

# Factors predicting pasireotide responsiveness in somatotroph pituitary adenomas resistant to first-generation somatostatin analogues: an immunohistochemical study

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## Abstract

**Aim:** To gather data regarding factors predicting responsiveness to pasireotide in acromegaly.

**Patients and methods:** SSTR2a, SSTR3, SSTR5, AIP, Ki-67 and the adenoma subtype were evaluated in somatotroph adenomas from 39 patients treated post-operatively with somatostatin analogues (SSAs). A standardized SSTR scoring system was applied (scores 0–3). All patients received first-generation SSAs, and 11 resistant patients were subsequently treated with pasireotide LAR.

**Results:** None of the patients with negative or cytoplasmic-only SSTR2a expression (scores 0–1) were responsive to first-generation SSAs, as opposed to 20% (score 2) and 50% of patients with a score of 3 ( $P=0.04$ ). None of the patients with an SSTR5 score of 0–1 were responsive to pasireotide, as opposed to 5/7 cases with a score of 2 or 3 ( $P=0.02$ ). SSTR3 expression did not influence first-generation SSAs or pasireotide responsiveness. Tumours with low AIP were resistant to first-generation SSAs (100 vs 60%;  $P=0.02$ ), while they had similar responsiveness to pasireotide compared to tumours with conserved AIP expression (50 vs 40%;  $P=0.74$ ). Tumours with low AIP displayed reduced SSTR2 (SSTR2a scores 0–1 44.4 vs 6.7%;  $P=0.006$ ) while no difference was seen in SSTR5 (SSTR5 scores 0–1 33.3 vs 23.3%;  $P=0.55$ ). Sparsely granulated adenomas responded better to pasireotide compared to densely granulated ones (80 vs 16.7%;  $P=0.04$ ).

**Conclusion:** The expression of SSTR5 might predict responsiveness to pasireotide in acromegaly. AIP deficient and sparsely granulated adenomas may benefit from pasireotide treatment. These results need to be confirmed in larger series of pasireotide-treated patients.

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## Introduction

Acromegaly is a complex disease that frequently requires a multi-modal treatment. Surgery is recommended as the primary treatment in patients with potentially surgically curable adenomas or in case of macroadenomas associated with local mass effects (1). However, even with an experienced neurosurgeon, surgical cure is only achieved in half of the cases (2), making medical treatment necessary for a large proportion of patients.

Somatostatin analogues (SSAs) represent the first choice in the medical treatment for acromegaly. While earlier studies reported a high success rate for first-generation SSAs, octreotide and lanreotide (3, 4), more recent studies show that control of growth hormone (GH) and insulin-like growth factor 1 (IGF1) levels using stricter criteria is achieved in <40% of patients (5, 6). Pasireotide is a multireceptor-targeted SSA that binds SSTR1, SSTR2, SSTR3 and SSTR5, with the highest affinity for SSTR5 (7). Pasireotide normalizes IGF1 in a higher proportion of patients compared to first-generation SSAs and in up to 20% of patients who are inadequately controlled on first-generation SSAs (8, 9). Markers that could predict responsiveness to pasireotide would then be extremely valuable in clinical practice.

Numerous factors are associated with resistance to first-generation SSAs (10), including reduced expression of SSTR2a (11, 12, 13, 14, 15, 16, 17, 18), sparsely granulated adenoma subtype (19, 20, 21, 22) and a higher Ki-67 proliferation index (23, 24). Adenomas that occur in patients with *AIP* mutations and *AIP* mutation-negative sporadic somatotroph adenomas with reduced *AIP* expression show increased invasiveness (25, 26, 27, 28) and are frequently resistant to first generation SSAs (29).

In the current study we have therefore evaluated clinical parameters and the immunohistochemical expression of SSTR2a, SSTR3, SSTR5, *AIP*, Ki-67 and the adenoma subtype in a series of acromegaly patients treated post-operatively with first-generation SSAs and pasireotide.

## Patients and methods

### Patients

The study population consisted of 39 patients (27 females, 12 males) treated post-operatively with SSAs who were evaluated retrospectively and identified from a series of 120 consecutive acromegaly patients submitted for

surgery and followed up at a referral Pituitary Centre (Policlinico 'A. Gemelli', Università Cattolica del Sacro Cuore, Rome) in the period 2000–2013. Patients whose archival tissue was not available or considered not enough for immunohistochemistry (IHC) were excluded ( $n=42$ ); of the remaining 78 patients, nine that had received medical treatment (either SSAs, dopamine-agonists or GH receptor antagonist) prior to surgery and further 30 patients who were considered disease-free following surgery were excluded. None of the patients had received radiotherapy prior to surgery. *AIP* mutations were ruled out in patients with disease onset below 30 years, as previously described (27). Persistence of acromegaly was assessed based on the recent consensus criteria (30), i.e. persistence of elevated age-adjusted IGF1 levels and lack of suppression of GH levels during the oral glucose tolerance test (OGTT, <0.4 ng/ml), assessed after 4 weeks from surgery. All patients were then treated with long-acting first-generation SSAs, either octreotide LAR or lanreotide ATG. Treatment was started at the dosage of 20 mg/4 weeks for octreotide LAR and 90 mg/4 weeks for lanreotide ATG and was titrated up, if necessary, to 30 mg or 120 mg respectively, based on random GH and age-adjusted IGF1 levels. Responsiveness (random GH <1 ng/ml and normal age-matched IGF1), including partial responsiveness (>50% decrease of both GH and IGF1 levels), was assessed after 6 months of continuous treatment. Eleven patients resistant to first generation SSAs have been subsequently treated with pasireotide LAR (40–60 mg/4 weeks) in the setting of clinical trials (8, 9) or compassionate use programme. These 11 patients (ten patients from the two clinical trials and one as compassionate use) were treated with pasireotide, while for the other 16 patients alternative treatment choice was made by the patient or the clinician. Responsiveness to pasireotide has been evaluated using the same criteria. Median follow-up was 9 years (interquartile range (IQR) 5–11). The study was approved by the local Ethics Committee and all patients gave written informed consent.

### Biochemical evaluations

GH and IGF1 were measured using chemiluminescent immunometric assays (Immulite 2000, Siemens Healthcare, Erlangen, Germany). The standard for GH was IS 80/505 until 2010, IS 98/574 afterwards. The standard for IGF1 was IS 02/254. Coefficients of variation were below 5% for both assays.

## Imaging studies

The maximum tumour diameter was evaluated based on the preoperative MRI. Cavernous sinus extension was assessed using the Knosp's classification; grade 3 and 4 defined cavernous sinus extension (31, 32).

## Histopathological studies

The haematoxylin and eosin (H&E)-stained sections from routine formalin-fixed paraffin-embedded tissue blocks were reviewed. One-millimetre cores were taken from three representative areas and used to construct tissue microarrays (TMAs). Four micrometer sections from the TMAs were used for immunoperoxidase IHC. Antigen unmasking was performed by heating the sections in citrate buffer (pH 6) for 12 min in a microwave oven at 650 W. Primary antibodies were incubated for 30 min at room temperature. Source and dilution of the primary antibodies are reported in Table 1. The sections were incubated with a species-specific biotinylated secondary antibody (Vector Laboratories, Burlingame, CA, USA), washed in PBS and incubated with the Avidin/Biotin Complex (Vector Laboratories) for 30 min at room temperature. The reactions were visualized using DAB as a chromogen (Vector Laboratories). Normal post-mortem pituitary gland was used as positive control. One section was processed with omission of primary antibodies as negative control. Images were obtained using a Leica DM5500 microscope coupled with the Leica DFC295 digital camera. All samples were anonymized and the immunohistochemical expression was scored by two observers (E C and D I) that were blind to the clinical data. Cases with discordant results were evaluated by a third observer (F R).

SSTR expression was scored as previously proposed by Volante *et al.* (33), taking into account both the subcellular localization and the extent of the staining: score 0, no immunoreactivity; score 1, cytoplasmic immunoreactivity; score 2, membranous staining in

<50% of cells or incomplete membranous staining; score 3, circumferential membranous staining in more than 50% of tumour cells.

Expression of AIP was scored semi-quantitatively (H-score), taking into account both the percentage of positive cells (0–100%) and the staining intensity (0–3), total range 0–300 (34).

Ki-67 labelling index (LI) was evaluated, in each case, in at least five non-overlapping representative areas counting at least 1000 cells, as previously reported (23).

Adenomas were classified as previously suggested (35) in sparsely granulated somatotroph adenomas (SGSAs), densely granulated somatotroph adenomas (DGSAs) or intermediate phenotype based on their morphology and cytokeratin pattern. Briefly, fibrous bodies in over 70% of neoplastic cells, irrespective of the percentage of transitional cells, defined SGSAs; DGSAs showed perinuclear cytokeratin in over 70% of cells or fibrous bodies in <8%, irrespective of the percentage of transitional pattern cells. Adenomas that could not be classified as DGSAs or SGSAs were defined as intermediate type. As previously suggested, intermediate type adenomas were grouped with DGSAs (35).

## Statistical analysis

Data are presented as mean (s.d.) when normally distributed, and as median (IQR) when not normally distributed, and were analysed using Student's *t* test, Mann–Whitney,  $\chi^2$  and ANOVA as appropriate, using the software Prism v5 for Windows. Significance was set as  $P < 0.05$ .

## Results

### Clinical data

Patients' characteristics are summarized in Table 2. All patients harboured macroadenomas. All patients were operated with a transsphenoidal approach except one, who was operated transcranially due to extensive

**Table 1** Details of the primary antibodies used for IHC.

Antibody	Code	Dilution	Supplier
SSTR2a (UMB-1) rabbit monoclonal	ab134152	1:500	Abcam, Cambridge, UK
SSTR3 (UMB-5) rabbit monoclonal	ab137026	1:750	Abcam, Cambridge, UK
SSTR5 (UMB-4) rabbit monoclonal	ab109495	1:100	Abcam, Cambridge, UK
AIP (35-2) mouse monoclonal	NB100-127	1:1500	Novus Biologicals, Littleton, CO, USA
Ki-67 (MIB-1) mouse monoclonal	F7268	1:75	Dako, Glostrup, Denmark
Cytokeratin 7/8 (CAM5.2) mouse monoclonal	345779	Prediluted	Becton Dickinson, Franklin Lakes, NJ, USA

**Table 2** Patients' characteristics and responsiveness to first-generation SSA treatment.

	Responsive	Resistant	P value
Age (years) (IQR)	49.5 (42.5–62.7)	37 (27–39)	<b>0.004</b>
Sex – females (%)	6/12 (50%)	21/27 (77.8%)	0.13
Maximum tumour diameter (mm) (IQR)	23 (15–25)	26 (20–30.2)	0.38
Cavernous sinus invasion (%)	2/12 (16.7%)	13/27 (48.1%)	0.08
GH (ng/ml) at diagnosis (IQR)	17.7 (8.3–83)	23.2 (14.9–52.6)	0.61
IGF1 ( $\times$ ULN) at diagnosis (IQR)	4.3 (3.7–5.6)	3.7 (2.8–4.6)	0.45
GH (ng/ml) after surgery (IQR)	4.6 (2.3–5.9)	3.4 (1.4–11)	0.88
IGF1 ( $\times$ ULN) after surgery (IQR)	2.6 (1.5–3.6)	2.1 (1.6–2.7)	0.32

suprasellar disease. Post-operative age-adjusted IGF1 levels were elevated in all patients. GH levels did not suppress below 0.4 ng/ml during the OGTT in 38/39 patients, while a 50-year-old female patient had suppressed GH levels but persistently high IGF1. All patients were treated post-operatively with long-acting first-generation SSAs (19 with octreotide LAR, 20 patients with lanreotide ATG). Twelve patients (30.8%) were considered responsive (including partially responsive patients) to the treatment, while 27 (69.2%) were considered resistant. Patients who were resistant to first-generation SSAs were significantly younger (37 vs 49.5 years old;  $P=0.004$ ). No difference was observed regarding sex distribution, tumour size, cavernous sinus invasion, GH and IGF1 levels at diagnosis or after surgery.

### Somatostatin receptors expression

SSTR2a, SSTR3 and SSTR5 were expressed in the majority of the samples. Representative images of the different scores for SSTR2a, SSTR3 and SSTR5 are shown in Fig. 1. The scoring results are summarized in Fig. 2.

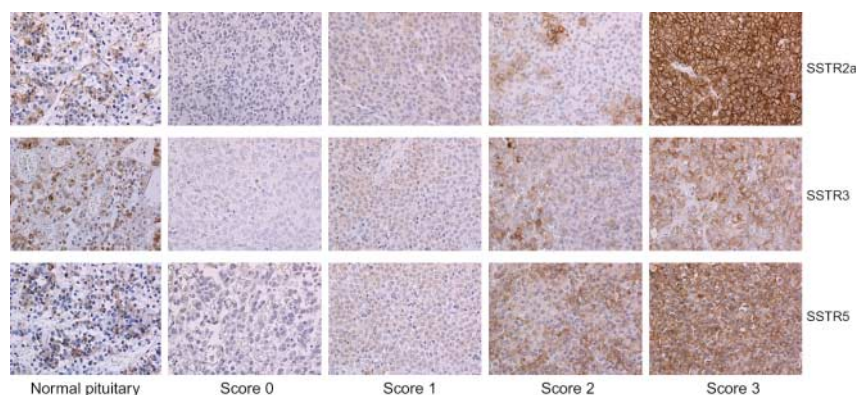
### First-generation SSAs

None of the patients with negative or purely cytoplasmic SSTR2a expression (scores 0–1) were responsive to first generation SSAs, while 20% of adenomas with a score of 2 and 50% of patients with a score of 3 were considered responsive ( $P=0.04$ ) (Fig. 3A).

The mean IGF1 change for patients with an SSTR2a score of 0–1 was  $10.1\% \pm 10.8$ ,  $-20.2\% \pm 7.1$  for score 2 and  $-37.6\% \pm 7.1$  in case of score 3 ( $P=0.005$  (score 0-1 vs score 3)) (Fig. 3B). No significant difference was observed in terms of SSTR3 (responsive tumours, score 0–1 16.7%, score 2 40%, score 3 40%;  $P=0.37$ ) and SSTR5 (responsive tumours, score 0–1 10%, score 2 44.5%, score 3 35%;  $P=0.22$ ) expression between responsive and resistant patients.

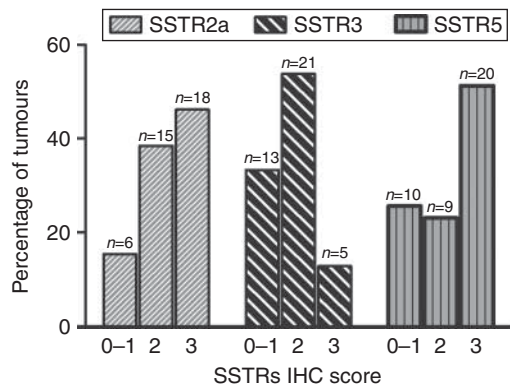
### Pasireotide

Among the 11 patients resistant to first-generation SSAs who have been treated with pasireotide LAR, five were considered responsive to the treatment and six were resistant. No difference was observed in age at diagnosis,

**Figure 1**

Representative images of SSTR2a, SSTR3 and SSTR5 staining scores in the normal human pituitary and somatotroph adenomas (immunoperoxidase, 40 $\times$ ).



**Figure 2**

SSTR2a, SSTR3 and SSTR5 IHC scores.

sex, tumour size, cavernous sinus invasion and in GH and IGF1 levels at diagnosis or after surgery. Patients' characteristics are summarized in Table 3.

None of the patients with negative or cytoplasmic-only SSTR5 expression (scores 0–1) were considered responsive to pasireotide, as opposed to 5/7 cases with membranous expression of SSTR5 (scores 2 or 3) ( $P=0.02$ ) (Fig. 4A).

The mean IGF1 change for patients with an SSTR5 IHC score of 0–1 was  $-15.3\% \pm 10$  and  $-58.2\% \pm 12.7$  in case of score 2 or 3 ( $P=0.048$ ) (Fig. 4B).

No significant difference was observed between pasireotide-responsive and -resistant samples in terms of SSTR2a (responsive tumours, scores 0–1: 2/4, scores 2–3: 3/7;  $P=0.82$ ) and SSTR3 (responsive tumours, scores 0–1: 2/4, scores 2–3: 3/7;  $P=0.82$ ) expression.

### AIP

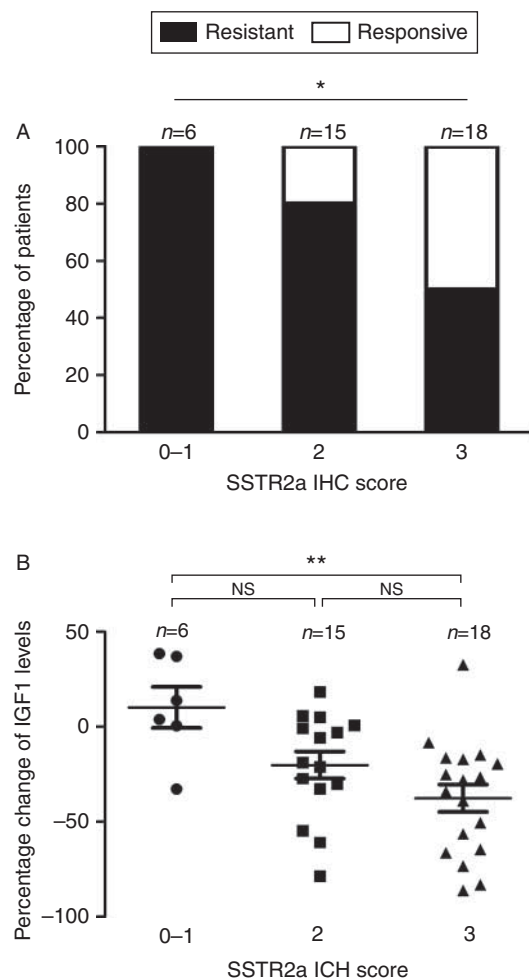
Low AIP expression (score  $\leq 50/300$ ) was found in 9/39 samples (23.1%). Tumours with low AIP were invariably resistant to first-generation SSAs compared to tumours with conserved AIP expression (100 vs 60%;  $P=0.02$ ) (Fig. 5A). Among pasireotide-treated patients, no difference was seen in responsiveness between AIP deficient and AIP proficient tumours (3/6 vs 2/5;  $P=0.74$ ) (Fig. 5B).

Regarding the expression of SSTR2a, tumours with low AIP were found to express SSTR2a at a lower score compared to AIP proficient tumours (scores 0–1, 44.4 vs 6.7% respectively;  $P=0.006$ ). The expression of SSTR3 was also reduced in tumours with low AIP (scores 0–1, 66.7 vs 23.3%;  $P=0.02$ ). No difference was observed in terms of SSTR5 expression (scores 0–1, 33.3 vs 23.3%;  $P=0.55$ ).

Tumours with low AIP were not different in regards to age at diagnosis, tumour size, prevalence of cavernous sinus invasion, GH and IGF1 levels at diagnosis or after surgery.

### Ki-67

Median Ki-67 labeling index (LI) was 0.6% (0.2–1.2). The median Ki-67 LI was not significantly different between tumours that were responsive or resistant to first generation SSAs (median: 0.2%, IQR: 0.2–0.7 vs median: 0.8%, IQR: 0.4–1.2;  $P=0.14$ ). No Ki-67 LI difference was found between cases with different SSTR2a, SSTR3 and SSTR5

**Figure 3**

(A) SSTR2a IHC expression and responsiveness to first generation SSA treatment ( $\chi^2$  test); (B) percentage change of IGF1 levels during first generation SSA treatment and SSTR2a IHC score (ANOVA and Bonferroni *post-hoc* test; data presented as individual values and mean and s.e.m.). \* $P<0.05$ ; \*\* $P<0.01$ ; NS, not significant.

**Table 3** Characteristics of patients treated with pasireotide.

	Responsive	Resistant	P value
Age (years) (IQR)	37 (33–52)	36.5 (22.7–46.7)	0.85
Sex – females (%)	4/5 (80%)	4/6 (66.7%)	0.62
Maximum tumour diameter (mm) (IQR)	30 (30–39)	25 (16–30.5)	0.22
Cavernous sinus invasion (%)	3/5 (60%)	4/6 (66.7%)	0.82
GH (ng/ml) at diagnosis (IQR)	41 (21.3–99.6)	15.9 (13.6–20.3)	0.25
IGF1 ( $\times$ ULN) at diagnosis (IQR)	4.4 (3.3–6.2)	4.2 (2.9–4.8)	0.54
GH (ng/ml) after surgery (IQR)	8.7 (5.2–16)	7.8 (3.9–17.3)	0.9
IGF1 ( $\times$ ULN) after surgery (IQR)	3.4 (2–5.2)	1.9 (1.5–2.4)	0.08

IHC scores. Ki-67 LI was significantly higher in SGSAs compared with DGSA (median: 1%, IQR: 0.3–1.4 vs median: 0.4%, IQR: 0.1–0.6;  $P=0.02$ ). No difference was found between pasireotide responsive and resistant tumours (median: 0.8%, IQR: 0.3–2 vs median: 0.2%, IQR: 0–0.9;  $P=0.19$ ). Ki-67 LI did not differ between AIP deficient and AIP proficient tumours.

### Adenoma subtype

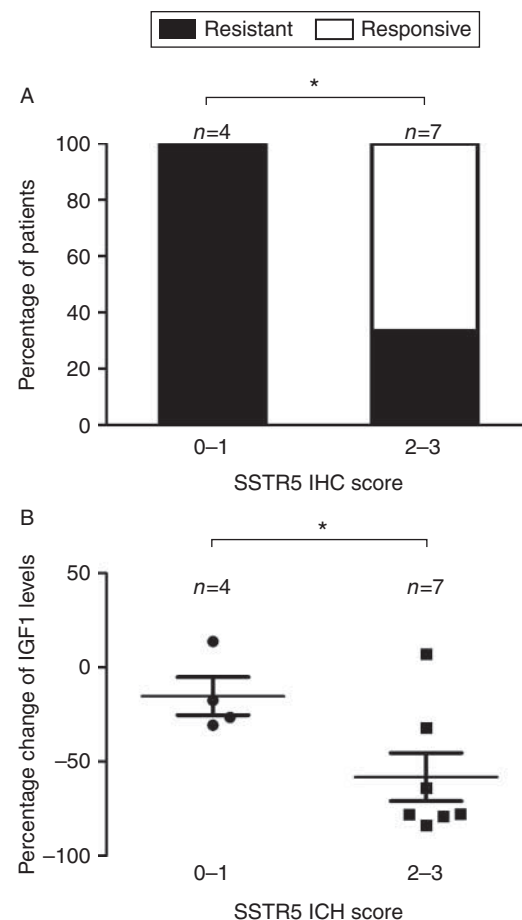
Nineteen tumours were classified as SGSAs (48.7%), 12 tumours were DGSA (30.8%), six had intermediate features (15.4%) and two (5.1%) showed no cytochrome expression.

The adenoma subtype did not correlate with responsiveness or resistance to first-generation SSA treatment. The mean IGF1 reduction was not statistically different in SGSAs as compared to DGSA ( $-20.1\% \pm 7$  vs  $-33.7\% \pm 9.2$ ;  $P=0.24$ ). SGSAs had a tendency to express lower SSTR2a compared to DGSA (scores 0–1, 26.3% vs 5.6%;  $P=0.09$ ). No difference was observed in SSTR3 (scores 0–1, SGSAs 50% vs DGSA 46.2%;  $P=0.91$ ) and SSTR5 expression (scores 0–1, SGSAs 21% vs DGSA 27.9%;  $P=0.71$ ). SGSAs responded better to pasireotide than DGSA (80% vs 16.7%;  $P=0.04$ ). SGSAs showed higher mean IGF1 reduction compared to DGSA ( $-58\% \pm 12.8$  vs  $-15.6\% \pm 10.2$ ;  $P=0.05$ ).

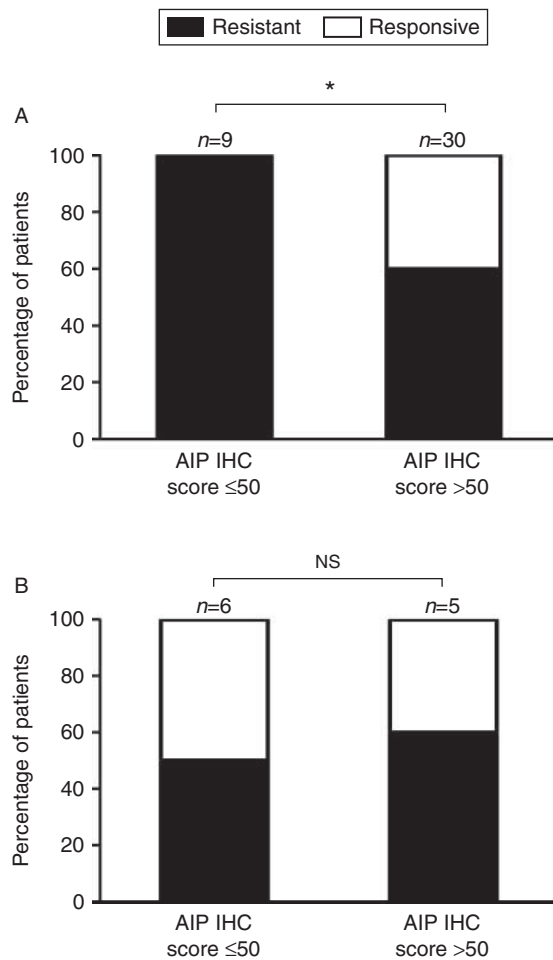
### Discussion

We studied a cohort of 39 acromegaly patients requiring post-operative SSA treatment that were selected from a large series of consecutive acromegaly patients submitted for surgery. In order to avoid biases, patients who received medical treatment prior to surgery were excluded, as pre-treatment has been shown to affect SSTR2 expression (17). Eleven patients resistant to first-generation SSAs have been treated with the multi-receptor analogue pasireotide LAR.

The introduction of highly specific rabbit monoclonal antibodies has allowed the reassessment of the expression of SSTR1, SSTR2a, SSTR3 and SSTR5 in normal and neoplastic tissues (36, 37, 38, 39), including pituitary adenomas (15, 17, 40, 41). We confirmed a positive

**Figure 4**

(A) SSTR5 IHC expression and responsiveness to pasireotide ( $\chi^2$  test); (B) percentage change of IGF1 levels during pasireotide treatment and SSTR5 IHC score (Student's  $t$  test; data presented as individual values and mean and s.e.m.).  $*P<0.05$ .

**Figure 5**

(A) Responsiveness to first generation SSAs according to AIP expression ( $\chi^2$  test); (B) responsiveness to pasireotide according to AIP expression ( $\chi^2$  test). \* $P < 0.05$ ; NS, not significant.

correlation between the expression of SSTR2a and responsiveness to first-generation SSAs. The staining scoring system we have used predicted responsiveness to first-generation SSA treatment: a score of 3 (diffuse circumferential membrane-associated expression) is associated to the highest likeliness of response and a greater reduction of IGF1 levels following medical treatment compared to a score of 2 (incomplete membranous staining or circumferential staining in  $< 50\%$  of the cells) and a score of 1 (cytoplasmic only) and 0 (no expression). None of the cases with scores 0–1 were considered responsive to first-generation SSA treatment, confirming that the membranous expression of SSTR2a is a prerequisite for the responsiveness to these drugs. It could be argued that cytoplasmic expression represents an unspecific finding

and these cases could be grouped with tumours not expressing SSTRs, restricting then the positivity to membrane-associated staining. These results are in line with previous studies where SSTR2a expression was investigated using IHC (12, 13, 14, 15, 16, 17). These studies employed different scoring systems, mainly accounting for the intensity and the extent of the staining. A standardized scoring system could facilitate the routine evaluation of SSTR expression of somatotroph adenomas and would help to provide straightforward information for the clinicians. The scoring system we have used takes into account both the subcellular localization and the extent of the staining, and has been shown to represent a clinically meaningful parameter in neuroendocrine tumours (33, 42). Based on our data, we propose that this could be extended to pituitary adenomas as well.

Pasireotide-responsive patients were similar to resistant ones for all the clinical parameters that were analysed. The expression of SSTR5 could predict responsiveness to the treatment: none of the patients lacking SSTR5 were responsive, and cases with a higher SSTR5 staining score had a greater reduction in IGF1 levels. No difference was observed in the expression of SSTR2a and SSTR3, suggesting that SSTR5 is a major determinant of the biochemical response to pasireotide. We have not evaluated the effect of SSA treatment on tumour volume, and further studies will be required to explore the role of SSTR expression in mediating the anti-proliferative effects of pasireotide.

In accordance with previous studies (29), we have confirmed that tumours with reduced expression of AIP are frequently resistant to first-generation SSAs. Samples with low AIP expressed SSTR2a at a lower score compared to tumours with conserved AIP expression. Interestingly, AIP deficient tumours were equally responsive to pasireotide compared to AIP proficient tumours, and no difference was observed in SSTR5 expression. The reduced membranous expression of SSTR2a in AIP deficient tumours might account for their resistance to first-generation SSAs. Previous studies did not show differences in SSTR2a expression between sporadic somatotroph adenomas with low or conserved AIP expression (29) or between samples from AIP mutation-positive patients and AIP mutation-negative controls (43). Our findings might be more sensitive due to the adoption of a scoring system that takes into account the subcellular localization rather than the staining intensity, and might provide an explanation for treatment resistance in tumours with reduced AIP expression. Recent data suggests that SSTR2a is down-regulated in pituitary somatotroph adenomas

from *AIP* mutation carriers (44). It would be interesting to evaluate the responsiveness to pasireotide in acromegaly patients carrying *AIP* mutations. Preliminary data from a somatotroph specific *AIP* knock-out mouse model suggests that pasireotide is effective in lowering IGF1 levels while octreotide treatment did not result in a significant effect (45).

In this case series, the proliferation marker Ki-67 was not significantly different in somatotroph adenomas resistant to first-generation SSAs compared to responsive cases. Previous results from our group showed that the Ki-67 is significantly higher in resistant tumours (23). The absence of adenomas with higher Ki-67 in this independent case series might account for these results.

SGSAs responded to pasireotide treatment better than DGSAs. In line with previous studies (21, 46), SGSAs had a tendency for a lower *SSTR2a* expression compared to DGSAs, while no difference was observed for *SSTR5*. This somatostatin receptor profile might explain the better responsiveness of SGSAs to pasireotide. We did not find that SGSAs were significantly more frequently resistant to first-generation SSAs compared to DGSAs. The sample size and stricter criteria of responsiveness used in this study might explain these findings.

Our study had several limitations: it is a retrospective study including a relatively small number of pasireotide-treated patients. Moreover, tissue samples were not available for all patients, thus restricting the number of cases eligible for IHC. Further studies are needed to confirm these findings in larger series of patients.

In conclusion, the evaluation of the expression of *SSTR5* in somatotroph adenomas might predict responsiveness to pasireotide. SGSAs and adenomas with low *AIP* expression may benefit from pasireotide treatment. Our findings suggest that the expression of *SSTR2a* and *SSTR5* should be routinely evaluated in somatotroph adenomas.

#### Declaration of interest

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