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

Adriana Albani1, Luis Gustavo Perez-Rivas1, Sicheng Tang1, Julia Simon1, Kristin Elisabeth Lucia1,7, Paula Colón-Bolea1, Jochen Schopohl1, Sigrun Roeber2, Michael Buchfelder3, Roman Rotermund4, Jörg Flitsch4, Jun Thorsteinsdottir5, Jochen Herms2, Günter Stalla1,6, Martin Reincke1, Marily Theodoropoulou1

1 Medizinische Klinik und Poliklinik IV, Ludwig-Maximilians-Universität München, Munich, Germany
2 Center for Neuropathology and Prion Research, LMU Munich, Munich, Germany
3 Department of Neurosurgery, University of Erlangen-Nürnberg, Erlangen, Germany
4 Department of Neurosurgery, Universitätskrankenhaus Hamburg-Eppendorf, Hamburg, Germany
5 Neurochirurgische Klinik und Poliklinik, LMU Klinikum, Munich, Germany
6 Medicover Neuroendocrinology, Munich, Germany
7Current address: Department of Neurosurgery, University Hospital Frankfurt, Frankfurt am Main, Germany.

*equal contribution

**Correspondence**: Medizinische Klinik und Poliklinik IV, LMU Klinikum, Ludwig-Maximilians-Universität München, Ziemssenstr. 5, 80336 Munich, Germany

Email: marily.theodoropoulou@med.uni-muenchen.de

**Short title**: Pasireotide in *USP8* mutant corticotroph tumours

**Key words**: Cushing’s disease, corticotroph tumours, *USP8*, somatostatin receptor, pasireotide

**Word count**: 3,466
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 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 ACTH 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 mediate their action by stimulating AP-1 transciptional 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.

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). The 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 persistent disease is around 10 times higher compared with patients in remission (Fleseriu et al., 2021).
The first line treatment in Cushing’s disease is 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 (Tritos and Biller, 2018, Pivonello et al., 2015, Feelders et al., 2019, Ritzel et al., 2013).

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 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, Batista et al., 2006, van der Hoek et al., 2005). Clinical trials and real world evidence have demonstrated pasireotide efficacy in normalizing 24-hours urinary free cortisol (24hUFC) levels in patients with Cushing’s disease, but also higher prevalence and severity of hyperglycaemia (Boscaro et al., 2009, Colao et al., 2012, Boscaro et al., 2014, 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 (Reincke et al., 2015, Ma et al., 2015, Perez-Rivas 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 wild-type tumours (Reincke et al., 2015, Ma et al., 2015, Perez-Rivas et al., 2015, Hayashi et al., 2016, Faucz et al., 2017, Albani et al., 2018b). 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 the USP8 mutational status could be utilized as a marker of pasireotide response (Fleseriu et al., 2021). The aim of the
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 (that were used for primary cell culture) and 51 archived formalin fixed paraffin embedded (FFPE) tumours (that were used for immunohistochemistry). Part of the FFPE cohort was described elsewhere (Albani et al., 2018b)(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 late-night salivary cortisol (Dimopoulou et al., 2014). All the patients with suspected central hypercortisolism, based on the baseline 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 magnetic resonance imaging (MRI). When the tumour was not clearly visible at the MRI or in case of inconclusive tests 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 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 Na2HPO4, 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 in -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 Medium (DMEM; Gibco) supplemented with 10% heat
inactivated foetal calf serum (FCS; Gibco) and 10^5 IU/l penicillin-streptomycin; Invitrogen) and left to recover for three days, before being treated with 10nM pasireotide in low serum medium (1% FCS DMEM) for three days. For each tumour culture, each condition was in triplicates.

**ACTH determination**

ACTH was determined in the primary cell culture supernatants using a radioimmunoassay 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 450nm (Roche Molecular Biochemicals) and are presented as (pg/ml)/OD450nm. 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, Perez-Rivas et al., 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 4µm 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 pH6.0, 3x 5min. 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, Burlingame, CA) 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 x percentage of cells with absent staining + 1 x percentage of cells with weak staining + 2 x percentage of cells with moderate staining + 3 x 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 in 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 wild-type and mutant USP8 were previously described (Reincke et al., 2015). The POMC-luc reporter vector has ~800bp of the human POMC promoter upstream to the luciferase gene (Panomics). The AP1-luc reporter vector has seven repeats of the AP1-responsive sequence upstream to luciferase (Stratagene). pcDNA-3xHA-SSTR5 expresses N-terminal 3x hemagglutinin (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 (Manassas, VA, USA) and authentized. Cells were cultured in DMEM supplemented with 10% heat inactivated foetal calf serum (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 (Karlsruhe, Germany), Nunc (Wiesbaden, Germany) and Sigma-Aldrich (St. Louis, MO, USA).

For luciferase assays and RNA extraction, cells were plated and the day after they were transfected using SuperFect (Qiagen GmbH, Hilden, Germany) following the manufacturer’s instructions. For experiments with reporter plasmids, cells were assayed 24 hours 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 hours. 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 hours after transfection as indicated for each experiment using Trizol (Invitrogen) per manufacturer’s instructions. 1µg RNA was reverse transcribed using QuantiTect reverse transcription kit (Qiagen, Hilden, Germany) 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’-GAATTGCCAAACTCATCAAAACT-3’). Primers against mouse Fos (5’-CATCCTCCCGCTGCAGTAG-3’ and 5’-GCGCAAAAGTCCTGTGTGTT-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 hours respectively. Then cells were treated with the SSTR5 ligand BIM 23052 (#2842, Tocris) or vehicle for 1 hour at a final concentration of 1 µM. Cells were scraped in cold lysis buffer (20mMTris-HCl (pH7.4), 150 mM
NaCl, 50 mM NaF, 1% Nonidet P-40, 1 mM EDTA, 1mM phenylmethylsulfonyl fluoride 1 µg/mL aprotinin, 1 µg/mL leupeptin, and 1 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 a 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 with 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 wild-type tumour groups in terms of ACTH suppression after treatment and SSTR5 immunoreactivity, respectively. Data are represented as mean ± standard deviation (SD) 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 wild-type (median [IQR] 0.5 [0.3-0.85] vs 0.2 [0-0.3], \( P=0.0007 \); Figure 1A) and IRS (median [IQR] 5 [4-9] vs. 4 [1.5-4.5], \( P=0.0115 \); Supplementary Figure 1). The most frequent mutation was the substitution c.2159C>G (p.720P>R), 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 wild type 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 wild-type cases (3 female, 1 male; all microadenomas) presented with higher SSTR5 levels, but no clinical differences were observed compared to the rest of wild-type 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), 24h-UFC (P=0.844) or cortisol after dexamethasone suppression test (P=0.522)].

To examine the impact of USP8 mutational status on the antisercretory response to pasireotide, we studied a separate cohort of 24 corticotroph tumours in primary culture: 11 USP8 wild-type and 13 mutant [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 (% ACTH suppression USP8 mutant 36±26 vs. wild-type 17±13; t-test P=0.044; Figure 1B). Pasireotide suppressed ACTH secretion by more than 20% in 14 cases: 5 USP8 wild-type and 9 mutant (1 p.720P>R, 4 p.718S>P and 4 deletion). We observed more responders than non-responders in the USP8 mutant group (9/14, 64%) than in the wild type group (5/11, 45.5%) but the difference was not statistically significant (χ-square P=0.408). Focusing in the pasireotide responders, there was a trend towards higher ACTH suppression in the USP8 mutant tumours compared to wild-type, which did not reach statistical significance (% ACTH suppression in USP8 mutant 49±21 vs. wild-type 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 to 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 wild-type (ANOVA \( P=0.0007 \); Figure 2A).

**USP8** mutant overexpression in AtT-20 cells resulted in significantly higher endogenous *Sstr5* expression (ANOVA \( P<0.0001 \); Figure 2B). In contrast, no effects were observed on the expression of *Sstr2*, a somatostatin receptor that is highly expressed in AtT-20 cells (Figure 2C). The murine *Sstr5* promoter has two binding sites for the activating protein 1 (AP-1) (Gordon et al., 1999). USP8 mutants stimulated AP-1 transcriptional activity significantly more compared to wild-type (ANOVA \( P=0.0325 \); Figure 2D) and knocking down the gene encoding for the AP-1 subunit Fos, abolished their stimulatory action on *Sstr5* transcription (Figure 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 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, indicating lack of ubiquitination (Figure 3A). In addition, co-immunoprecipitation did not detect any physical association between wild type or mutant USP8 and overexpressed human SSTR5 (Figure 3B).

**DISCUSSION**

Pasireotide responder patients experience significant clinical benefits, such as improvement of arterial blood pressure, body mass index (BMI), lipid profile, quality of life and reduced cardiometabolic risk, but also side effects like hyperglycaemia (Boscaro et al., 2009, Colao et al., 2012, Albani et al., 2018a, Henry et al., 2013, Silverstein, 2016, Petersenn et al., 2017, 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.,...
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 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.

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 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, Neou et al., 2020, Castellnou 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 wild-type 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 remains also 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 and 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 of pasireotide response in patients with Cushing’s disease. In our cohort, five patients were treated with pasireotide: four had USP8 wild-type 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 (subcutaneous, up to 1200µg bid), which had to be terminated because of severe hyperglycaemia and gastrointestinal complains.

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 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 predictor of successful pasireotide treatment response in Cushing’s disease patients for whom surgery is contraindicated, not feasible or not successful.

**Declaration of interest**
AA, LGPR, ST, JS, KL, PCB, SR, MB, RR, JF, JT, JH, MR and MT report no conflict of interest that could be perceived as prejudicing the impartiality of the research. GKS received consultancy and speaker fees from Recordati. JSc received grants and fees from Novartis, Ipsen, Pfizer and Recordati.

**Funding**

Supported by the Deutsche Forschungsgemeinschaft (DFG) (Project number: 314061271-TRR 205) to MR and MT. AA was supported by the Munich Excellence Training Initiative for Physician Scientists (Metiphys). MR was supported by the Else Kröner-Fresenius Stiftung.

**Acknowledgements**

We thank J Stalla and JL Monteserin-Garcia for their excellent technical assistance.
REFERENCES


somatostatin analogue SOM230 inhibits ACTH secretion by cultured human corticotroph adenomas via somatostatin receptor type 5 *European Journal of Endocrinology* 152 645-54.


Silverstein JM 2016 Hyperglycemia induced by pasireotide in patients with Cushing's disease or acromegaly *Pituitary* 19 536-43.


diagnosis in patients with Cushing's syndrome: results from the ERCUSYN registry

*European Journal of Endocrinology* 181 461-472.


LEGENDS

**Figure 1.** A. SSTR5 immunoreactivity score in 51 FFPE corticotroph tumour cohort: USP8 wild-type (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 wild type (n=11) and mutant (n=13) corticotroph tumours. For each culture, each condition (control or pasireotide treatment) was 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).

**Figure 2.** A. Suppressive action of pasireotide on human *POMC* promoter activity in immortalized mouse corticotroph tumour AtT-20 cells overexpressing USP8 wild-type (WT), p.Ser718Pro, p.Pro720Arg, p.Ser718del or empty pME vector (mock). Cells were transfected for 24 hours before being treated with 10nM pasireotide for 6 hours in serum-free medium. Data are calculated as luciferase to β-galactosidase ratio and are means ± SD of 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-C. Real time qRT-PCR data showing the impact of USP8 mutants on endogenous *Sstr5* (B) and *Sstr2* (C) expression. Data are *Sstrx*/TfIIb obtained in triplicates. D. Effect of USP8 mutants versus wild-type (wt) on AP-1 transcriptional activity. Data are calculated as luciferase to β-galactosidase ratio, are means ± SD of 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 hours. Data are *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 SD.

**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 (6xHist-Ubiquitin) and left for 48 hours 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 10nM BIM 23052 for 1 hour 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 ~55-100 kDa due to glycosylation (the size of the unglycosylated SSTR5 is ~40kDa; Lupp et al., 2011). In 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.
Table 1. Clinical, radiological and biochemical presentation of the patients included in the FFPE cohort.

<table>
<thead>
<tr>
<th>Variable</th>
<th>total n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at diagnosis (years), mean ± SD</td>
<td>48</td>
</tr>
<tr>
<td>Female patients, n (%)</td>
<td>41</td>
</tr>
<tr>
<td>Disease presentation, n (%)</td>
<td>51</td>
</tr>
<tr>
<td>Cushing’s disease</td>
<td>47</td>
</tr>
<tr>
<td>CTP-BADX/NS</td>
<td>4</td>
</tr>
<tr>
<td>USP8 mutant tumours, n (%)</td>
<td>21</td>
</tr>
<tr>
<td>Macroadenomas, n (%)</td>
<td>17</td>
</tr>
<tr>
<td>Cavernous sinus invasion, n (%)</td>
<td>8</td>
</tr>
<tr>
<td>Postoperative biochemical remission, n (%)</td>
<td>38</td>
</tr>
<tr>
<td>Basal plasma ACTH (pg/ml), median [IQR]</td>
<td>70</td>
</tr>
<tr>
<td>Basal levels of cortisol (µg/dl), median [IQR]</td>
<td>24</td>
</tr>
<tr>
<td>24h urinary free cortisol (µg/24 hrs), median [IQR]</td>
<td>584</td>
</tr>
<tr>
<td>Cortisol after low-dose dexamethasone suppression test (µg/dl), median [IQR]</td>
<td>25</td>
</tr>
</tbody>
</table>
Figure 1. SSTR5 immunoreactivity score in 51 FFPE corticotroph tumour cohort and antisecretory response to in vitro pasireotide treatment in a separate cohort of 24 primary cultures of human corticotroph tumours.
Figure 2. Action of pasireotide on human POMC promoter activity

176x149mm (300 x 300 DPI)
Figure 3. Immunoprecipitation experiment showing lack of SSTR5 ubiquitination and interaction between SSTR5 and USP8 forms.

144x196mm (300 x 300 DPI)
**Supplementary Table 1.** Categorical SSTR5 immunoreactivity in *USP8* wild type and mutant corticotroph tumours.

<table>
<thead>
<tr>
<th>SSTR5 IRS categories</th>
<th>USP8 status</th>
<th>Wild type</th>
<th>Mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td></td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>Low</td>
<td></td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Medium</td>
<td></td>
<td>13</td>
<td>12</td>
</tr>
<tr>
<td>High</td>
<td></td>
<td>4</td>
<td>6</td>
</tr>
</tbody>
</table>

IRS categories are defined as: negative, IRS 0-1; low, IRS 2-3; medium, IRS 4-8; high, IRS 9-12. Fisher exact test P=0.073.
Supplementary Figure 1. SSTR5 IRS in 51 FFPE corticotroph tumour cohort: USP8 wild-type (n= 30) and mutant (n=21) tumours. Data are median [IQR]. *P=0. 0115.