RESEARCH

Improved pasireotide response in *USP8* mutant corticotroph tumours *in vitro*

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Abstract

Cushing's disease is a rare but devastating and difficult to manage condition. The somatostatin analogue pasireotide is the only pituitary-targeting pharmaceutical approved for the treatment of Cushing's disease but is accompanied by varying efficacy and potentially severe side effects. Finding means to predict which patients are more likely to benefit from this treatment may improve their management. More than half of corticotroph tumours harbour mutations in the USP8 gene, and there is evidence of higher somatostatin receptor 5 (SSTR5) expression in the USP8-mutant tumours. Pasireotide has a high affinity for SSTR5, indicating that these tumours may be more sensitive to treatment. To test this hypothesis, we examined the inhibitory action of pasireotide on adrenocorticotrophic hormone synthesis in primary cultures of human corticotroph tumour with assessed *USP8* mutational status and in immortalized murine corticotroph tumour cells overexpressing human USP8 mutants frequent in Cushing's disease. Our *in vitro* results demonstrate that pasireotide exerts a higher antisecretory response in USP8-mutant corticotroph tumours. Overexpressing USP8 mutants in a murine corticotroph tumour cell model increased endogenous somatostatin receptor 5 (*Sstr5*) transcription. The murine *Sstr5* promoter has two binding sites for the activating protein 1 (AP-1) and USP8 mutants possibly to mediate their action by stimulating AP-1 transcriptional activity. Our data corroborate the *USP8* mutational status as a potential marker of pasireotide response and describe a potential mechanism through which USP8 mutants may regulate *SSTR5* gene expression.

Key Words

- Cushing's disease
- corticotroph tumours
- USP8
- somatostatin receptor
- pasireotide

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Introduction

Cushing's disease is caused by corticotroph tumours and is the most common form of endogenous hypercortisolism (Newell-Price *et al.* 2006, Lacroix *et al.* 2015). Chronic exposure to elevated cortisol levels is associated with increased morbidity and overall mortality (Dekkers *et al.* 2013, Clayton *et al.* 2016, Valassi *et al.* 2019). Early diagnosis and successful treatment are important goals to achieve, as the mortality rate in patients with the persistent disease is around ten times higher compared to patients in remission (Fleseriu *et al.* 2021).

The first-line treatment for Cushing's disease is the surgical removal of the pituitary tumour with a success rate varying between 79% in microadenomas and 40% in macroadenomas (Dimopoulou *et al.* 2014, Petersenn *et al.* 2015). Other therapeutic options in patients with contraindication to surgery or with either persistent or recurrent disease are medical therapy, pituitary radiotherapy and bilateral adrenalectomy (Ritzel *et al.* 2013, Pivonello *et al.* 2015, Tritos & Biller 2018, Feelders *et al.* 2019).

The somatostatin analogue pasireotide is the only pituitary-targeting pharmaceutical that is approved for the treatment of patients with persistent or recurrent Cushing's disease (Pivonello *et al.* 2015, Fleseriu *et al.* 2021). Pasireotide has a high affinity for the somatostatin receptor (SSTR) 5, which is abundantly expressed in corticotroph tumours and whose expression is not affected by the high plasma cortisol levels (Hofland *et al.* 2005, van der Hoek *et al.* 2005, Batista *et al.* 2006). Clinical trials and real-world evidence have demonstrated pasireotide efficacy in normalizing 24-h urinary free cortisol (24hUFC) levels in patients with Cushing's disease and also higher prevalence and severity of hyperglycaemia (Boscaro *et al.* 2009, 2014, Colao *et al.* 2012, Schopohl *et al.* 2015, Petersenn *et al.* 2017, Lacroix *et al.* 2018, Pivonello *et al.* 2019). Furthermore, a meta-analysis reported biochemical normalization in 41% of patients, clearly demonstrating resistance to pasireotide treatment in more than half of cases (Broersen *et al.* 2018).

Almost half of corticotroph tumours carry somatic mutations in the ubiquitin-specific protease 8 (*USP8*) gene (Ma *et al.* 2015, Perez-Rivas *et al.* 2015, Reincke *et al.* 2015, reviewed in Sbiera *et al.* 2019). Studies reported *USP8*-mutant tumours to be smaller in size but with worse postoperative outcome in the long-term compared with WT tumours (Ma *et al.* 2015, Perez-Rivas *et al.* 2015, Reincke *et al.* 2015, Hayashi *et al.* 2016, Faucz *et al.* 2017, Albani *et al.* 2018*b*). There is evidence that USP8-mutant corticotroph tumours have higher SSTR5 immunoreactivity score, suggesting a better response to pasireotide (Hayashi *et al.* 2016, Castellnou *et al.* 2020). Based on this, a recent consensus suggested that thse *USP8* mutational status could be utilized as a marker of pasireotide response (Fleseriu *et al.* 2021). The aim of the present study was to determine the impact of *USP8* mutations on pasireotide response in human and murine corticotroph tumour *in vitro*.

Material and methods

Patients and samples

The study included 24 corticotroph tumours freshly obtained after transsphenoidal surgery (used for primary cell culture) and 51 archived formalin-fixed paraffin-embedded (FFPE) tumours (used for immunohistochemistry). A part of the FFPE cohort was described elsewhere (Albani *et al.* 2018*b*) (Table 1). Biochemical diagnosis of Cushing's syndrome was based on abnormal 24hUFC, 1 mg dexamethasone suppression test overnight (low-dose suppression test LDDST) and

Table 1 Clinical, radiological and biochemical presentation of the patients included in the FFPE cohort.

Variable			Total, *n*
Age at diagnosis (years), mean ± s.d.	48	13	51
Female patients, *n* (%)	41	(80)	51
Disease presentation, *n* (%)			51
Cushing's disease	47	(92)	
CTP-BADX/NS	4	(8)	
USP8 mutant tumours, *n* (%)	21	(41)	51
Macroadenomas, *n* (%)	17	(34)	50
Cavernous sinus invasion, *n* (%)	8	(25)	32
Postoperative biochemical remission, *n* (%)	38	(81)	47
Basal plasma ACTH (pg/mL), median (IQR)	70	48	33
Basal levels of cortisol (µg/dL), median (IQR)	24	10	33
24-h urinary free cortisol (µg/24 h), median (IQR)	584	1099	25
Cortisol after low-dose dexamethasone suppression test (µg/dL), median (IQR)	25	14	12

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late-night salivary cortisol (Dimopoulou *et al.* 2014). All the patients with suspected central hypercortisolism, based on the baseline adrenocorticotrophic hormone (ACTH) level and on the response to the high-dose dexamethasone suppression test and to the corticotrophin-releasing hormone (CRH) stimulation test (100 µg human CRH i.v.), underwent MRI. When the tumour was not clearly visible in the MRI or in case of inconclusive test results, the inferior petrosal sinus sampling (IPSS) was performed. Once the diagnosis of CD was made, transsphenoidal surgery was performed as first-line therapy. The study was approved by the ethics committee of the LMU Munich (Nr. 643-16), and all subjects gave written informed consent.

Primary cell culture

Primary cultures were derived from the 24 corticotroph tumours freshly obtained after transsphenoidal surgery. Tumours were washed with HDB buffer (15 mmol/L HEPES (pH 7.4), 137 mmol NaCl, 5 mmol/L KCl, 0.7 mmol/L Na_2HPO_4, 10 mmol/L glucose, 2.5 mg/mL amphotericin B, 10^5 units/L penicillin/streptomycin). Part of each tumour was snap-frozen and stored at −80°C for DNA extraction, while the rest was enzymatically dispersed in collagenase, as previously described (Stalla *et al.* 1988). Cell viability was determined by trypan blue staining and was considered acceptable when above 80%. Cells were seeded in 96-well plates (20,000 cells per well) in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% heat-inactivated fetal calf serum (FCS; Gibco) and 10^5 IU/L penicillin–streptomycin; Invitrogen and left to recover for 3days, before being treated with 10 nM pasireotide in low serum medium (1% FCS DMEM) for 3 days. For each tumour culture, each condition was in triplicates.

ACTH determination

ACTH was determined in the primary cell culture supernatants using a RIA as previously described (Stalla *et al.* 1988). ACTH values were normalized with cell viability values obtained with the non-radioactive colorimetric WST-1 assay at 450 nm (Roche Molecular Biochemicals) and are presented as pg/mL/OD 450 nm. A physiologically relevant response was arbitrarily set at ≥20% secretion suppression compared to vehicle control.

USP8 sequencing

DNA extraction from the 24 frozen tumours was performed using a Maxwell Instrument and Maxwell Tissue DNA Kit (Promega). The FFPE DNA mini kit (Qiagen) was used for DNA extraction from the 51 FFPE specimens. Exon 14 of USP8 containing the mutational hotspot was amplified by PCR and sequenced as previously described (Perez-Rivas *et al.* 2015, 2017). Chromatograms were analysed with the Mutation Surveyor software version v4.09 (Soft Genetics).

Immunohistochemistry

SSTR5 immunoreactivity was assessed on the 51 FFPE corticotroph tumours by peroxidase immunohistochemistry on 4 µm formalin-fixed paraffin-embedded sections using the rabbit monoclonal UMB4 antibody (#ab109495, Abcam). After deparaffinization and consecutive rehydration, antigen retrieval was done by microwaving in citrate buffer of pH 6.0 for 3 × 5 min. Sections were blocked in diluted goat serum for 30 min and incubated with the primary antibody diluted 1:80 in Tris buffer saline (TBS) overnight at 4°C. After washing in TBS, they were incubated in biotinylated goat anti-rabbit IgG (Vector Laboratories) for 30 min, washed and incubated in avidin–biotin complex (Vectastain Elite Kit, Vector Laboratories) for 30 min. Immunoreactivity was visualised with 1 mg/mL diaminobenzidine (DAB, Sigma) as chromogen and 0.01% hydrogen peroxide as substrate, and sections were counter-stained in Toluidine blue, dehydrated and mounted with Entellan (Sigma).

SSTR5 immunoreactivity was mainly cytoplasmic and was scored based on staining intensity as absent (0), weak (1), moderate (2) and strong (3). Immunoreactivity score was calculated as follows: 0 × percentage of cells with absent staining + 1 × percentage of cells with weak staining + 2 × percentage of cells with moderate staining + 3 × percentage of cells with strong staining (H-score; Detre *et al.* 1995). Each value was divided by 300, which is the hypothetical maximum score of 100% of cells with strong staining, giving an immunoreactivity score range between 0 (no immunoreactivity) and 1 (maximum immunoreactivity). SSTR5 immunoreactive score (IRS) was calculated as previously described and divided into four categories (negative (IRS 0–1), low (2–3), medium, (4–8) and high (9–12)) (Hayashi *et al.* 2016, Castellnou *et al.* 2020).

Plasmids

The pME-Flag-USP8 vectors expressing WT and mutant USP8 were previously described (Reincke *et al.* 2015). The *POMC*-luc reporter vector has ~800 bp of the human *POMC* promoter upstream to the luciferase gene (Panomics). The *AP1*-luc reporter vector has seven repeats

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of the AP1-responsive sequence upstream to luciferase (Stratagene). pcDNA-3×HA-SSTR5 expresses N-terminal 3× haemagglutinin (HA)-tagged human SSTR5 (cDNA Resource Center, Bloomsburg University). pCI-His-hUbi encodes for histidine-tagged human ubiquitin and was a gift from Astar Winoto (Addgene plasmid 31815; RRID:Addgene_31815) (Young *et al.* 2011).

Cell culture and luciferase assay

Mouse corticotroph tumour AtT20/D16vF2 (ATCC® CCL-89™) cells were obtained from the American Type Culture Collection and authentized. Cells were cultured in DMEM supplemented with 10% heat-inactivated FCS, 2 nmol/L glutamine and 10^5 IU/L penicillin–streptomycin at 37°C and 5% CO2. Cell culture materials were from Life Technologies, Nunc and Sigma–Aldrich.

For luciferase assays and RNA extraction, cells were plated and the day after, they were transfected using SuperFect (Qiagen) following the manufacturer's instructions. For experiments with reporter plasmids, cells were assayed 24 h after transfection. The transfection efficacy was monitored by cotransfection with the RSV-β-gal construct, and results are presented as luciferase: β-galactosidase activity ratio. The empty vector pME-Flag was used as 'mock' negative control.

AtT-20 cells were transfected with a *Fos* siRNA pool against mouse c-Fos (#sc-29222, Santa Cruz Biotechnology) for 48 h. Scrambled unspecific siRNA (# sc-37007, Santa Cruz) was used as control.

RNA extraction and quantitative qRT-PCR

RNA was extracted from AtT-20 cells 48 h after transfection as indicated for each experiment using Trizol (Invitrogen) per manufacturer's instructions. 1 µg RNA was reverse transcribed using QuantiTect RT kit (Qiagen), and qPCR was done with QuantiFast SYBR Green PCR kit (Qiagen) on LightCycler (Roche). Primers were against mouse *Sstr5* (5'-GTGGACAGCCGTGGAGAC-3' and 5'-TGAGCGCTCTGCTCTTTG-3') and *TfIIb* (5'-tggagatttgtccaccatga-3' and 5'-gaattgccaaactcatca-aaact-3'). Primers against mouse *Fos* (5'-CATCCTCC-CGCTGCAGTAG-3' and 5'- GCGCAAAAGTCCT-GTGTGTT-3') were used to validate *Fos* knockdown. Gene expression was normalized to *TfIIb* transcript levels.

SSTR5 Immunoprecipitation

AtT-20 cells overexpressing HA-tagged human SSTR5 (pcDNA-3xHA-SSTR5), ubiquitin (pCI-His-hUbi) and the different USP8 vectors (pME-Flag-USP8) or empty plasmid were pretreated with 25 nM MG132 and 200 µM chloroquine (both Sigma–Aldrich) for 3 and 1 h, respectively. Then, cells were treated with the SSTR5 ligand BIM 23,052 (#2842, Tocris) or vehicle for 1 h at a final concentration of 1 µM. Cells were scraped in cold lysis buffer (20 mM Tris–HCl (pH 7.4), 150 mM NaCl, 50 mM NaF, 1% Nonidet P-40, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 µg/mL aprotinin, 1 µg/mL leupeptin and1 µg/mL pepstatin A) supplemented with 25 nM MG132, 200 µM chloroquine and 10 mM N-ethylmaleimide (reagents from Sigma–Aldrich). Supernatants were collected after centrifugation, and immunoprecipitation and immunoblotting were performed following standard procedures. HA-tagged human SSTR5 was immunoprecipitated using an HA-Tag antibody (anti-HA.11 Epitope Tag Antibody, #901513, Biolegend) and protein A/G agarose (Sigma–Aldrich). Membranes were immunoblotted with antibodies against His-tag (HRP-conjugated His-Tag (27E8), #9991, Cell Signaling) to detect ubiquitin, USP8 (HPA004869, Sigma) to detect USP8 binding and SSTR5 (UMB4, #ab109495; Abcam) to monitor human SSTR5 overexpression. Human USP8 overexpression was monitored using Flag-Tag (M2, #F3165, Sigma–Aldrich). Signals were detected using ECL Clarity (Biorad).

Statistical analysis

Statistical analysis was performed using the statistical software package SPSS version 24.0 (IBM SPSS Statistics). Normal distribution of the data was examined using the Shapiro–Wilk test. Student's *t*-test and Mann–Whitney *U*-test were used to compare USP8 mutant and WT tumour groups in terms of ACTH suppression after treatment and SSTR5 immunoreactivity, respectively. Data are represented as mean ± standard deviation (S.D.) or median (interquartile range, IQR). Statistical significance was considered when $P < 0.05$.

Results

Screening 51 archival FFPE corticotroph tumours revealed significantly higher SSTR5 immunoreactivity score in 21 *USP8* mutant compared to 30 WT (median (IQR), 0.5 (0.3–0.85) vs 0.2 (0–0.3); $P = 0.0007$; Fig. 1A) and IRS (median (IQR), 5 (4-9) vs 4 (1.5–4.5); $P = 0.0115$; Supplementary Fig. 1, see section on supplementary materials given at the end of this article). The most frequent mutation was the substitution c.2159C>G (p.720P>R),

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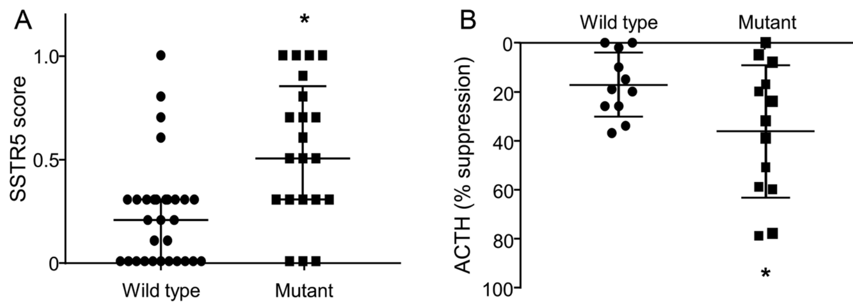

Figure 1
(A) SSTR5 immunoreactivity score in 51 FFPE corticotroph tumour cohort: USP8 WT (n = 30) and mutant (n = 21) tumours. Data are median (IQR). *P = 0.0007. (B) Antisecretory response to *in vitro* pasireotide treatment in a separate cohort of 24 primary cultures of human WT (n = 11) and mutant (n = 13) corticotroph tumours. For each culture, each condition (control or pasireotide treatment) was given in triplicates. Data are calculated as ACTH (pg/mL) to cell viability (WST-1 colorimetric assay; $OD_{450\ nm}$) and presented as % suppression to individual vehicle controls. *P = 0.044 (t-test).

which was found in 9/21 (43%) cases. The other variants were the deletion c.2155_2157delTCC in 7/21 (33%) and the substitutions c.2152T>C (p.718S>P) in 4/21 (19%) and c.2159C>A (p.720P>Q) in 1 case (5%). We observed higher SSTR5 immunoreactivity scores in the *USP8*-mutant tumour groups regardless of mutation type (median (IQR) for p.Ser718P, p.S718del and p.Pro720Arg/Gln: 0.9(0.6), 0.5(0.6) and 0.6(0.7), respectively versus WT 0.2(0.3)). These differences were significant for the p.Ser718P and p.S718del mutant groups (P = 0.013 and 0.041, respectively) but did not reach statistical significance for the p.Pro720Arg/Gln group (P = 0.229). Dividing in four IRS categories, we observed a trend towards higher incidence of *USP8* mutations in the tumours with higher SSTR5 IRS (P = 0.065; Supplementary Table 1).

Four *USP8* WT cases (three female and one male; all microadenomas) presented with higher SSTR5 levels, but no clinical differences were observed compared to the rest of WT tumours that had low SSTR5 immunoreactivity (age at diagnosis (P = 0.622), sex (P = 1.00), disease presentation (P = 1.00), tumour size (P = 0.216), invasion (P = 1.00), basal ACTH (P = 0.352), basal cortisol P = 0.595), 24hUFC (P = 0.844) or cortisol after dexamethasone suppression test (P = 0.522)).

To examine the impact of *USP8* mutational status on the antisecretory response to pasireotide, we studied a separate cohort of 24 corticotroph tumours in primary culture: 11 *USP8* WT and 13 mutants (c.2159C>G (p.720P>R; n = 4), c.2152T>C (p.718S>P; n = 5), S718 deletion c.2155_2157delTCC (n = 3) and long deletion c.2154_2172delCTCCCCAGATATAACCCA (n = 1)). Pasireotide treatment *in vitro* suppressed ACTH secretion to a significantly higher extent in the *USP8*-mutant corticotroph tumours (% of ACTH suppression *USP8* mutant, 36 ± 26 vs WT 17 ± 13; t-test P = 0.044; Fig. 1B). Pasireotide suppressed ACTH secretion by more than 20% in 14 cases: 5 *USP8* WT and 9 mutants (1 p.720P>R, 4 p.718S>P and 4 deletions). We observed more responders than non-responders in the USP8 mutant group (9/14, 64%) than in the WT group (5/11, 45.5%), but the difference was not statistically significant (χ-square P = 0.408). Focusing on the pasireotide responders, there was a trend towards higher ACTH suppression in the *USP8*-mutant tumours compared to WT, which did not reach statistical significance (% of ACTH suppression in *USP8* mutant, 49 ± 21 vs WT 29 ± 6; t-test P = 0.067).

To further study the mechanisms responsible for the improved response to pasireotide in *USP8*-mutant corticotroph tumour cells, we overexpressed the most common *USP8* mutants, p.Ser718Pro, p.Pro720Arg and p.Ser718del, in immortalized AtT-20 cells. All USP8 mutants significantly increased human *POMC* promoter activity (ANOVA P = 0.0145) similar to what we reported using the rat *Pomc* promoter (Reincke *et al.* 2015). In accordance with what we observed in primary human corticotroph tumour cultures, pasireotide suppressed human *POMC* promoter activity more potently in the USP8 mutant overexpressing cells compared to WT (ANOVA P = 0.0007; Fig. 2A).

USP8 mutant overexpression in AtT-20 cells resulted in significantly higher endogenous *Sstr5* expression (ANOVA P < 0.0001; Fig. 2B). In contrast, no effects were observed on the expression of *Sstr2*, a somatostatin receptor that is highly expressed in AtT-20 cells (Fig. 2C). The murine *Sstr5* promoter has two binding sites for the AP-1 (Gordon *et al.* 1999). USP8 mutants stimulated AP-1 transcriptional activity significantly more compared to WT (ANOVA P = 0.0325; Fig. 2D) and knocking down the gene encoding for the AP-1 subunit Fos abolished their stimulatory action on *Sstr5* transcription (Fig. 2E).

Considering the role of the USP8 deubiquitinase as a rescue protein (Mizuno *et al.* 2005), we investigated whether it may also regulate SSTR5 at the posttranslational level. Somatostatin-14 treatment was shown to induce SSTR3 ubiquitination in heterologous cell systems (Tulipano *et al.* 2004). However, in our study, we did not observe ubiquitin binding in human SSTR5 immunoprecipitates after treatment with the SSTR5 agonist BIM 23052,

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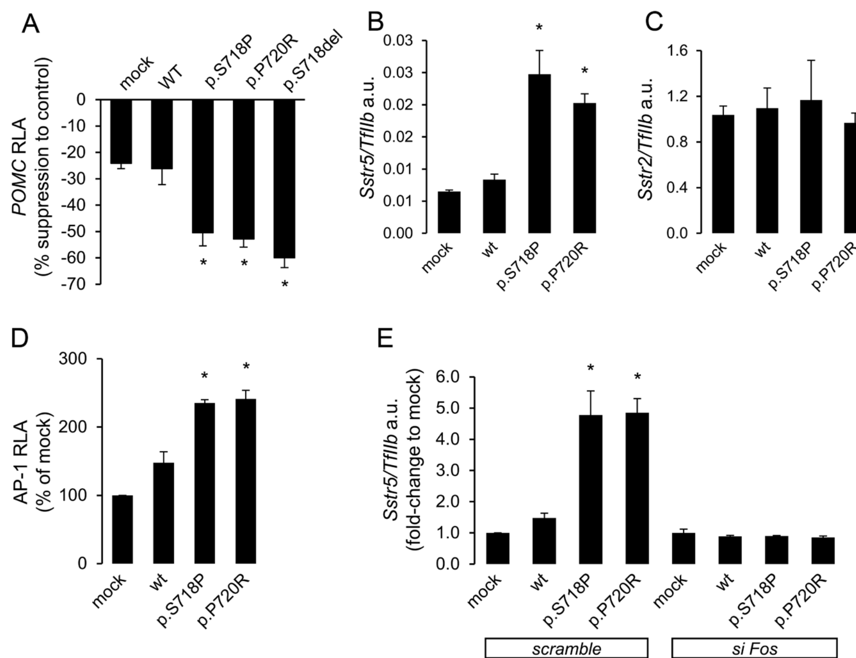

Figure 2
(A) Suppressive action of pasireotide on human *POMC* promoter activity in immortalized mouse corticotroph tumour AtT-20 cells overexpressing USP8 WT, p.Ser718Pro, p.Pro720Arg, p.Ser718del or empty pME vector (mock). Cells were transfected for 24 h before being treated with 10 nM pasireotide for 6 h in serum-free medium. Data are calculated as luciferase to β-galactosidase ratio and as means ± s.d. for three experiments with each transfection condition in triplicates presented as percentage of each vehicle treatment control. RLA, relative luciferase activity. $^*P < 0.05$ to mock (*t*-test). (B and C). Real-time qRT-PCR data showing the impact of USP8 mutants on endogenous *Sstr5* (B) and *Sstr2* (C) expression. Data of *Sstrx/TfIIb* obtained in triplicates. (D) Effect of USP8 mutants vs WT (wt) on AP-1 transcriptional activity. Data are calculated as luciferase to β-galactosidase ratio and as means ± s.d. for three experiments with each transfection condition in triplicates and presented as percentage of empty pME vector (mock). $^*P < 0.005$ to mock (*t*-test). (E) Real-time qRT-PCR data showing the impact of knocking down *Fos* on the stimulatory action of USP8 mutants on endogenous *Sstr5* transcription. RNA was extracted from AtT-20 cells transfected with the various USP8 vectors and siRNA against mouse Fos for 48 h. Data of *Sstr5/TfIIb* obtained in triplicates and presented as fold change to empty pME (mock)-scramble control. $^*P < 0.05$ to mock-scramble. All error bars are s.d.

indicating a lack of ubiquitination (Fig. 3A). In addition, co-immunoprecipitation did not detect any physical association between WT or mutant USP8 and overexpressed human SSTR5 (Fig. 3B).

Discussion

Pasireotide responder patients experience significant clinical benefits, such as improvement of arterial blood pressure, BMI , lipid profile, quality of life and reduced cardiometabolic risk and also side effects like hyperglycaemia (Boscaro *et al.* 2009, Colao *et al.* 2012, Henry *et al.* 2013, Silverstein 2016, Petersenn *et al.* 2017, Albani *et al.* 2018*a*, Samson *et al.* 2021). Therefore, finding ways to predict whether a patient would benefit from pasireotide treatment could greatly facilitate patient management. To this end, lower baseline UFC was linked to higher rates of UFC normalization under pasireotide treatment (Colao *et al.* 2012, Witek *et al.* 2018). Recently, a consensus statement has highlighted the potential of *USP8* mutational status as a molecular marker of pasireotide response (Fleseriu *et al.* 2021).

Our present *in vitro* study reports a significantly better response to pasireotide treatment in terms of suppressed ACTH secretion in human corticotroph tumours carrying *USP8* mutations. We have previously shown that pasireotide suppresses *POMC* promoter activity in a murine corticotroph tumour cell model (Castillo *et al.* 2011), and herein, we show that introducing human USP8 mutants improved this suppressive action.

These observations postulate a regulatory role of USP8 mutants specifically on SSTR5. We have previously reported that USP8 mutants rescue cell surface receptors like EGFR by deubiquitinating them (Reincke *et al.* 2015); therefore, we hypothesized that a similar mechanism may be responsible for the higher SSTR5 immunoreactivity observed in USP8-mutant corticotroph tumours. Receptor ubiquitination after somatostatin binding was previously observed for SSTR3, where it is involved in agonist-induced receptor internalization and endosomal sorting (Tulipano *et al.* 2004). However, we did not observe human SSTR5 ubiquitination after treatment with an SSTR5-specific ligand or any physical association between USP8 and this receptor. Accordingly, our results stipulate that SSTR5 is unlikely to be ubiquitinated and a direct client of the USP8 deubiquitinase.

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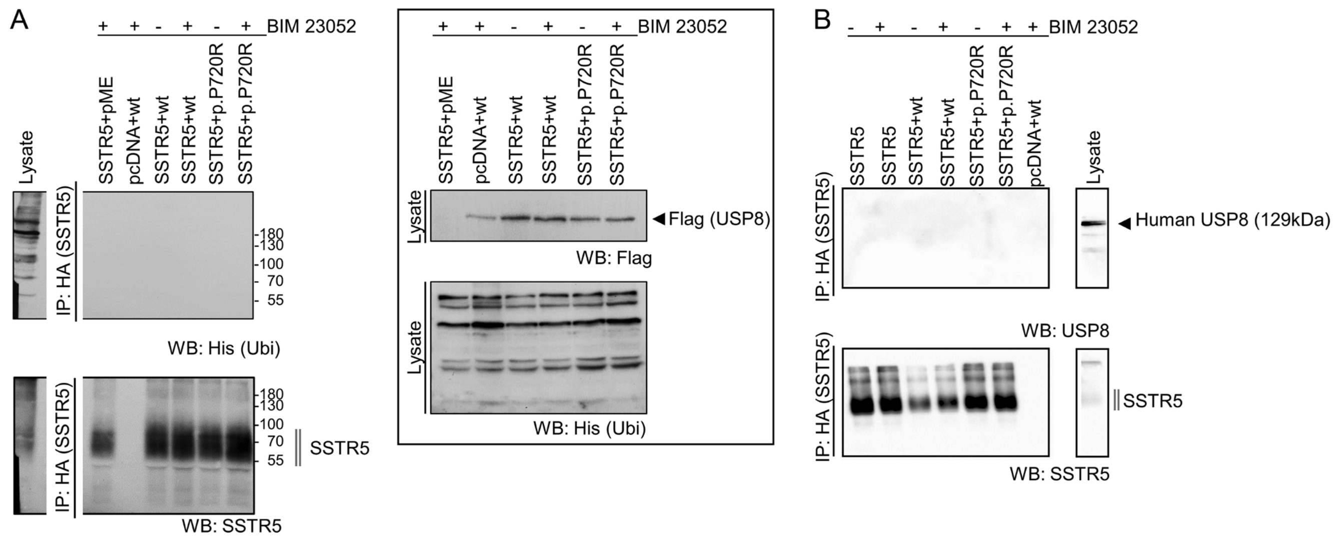

Figure 3
(A) SSTR5 immunoprecipitation experiment showing lack of ubiquitination. AtT-20 cells were transfected with overexpressing vectors for N-terminus HA-tagged human SSTR5 and/or different Flag-USP8 constructs plus ubiquitin (6×Hist-Ubiquitin) and left for 48 h in 2% FCS DMEM. The empty vectors pcDNA3.1+(HA) and pME(Flag) were used as mock controls for SSTR5 and Flag-USP8 constructs, respectively. Transfected cells were pretreated with MG132 and chloroquine before being treated with 10 nM BIM 23,052 for 1 h in serum-free medium. Overexpressed human SSTR5 was immunoprecipitated with the anti-HA-tag (C29F4) antibody and immunoblotted with anti-His-tag to detect overexpressed His-tagged ubiquitin. Blots were stripped with 100 mmol/L Tris–HCl pH2.0 and blotted with anti-SSTR5 (UMB4) to monitor human SSTR5 overexpression. SSTR5 is detectable as a broad band migrating between ~55 and 100 kDa due to glycosylation (the size of the unglycosylated SSTR5 is ~40 kDa; Lupp *et al.* 2011). In the inset, total lysate immunoblots showing overexpressed Flag-USP8 forms (blotted with the FLAG M2 antibody against Flag-tag) and His-tagged ubiquitin (blotted with the His-tag antibody). (B) Immunoprecipitation experiment showing lack of interaction between SSTR5 and USP8 forms. Overexpressed human SSTR5 was immunoprecipitated with the HA antibody and immunoblotted with anti-USP8 or SSTR5 (UMB4) antibodies.

Most studies, including ours, assess SSTR5 by immunohistochemistry, but transcriptomic and other analyses showed increased transcript levels in USP8 mutant tumours (Hayashi *et al.* 2016, Neou *et al.* 2020), indicating a transcriptional primary mode of action. Indeed, our study reveals an increase in endogenous *Sstr5* transcript levels in murine corticotroph tumour cells overexpressing USP8 mutants. This is in accordance with the higher SSTR5 expression and immunoreactivity score observed in human USP8-mutant corticotroph tumours (Hayashi *et al.* 2016, Castellnou *et al.* 2020, Neou *et al.* 2020). In contrast, we did not observe any effect on *Sstr2* transcription, reflecting the lack of significant differences in *SSTR2* transcript levels between USP8 WT and mutant human corticotroph tumours (Hayashi *et al.* 2016).

To understand how USP8 mutants may affect endogenous *Sstr5* transcription in AtT-20 cells, we reviewed the mouse *Sstr5* promoter and observed that it contains AP-1-binding site (Gordon *et al.* 1999). We have previously shown that USP8 mutants potentiate EGFR-induced AP-1 transcriptional activity (Reincke *et al.* 2015), and herein, we observed that their stimulatory action also remains in the absence of EGFR overexpression. AP-1 is a collective term that refers to dimers of transcription factors belonging to the JUN, FOS, ATF and MAF families of basic region and leucine zipper (bZIP) domain proteins (Eferl & Wagner 2003). There is evidence that Fos (also known as c-Fos) is regulated by ubiquitination that causes its degradation (Stancovski *et al.* 1995). A potential direct impact on c-Fos ubiquitination was out of the scope of the present study, nevertheless, the lack of stimulatory action on *Sstr5* transcription in *Fos* knockdown cells led us to conclude that USP8 mutants exert their effect via c-Fos.

A limitation in our study is the lack of clinical data on pasireotide response in patients with Cushing's disease. In our cohort, five patients were treated with pasireotide: four had USP8 WT corticotroph tumours and did not show improvement after pasireotide treatment. One patient had a USP8 mutant corticotroph tumour (p.718S>P) but showed no improvement after 3 months of treatment (s.c., up to 1200 µg bid), which had to be terminated because of severe hyperglycaemia and gastrointestinal complaints.

In conclusion, our *in vitro* results on human corticotroph tumours highlight for the first time the role of *USP8* mutational status on the antisecretory response to pasireotide and reveal a potential mechanism

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through which mutant USP8 forms may upregulate SSTR5 transcription. Extensive clinical studies are needed to consolidate the use of a positive *USP8* mutational status as a predictor of successful pasireotide treatment response in Cushing's disease patients for whom surgery is contraindicated, not feasible or not successful.

Supplementary materials
This is linked to the online version of the paper at https://doi.org/10.1530/ERC-22-0088.

Declaration of interest
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