroidism. *J Clin Invest*. 1996;97:910-914.

3. Brown EM, Gamba G, Riccardi D, et al. Cloning and characterization of an extracellular Ca (2+)-sensing receptor from bovine parathyroid. *Nature*. 1993;366:575-580.

4. Brown EM. Role of the calcium-sensing receptor in extracellular calcium homeostasis. *Best Pract Res Endocrinol Metab*. 2013;27:313-343.

5. Kifor O, Moore Jr FD, Delaney M, et al. A syndrome of hypocalciuric hypercalcemia caused by autoantibodies directed at the calcium-sensing receptor. *J Clin Endocrinol Metab*. 2003;88:60-72.

6. Hannan F, Thakker RV. Calcium-sensing receptor (CaSR) mutations and disorders of calcium, electrolyte and water metabolism. *Best Pract Res Clin Endocrinol Metab.* 2013;27:357-371.

7. Kifor O, McElduff A, Leboff MS, et al. Activating antibodies to the calcium-sensing receptor in two patients with autoimmune hypoparathyroidism. *J Clin Endocrinol Metab*. 2004;89:548-556.

8. Pallais JC, Kifor O, Chen Y-B, Slovik D, Brown EM. Acquired hypocalciuric hypercalcemia due to autoantibodies to the calcium-sensing receptor. *N Engl J Med*. 2004;351:362-369.

9. Pallais JC, Kemp EH, Bergwitz C, et al. Autoimmune hypocalciuric hypercalcemia unresponsive to glucocorticoid therapy in a patient with blocking antibodies against the calcium-sensing receptor. *J Clin Endocrinol Metab*. 2011;96:672-680.

10. Makita N, Sato J, Manaka K, et al. An acquired hypocalciuric hypercalcemia autoantibody induced allosteric transition among active human Ca-sensing receptor conformations. *Proc Natl Acad Sci*. 2007;104:5443-5448.

11. Kemp EH, Gavalas NG, Krohn KJE, Brown EM, Watson PF, Weetman AP. Activating autoantibodies against the calcium-sensing receptor in two patients with autoimmune polyendocrine syndrome type 1. *J Clin Endocrinol Metab*. 2009;94:4749-4756.

12. Gavalas NG, Kemp EH, Krohn KJE, Brown EM, Watson PF, Weetman AP. The calcium-sensing receptor is a target of autoantibodies in patients with autoimmune polyendocrine syndrome type 1. *J Clin Endocrinol Metab*. 2007;92:2107-2114.

13. Trinquet E, Fink M, Bazin H, et al. D-myo-inositol 1-phosphate as a surrogate of D-myo-inositol 1,4,5-tris phosphate to monitor G protein-coupled receptor activation. *Anal Biochem*. 2006;358:126-135.

14. Maltzman JC, Koretzy GA. Azathioprine; old drug, new actions. *J Clin Invest*. 2003;111:1122-1124.

15. Houssiau FA, D'Cruz D, Sangle S, et al. Azathioprine versus mycophenolate mofetil for long-term immunosuppression in lupus nephritis. *Ann Rheum Dis.* 2010;69:2083-2089.

16. Pagnoux C, Mahr A, Hamidou MA, et al. Azathioprine vs. methotrexate for ANCA-associated vasculitis. *N Engl J Med*. 2008;359:2790-2803.

17. Mayer A, Ploix C, Orgiazzi J, et al. Calcium-sensing receptor autoantibodies are relevant markers of acquired hypoparathyroidism *J Clin Endocrinol Metab*. 2004;89:4484-4488.

18. Tomar N, Gupta N, Goswami R. Calcium-sensing receptor autoantibodies and idiopathic hypoparathyroidism *J Clin Endocrinol Metab*. 2013;98:3884-3891.

19. Conigrave, A. The calcium-sensing receptor and the parathyroid: past, present, future. Front Physiol. 2016;7:563-599.

20. Brown EM. Control of parathyroid hormone secretion by its key physiological regulators. In: Bilezikian JP, Marcus R, Levine MA, eds. The Parathyroids: Basic and Clinical Concepts. Boston, MD: Elsevier; 2015:101-118.

21. Corbetta S, Lania A, Filopanti M, Vicentini L, Ballare E, Spada A. Mitogen-activated protein kinase cascade in human normal and tumoral parathyroid cells. J Clin Endocrinol Metab. 2002;87:2201-2205.

22. Nemeth EF, Shoback DM. Calcimimetic and calcilytic drugs for treating bone and mineral disorders. *Best Pract Res Clin Endocrinol Metab*. 2013;27:373-384.

23. Brown EM. Anti-parathyroid and anti-calcium-sensing receptor antibodies in autoimmune hypoparathyroidism. *Endocrinol Metab Clin N Am*. 2009;38:437-445.

24. Kuo E, Kemp EH, Sandhu HK, Brown EM, Weetman AP, Huang CL. Acquired hypocalciuric hypercalcemia in a patient with CKD. *Am J Kidney Dis*. 2013;62:1151-1154.

Table 1: Prednisone dose and total serum calcium levels over time

Week[a]	Prednisone dose (mg)	Total serum calcium (mg/dL)
0	60	9.3
2[b]	20	6.8
2.3	60	6
2.4	60	7.4
2.5	60	9.6
2.6	60	10.8
3	60	13.5
3.1	60	12
4	60	10.1
6	60	8.5
7	50	10.6
8	40	10.5
9	40	11
9.2	40	9.9
9.5[c]	40	6.9
9.6	40	7
10	60	8.6
10.3	60	13.0
10.5-13[d]	60	15.2-8.3
14-16	60	8-9.2
17-18	50	8.5-8.8
19-20	40	8.4
21-22	30	8.6-10
23-24	20	8.1
25-27	10	8.5
28-29	5	8.4
30	0	8.6

[a]Week 0 is the day of discharge after the patient's initial hospitalisation at our institution. The tenth place represents day of the week.

[b]Time of first relapse after prednisone was tapered down to 20 mg.

[c]Time of second relapse after prednisone was tapered down to 40 mg.

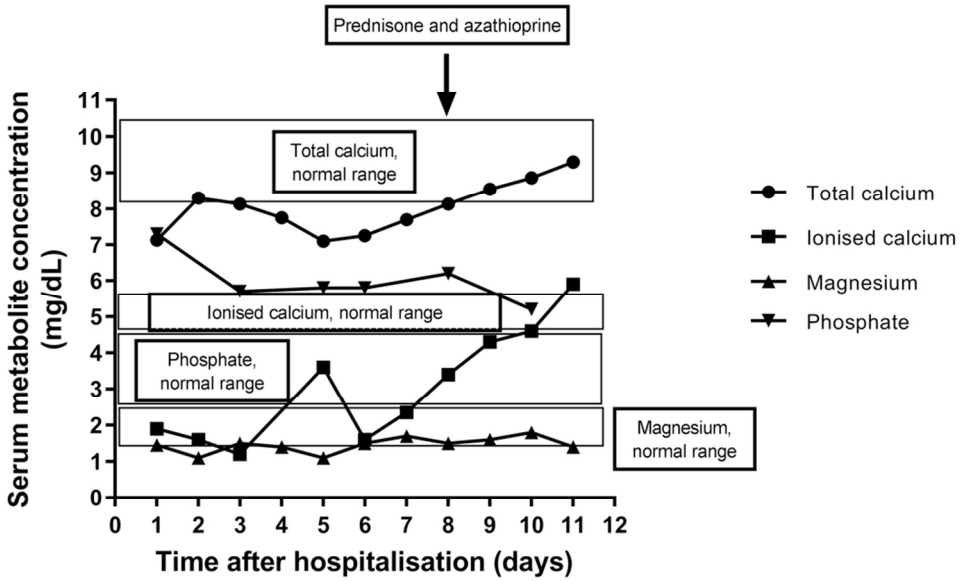
[d]Patient re-hospitalised with recurring acute kidney injury.

Figure Legends

Figure 1 Biochemical response to immunosuppressive therapy. The patient's serum levels of total calcium, ionised calcium, magnesium, and phosphate are shown over the course of treatment at our institution. The black arrow indicates the start of immunosuppressive therapy with prednisone (60 mg daily) and azathioprine (150 mg daily). Data are shown for total calcium (normal range, 8.3-10.6 mg/dL), ionised calcium (normal range, 4.6-5.4 mg/dL), magnesium (normal range, 1.8-2.mg/dL), and phosphate (normal range, 2.5-4.5 mg/dL).

Figure 2 Analysis of patient CaSR antibodies. Serum samples from the patient (n = 2), and healthy controls (n = 10) were analysed for CaSR antibodies in CaSR immunoprecipitation assays at a 1:100 dilution. The patient's serum samples were taken before and after prednisone and azathioprine treatment. The CaSR antibody index (± SD) of the pre-treatment sample is shown in panel (A) next to the CaSR antibody index value (± SD) for the patient's post-treatment serum sample, each representing the mean of three experiments. The mean CaSR antibody index (± SD) of the group of 10 healthy control sera is shown also. The upper limit of normal for the CaSR immunoprecipitation assay was a CaSR antibody index of 2.75. Serum samples from the patient (pre-treatment) and healthy controls were tested in CaSR peptide ELISAs to determine the binding site of the patient's CaSR antibodies. The antibody indices are in panel (B) and indicate reactivity against CaSR peptide 114-126. ELISA analysis of the IgG subclass of the patient's CaSR antibodies is shown in (C) and indicate they were of the IgG1 subclass. Changes in IP1 accumulation and ERK1/2

phosphorylation were measured in response to Ca^{2+} in HEK293-CaSR cells pre-incubated with the patient's IgG samples (n = 2) at a 1:100 dilution. The IgG samples were from serum taken before and after prednisone and azathioprine treatment. IgG samples from healthy controls (n = 10) were tested. HEK293-CaSR cells without pre-incubation with IgG were included. The results in panel (D) show IP1 accumulation (mean ± SD of three experiments) in Ca^{2+}-stimulated HEK293-CaSR cells that were pre-incubated with either IgG from the patient or IgG from a single control, or that were not pre-incubated with IgG. Only the patient's pre-treatment IgG sample significantly increased the levels of IP1 accumulation when compared with Ca^{2+}-stimulation of HEK293-CaSR cells not pre-incubated with IgG: *P* values were < 0.05 when comparing Ca^{2+}-stimulated IP1 accumulation at Ca^{2+} concentrations of 0.5, 1.5 and 3 mM. The results in panel (E) show ERK1/2 phosphorylation (mean ± SD of three experiments) in Ca^{2+}-stimulated HEK293-CaSR cells pre-incubated with either IgG from the patient or IgG from a single control, or not pre-incubated with IgG. Only the patient's pre-treatment IgG sample significantly increased the levels of ERK1/2 phosphorylation when compared with Ca^{2+}-stimulation of HEK293-CaSR cells not pre-incubated with IgG: *P* values were < 0.05 when comparing Ca^{2+}-stimulated ERK1/2 phosphorylation at a stimulatory Ca^{2+} concentration of 1.5 mM.



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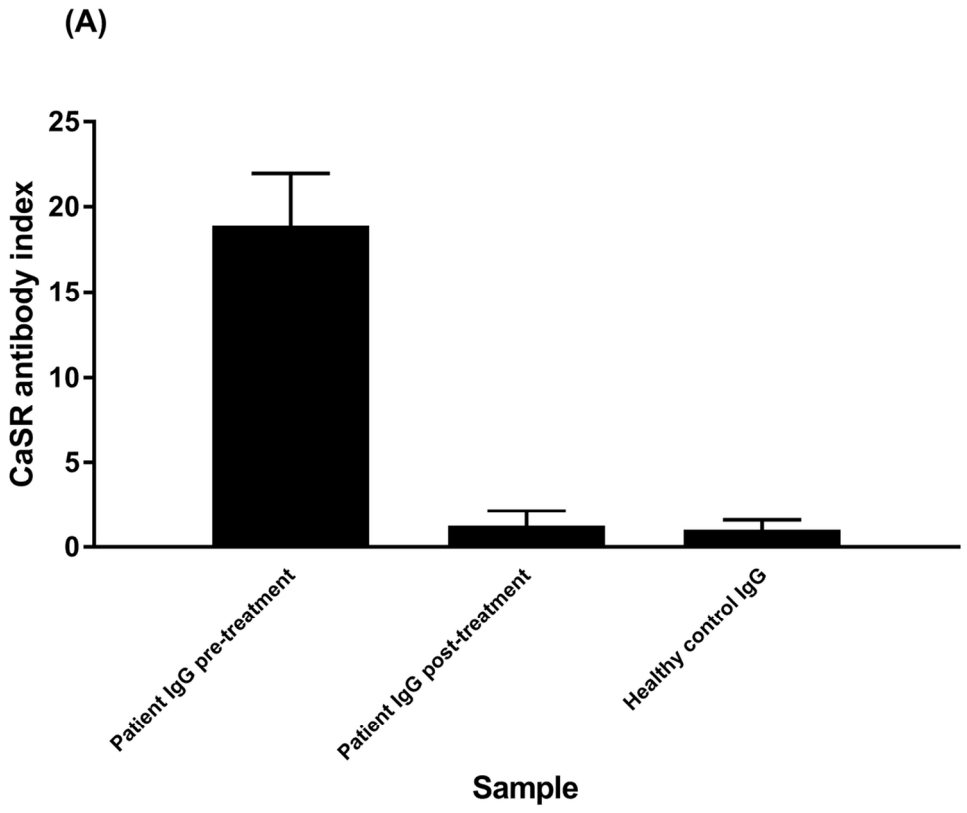

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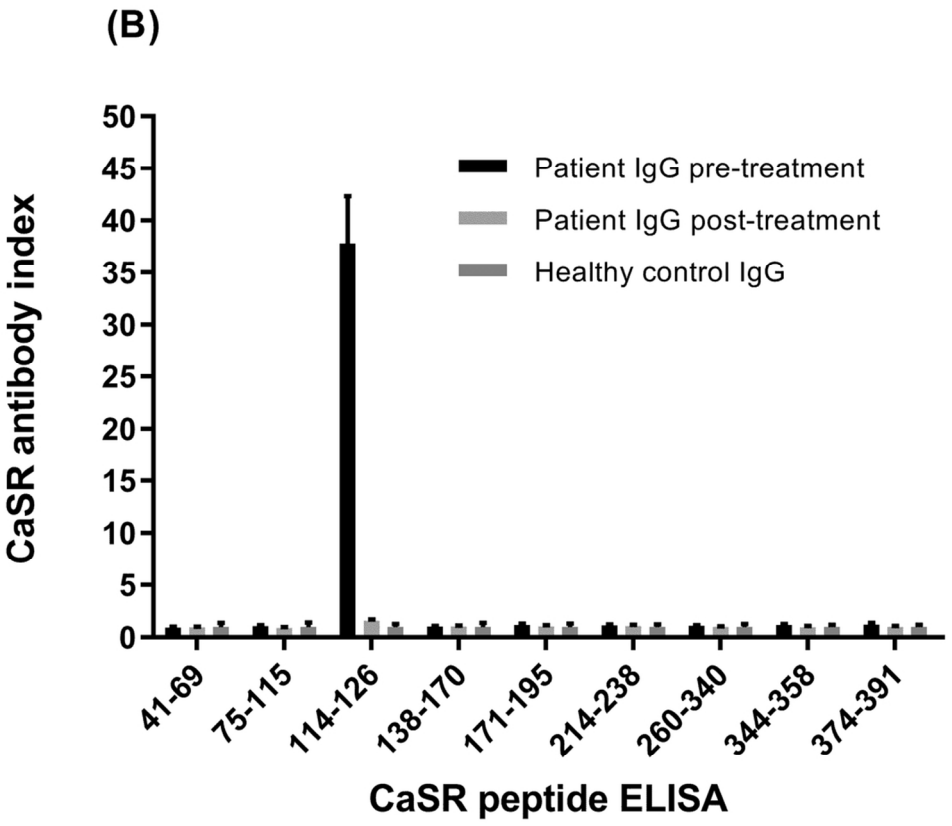

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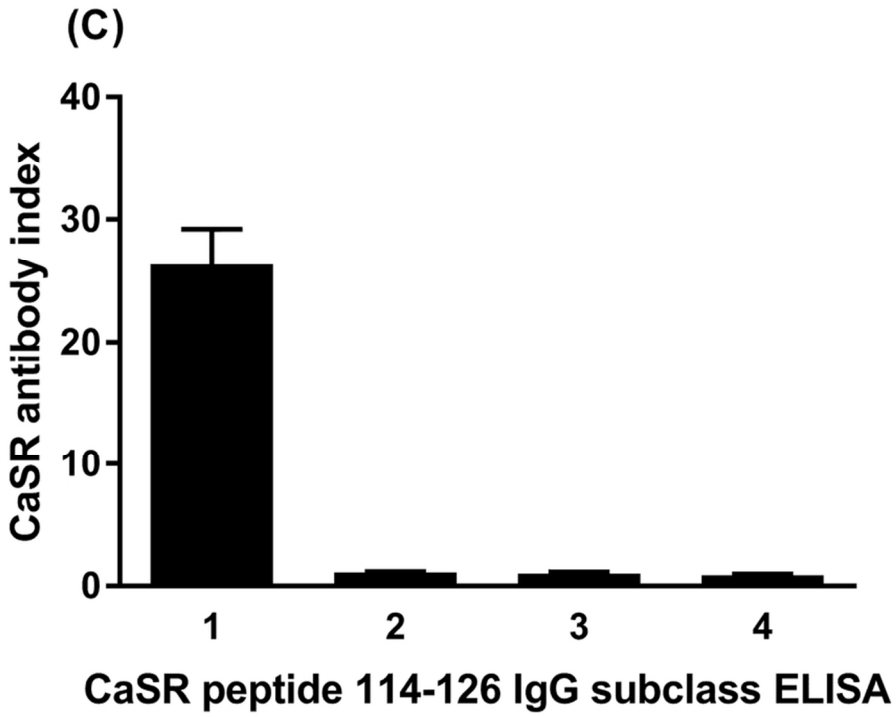

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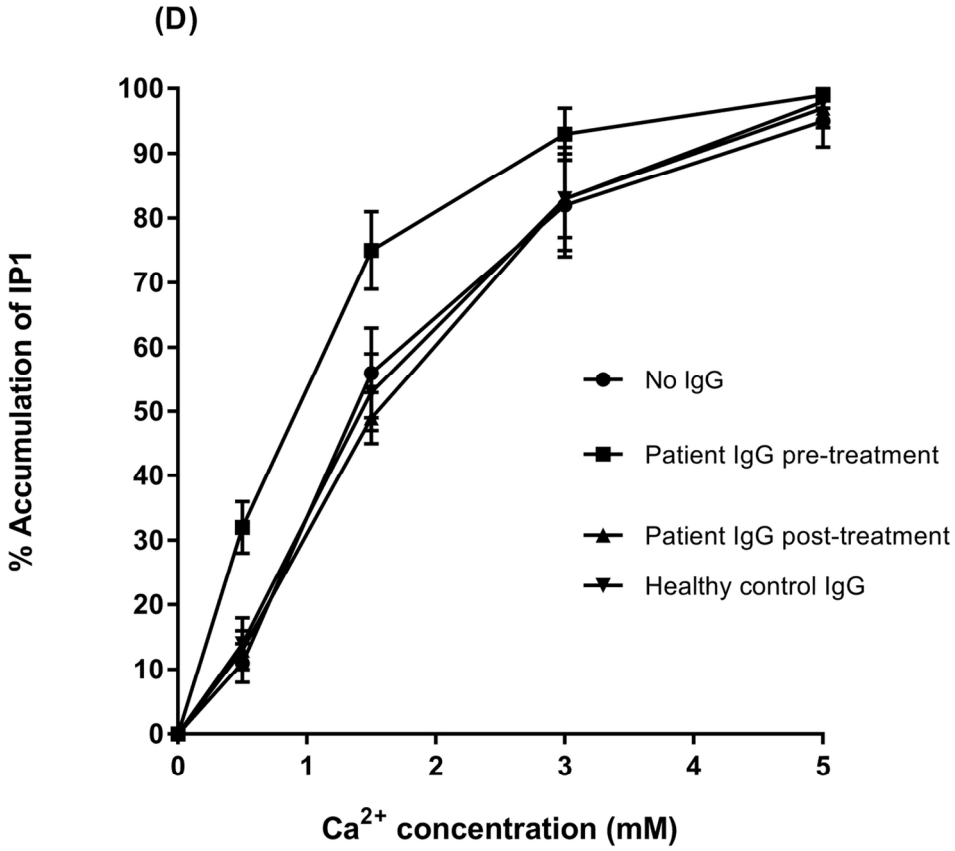

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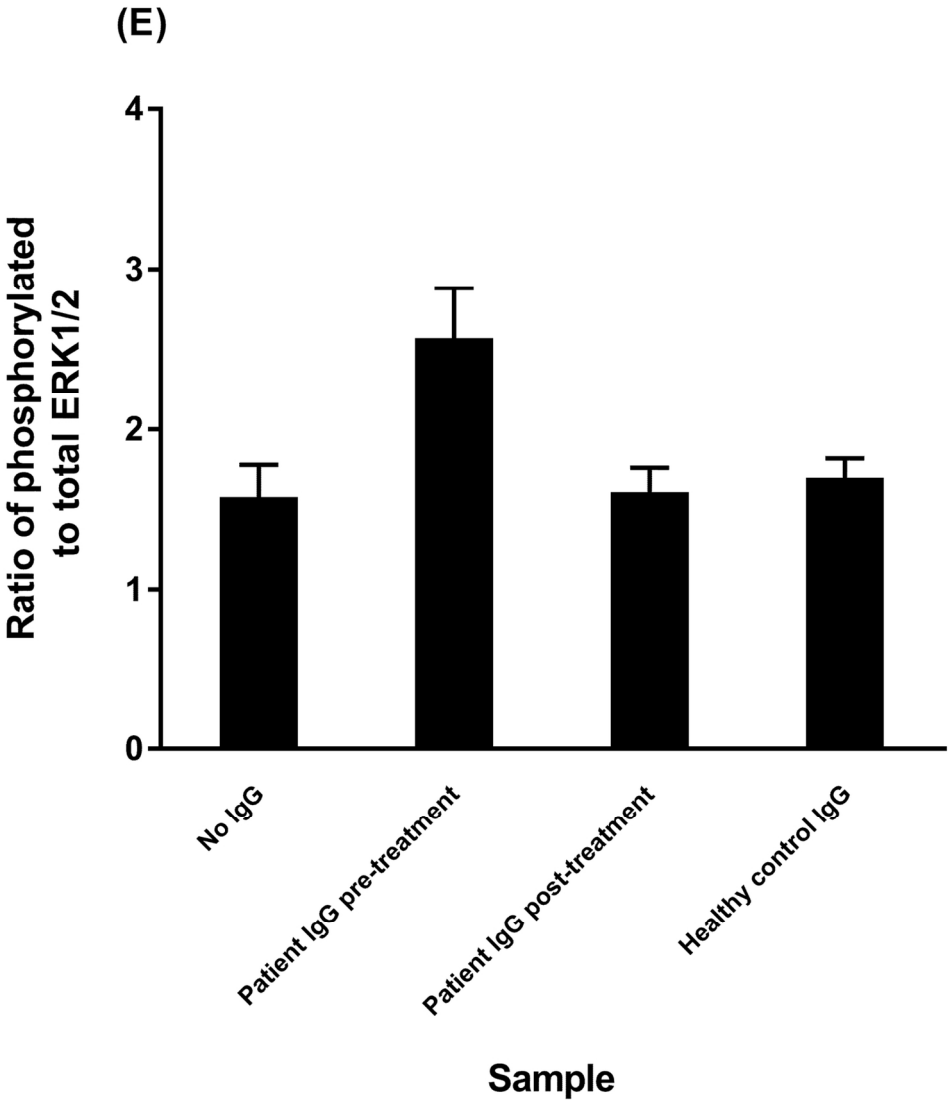

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