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**Brief Report**

# Digital quantification of somatostatin receptor subtypes 2 and 5 in growth hormone–secreting pituitary tumors

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## Abstract

Immunohistochemistry (IHC) of somatostatin receptor subtype 2 can predict response to first-generation somatostatin receptor ligands (fg-SRLs) in acromegaly. Recently, we validated an open-source digital image analysis (DIA) to quantify somatostatin receptor subtype 2 (SSTR2) expression. We aimed to validate the DIA also on somatostatin receptor subtype 5 (SSTR5) in a new cohort of growth hormone (GH)-secreting pituitary tumors, with IHC performed in a different laboratory, and to correlate fg-SRL response with SSTs expression. Somatostatin receptor subtype 2 and SSTR5 were assessed in 42 GH-secreting pituitary tumors, using a semiquantitative immunoreactivity score (IRS) and the DIA by use of the open-access software CellProfiler. The DIA calculates the staining intensity and the percentage of positive cells (%PC). We found a good correlation between IRS and DIA for both SSTR2 and SSTR5 ($P < .001$), demonstrating the reliability of the DIA in this setting. Response to fg-SRL treatment correlated with SSTR2, but not SSTR5, expression. Somatostatin receptor subtype 2 expression predicted response to fg-SRL. In particular, the identified cut-offs were IRS ≥ 5 (area under the curve [AUC] 0.763; sensitivity 77%; specificity 83%); intensity/area ≥0.106 (AUC 0.833; sensitivity 92%; specificity 83%); and %PC-DIA ≥63.7% (AUC 0.917; sensitivity 92%; specificity 83%). The SSTR2 %PC correlated with treatment response only when evaluated using the DIA, showing a better performance of this method.

**Keywords:** acromegaly, GH-secreting pituitary adenomas, immunohistochemistry, digital image analysis, IRS

### Significance

We have validated a digital image analysis (DIA) method to quantify somatostatin receptor subtype 5 (SSTR5) immunoreactivity in somatotroph tumors and confirmed its reliability for SSTR2 quantification. The DIA shows a good performance in detecting immunoreactivity and the percentage of positive cells (PCs) for both receptors, compared with the gold-standard semiquantitative immunoreactivity score (IRS). We confirm a good direct correlation between SSTR2 expression (but not SSTR5) and the response to first-generation somatostatin receptor ligands in patients with acromegaly. In particular, the percentage of SSTR2 PC correlates with treatment response only when evaluated using the DIA, thus showing a better performance of this method compared with the IRS.

## Introduction

Acromegaly is a systemic endocrine disease, in the vast majority of cases caused by a pituitary tumor-secreting growth hormone (GH), causing high circulating levels of GH and insulin-like growth factor 1 (IGF-1). If untreated, acromegaly leads to numerous systemic complications, reduced quality of life, and increased mortality compared with the general population.[1] Surgical resection of the tumor is the recommended first-line treatment in the majority of patients and can lead to disease remission in up to 75%-90% of patients with microadenomas and in 40%-60% of macroadenomas.[1] However, surgery can be contraindicated due to patients' comorbidities, have a low likelihood of complete resection due to the tumor's invasiveness, or it can be refused by the patient. In these cases, medical treatment with first-generation somatostatin receptor ligands (fg-SRLs, ie, octreotide and lanreotide), is recommended.[2] Fg-SRLs lead to biochemical control in 35%-55% of patients and to tumor shrinkage in 50%.[1] In this light, protein expression of SSTR2 evaluated by immunohistochemistry (IHC), among other factors, has been widely shown to predict the biochemical response to fg-SRLs.[3-6] On the other hand, SSTR5 expression did not seem to have a role in fg-SRL response, consistent with the receptor binding affinity of

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fg-SRL. To date, assessment of SSTR2 expression is not routinely recommended by consensus statements and guidelines due to the high heterogeneity of staining protocols and quantification methods reported in the different studies. Moreover, most of the scoring systems used to quantify SSTR2 expression rely on the pathologist/researcher's subjective estimation.[7] Recently, the immunoreactivity score (IRS) has been proposed as the gold standard for SSTR2 quantification, being the only one validated in both retrospective and prospective studies.[3-5,8] However, an intrinsic limit of the IRS is its semiquantitative nature. Trying to overcome this limitation, our group recently validated a novel method using DIA to quantify SSTR2 protein expression.[9] Here, we aim to validate, in a retrospective study, the DIA protocol also for the quantification of SSTR5 expression, showing that the same pipeline can be used for multiple receptors. Furthermore, we provide further validation of the SSTR2 DIA reliability on a new cohort of GH-secreting pituitary tumors, in which IHC staining was performed in a different laboratory facility compared with the previous study.

## Methods

### Patients, tumors, and assays

Tumor samples were obtained from 42 consecutive patients with acromegaly who underwent trans-sphenoidal surgery at the IRCCS Ospedale Policlinico San Martino, Genova (Italy), between 1996 and 2021. The exclusion criteria were the following: acromegaly caused by a pituitary carcinoma, having performed surgery in a different center, patients referred to our institution after surgery and/or after 6 months of adjuvant treatment with fg-SRL, radiotherapy performed before completing the 6 months of adjuvant treatment with fg-SRL, and the lack of sufficient paraffin-embedded tissue to perform SSTR2 and SSTR5 IHC analysis.

Diagnosis of acromegaly was made based on clinical features, biochemical evidence of GH hypersecretion (lack of suppression of GH to <1 µg/L after a 2-h oral glucose tolerance test in non-diabetic patients), IGF-1 levels above the age-adjusted upper limit of normality (>1 xULN), and the presence of a pituitary adenoma at magnetic resonance imaging. The majority of the patients were female (57%, $n = 24$) and the median age at the time of diagnosis was 49.0 years (interquartile range [IQR] 37.25-56.25). In the vast majority of cases, the lesion was a macroadenoma (86%, $n = 36$). Moreover, 64% of patients ($n = 27$) received fg-SRL treatment before surgery, and 57% of the patients ($n = 24$) needed adjuvant treatment with fg-SRL after surgery due to disease persistence or recurrence (Table 1). Indications for pre-treatment were the patient's preference, delayed surgery, or low likelihood to achieve a cure after surgery. Patients started adjuvant treatment after surgery if they showed persistent or recurrent disease after surgery (defined as lack of suppression of GH to <1 µg/L after a 2-h oral glucose tolerance test in non-diabetic patients and IGF-1 ULN >1 at least 3 months after surgery).

To determine the biochemical response to fg-SRL, GH and IGF-1 xULN were evaluated before (baseline) and after 6 months of treatment. These data were used to calculate the GH and IGF-1 xULN % decrease. As previously reported by Ilie and colleagues, patients were considered full responders if the IGF-1 xULN % decrease was >50%, and partial responders if the IGF-1 xULN % decrease was between 20% and 50%. Patients showing an IGF-1 xULN % decrease ≤20% were considered as resistant.[5] Due to the relatively low number of cases, we combined partial responders and full responders into a single group, defined as responders (IGF-1 xULN % decrease >20%), to be compared with resistant patients (IGF-1 xULN % decrease ≤20%).

**Table 1.** General and clinical characteristics of acromegaly patients included in the study.

| Data | Number |
|---|---|
| Patients (*n*) | 42 |
| F:M (*n*, %) | 24 (57%): 18 (43%) |
| Age (years) | 49.00 (37.25-56.25) |
| Microadenoma/macroadenoma (*n*, %) | 6 (14%): 36 (86%) |
| **Hormonal data at diagnosis** | |
| GH (µg/L) | 13.60 (6.2-39.80) |
| IGF-1 (µg/L) | 713 (569-858) |
| IGF-1 xULN | 2.52 (1.95-3.68) |
| **fg-SRL treatment** | |
| Pre-treatment (before surgery) | 27 (64%) |
| Adjuvant (after surgery) | 24 (57%) |
| **Hormonal data before adjuvant fg-SRL treatment** | |
| GH (µg/L) | 3.74 (2.65-6.26) |
| IGF-1 (µg/L) | 415 (324-483) |
| IGF-1 xULN | 1.66 (1.35-1.89) |
| **Hormonal data after adjuvant fg-SRL treatment**[a,b] | |
| GH (µg/L) | 1.59 (0.78-3.35) |
| IGF-1 (µg/L) | 209 (169-340) |
| IGF-1 xULN | 0.95 (0.76-1.63) |
| **Response to adjuvant fg-SRL treatment** | |
| IGF-1 xULN < 1 (*n*, %)[b] | 14 (63.6%) |
| GH % decrease | 51.5 (28.9-66.4) |
| IGF-1 xULN % decrease | 37.2 (13.0-41.5) |
| **IHC** | |
| SSTR2 IRS %PC | 4.0 (3.0-4.0) |
| SSTR2 IRS | 6.0 (4-8) |
| SSTR2 DIA %PC | 73.6 (41.5-87.0) |
| SSTR2 DIA intensity/area | 0.169 (0.051-0.136) |
| SSTR5 IRS %PC | 3.0 (2.0-4.0) |
| SSTR5 IRS | 3.0 (2.0-8.0) |
| SSTR5 DIA %PC | 26.7 (0.2-76.4) |
| SSTR5 DIA intensity/area | 0.029 (0.001-0.136) |

DIA, digital image analysis; F, female; fg-SRLs, first generation somatostatin receptor ligands; GH, growth hormone; IGH-1. insulin-like growth factor 1; IHC, immunohistochemistry; IRS, immunoreactivity score; M, males; *n*, number; sg-SRL, second generation somatostatin receptor ligand; ULN, upper limit of normality.
[a]Six-month treatment.
[b]Not all data were available for all patients, IGF-1 xULN after treatment was available only on 19 patients. Where applicable, data are presented as median (IQR).

Both GH and IGF-1 levels were determined using a 2-site chemiluminescent immunometric assay (Immulite 2000, Siemens Healthcare Diagnostics Products), as previously described.[10]

Due to the retrospective nature of the study, not all data were available for each patient at all time points.

The study was conducted in accordance with the recommendations of the Declaration of Helsinki, and all patients gave written informed consent to use clinical data for research purposes. This study was approved by the local ethical committee (Comitato Etico Territoriale Regione Liguria, CET-Liguria register number: 360/2019).

### Immunohistochemistry

IHC was performed on 5-µm thick slide sections from formalin-fixed and paraffin-embedded tissue blocks using

the Dako EnVision®+ Dual Link System-HRP (DAB+) kit (Dako/Agilent, Santa Clara, CA, USA) according to the manual protocol previously reported.[3] SSTR2 monoclonal antibody UMB-1 (RRID: AB_2737601, Abcam, Cambridge, UK) was used at a 1:200 dilution. The same dilution was used for the SSTR5 monoclonal antibody UMB-4 (RRID: AB_10859946, Abcam, Cambridge, UK). Slides were counterstained with hematoxylin.

The stained tissue slides were digitalized using the NanoZoomer 2.0 HT (Hamamatsu, Naka-ku, Hamamatsu City, Japan). Receptor staining was assessed with both the semi-quantitative IRS and the quantitative DIA methods, as previously reported.[9] Briefly, IRS was obtained by multiplying the score of the percentage of positive stained cells (%PC-IRS) (0: no positive cells; 1: <10%; 2: 10%-50%; 3: 51%-80%; 4: >80%) with the score of the staining intensity (0: no staining; 1: weak staining; 2: moderate staining; 3: strong staining), thus resulting in a total score ranging from 0 to 12. The IRS was performed independently by 2 investigators (J.A. and C.C.).

As concerns the DIA, we used the open-source software CellProfiler version 4.0.7, taking into consideration the high inter-observer agreement previously shown,[9] the image selection and the definition of the region of interest (ROI; ie, the outline of the tumor area) were performed by a single investigator. The pipeline previously reported[11] was optimized for the current cohort, based on the intensity of the DAB and the hematoxylin staining, by calibration on a negative sample, a sample with intermediate staining, and 1 with high staining. In particular, since the current cohort had a weaker hematoxylin staining compared with the previous study, the threshold for the hematoxylin signal was lowered until, in the test samples, all nuclei were detected. The same process was repeated for the DAB staining, increasing the threshold due to the presence of a higher background noise. The pipeline was then run on the whole cohort. Two measures were obtained with the DIA: the %PC-DIA (calculated as [number of stained cells/total number of cells] × 100) and the intensity/area (total intensity of immunoreactivity/ROI area). The intensity/area values ranged between 0 (no staining) and 1 (maximum staining) arbitrary units/pixel. We validated the DIA using the currently proposed gold-standard method for SSTR2 quantification by IHC (IRS).[6] In particular, we correlated the "total" receptor expression defined for the DIA as the intensity/area with the total IRS, while the %PC calculated by the DIA was correlated with the %PC component of the IRS.

### Statistical analysis

SPSS 25.0 for Windows (SPSS, Chicago, IL, USA) was used for statistical analyses, whereas graphs were created using GraphPad Prism software version 9.0 (GraphPad Software, San Diego, CA, USA). Categorical data are presented as frequencies and percentages, while quantitative data are reported as median and IQR. Comparison between receptor expression in pre-treated and naïve patients and between SSTR2 and SSTR5 expression levels was performed with the Mann–Whitney test. Spearman and Pearson's correlations were used, as appropriate, to validate the DIA with respect to the IRS and to investigate the potential association of SSTR2 and SSTR5 expression with the biochemical response to fg-SRLs. The receiver-operating characteristic (ROC) curve was used to assess the predictive discrimination of SSTR2 expression to biochemical response during treatment with fg-SRLs. The best-fitting cut-offs were then computed using the Youden index, and the different ROC curves were compared using the DeLong's test. Differences were considered statistically significant at $P < .05$.

## Results

### SST expression

All analyzed samples expressed SSTR2, although with high variability between tumors. Median SSTR2 IRS was 6 (IQR 4-8), median DIA intensity/area was 0.169 (IQR 0.051-0.230), and median %PC-DIA was 73.6% (IQR 41.5%-87.0%).

Similarly, a high variability between samples was observed for SSTR5 expression as well. Median IRS was 3 (IQR 2-8), median DIA intensity/area was 0.029 (IRQ 0.001-0.136), and median %PC-DIA was 26.7% (IQR 0.2%-76.4%; Table 1). Figure 1 shows representative images of immunohistochemical staining and their corresponding scores.

Despite the high heterogeneity observed between samples for both receptors, SSTR2 expression was higher than SSTR5, independent of the method used (IRS: $P = .018$, DIA intensity/area: $P < .001$, %PC-DIA: $P = .004$; Figure S1).

Consistent with our previous study,[9] also in this patient cohort, we observed a strong positive correlation between the semi-quantitative IRS and the quantitative DIA for the "total" SSTR2 expression ($\rho = 0.924$, $P < .001$; Figure 2A). Accordingly, a significant positive correlation was observed between the %PC quantified by DIA and the %PC component of the IRS ($\rho = 0.649$, $P < .001$; Figure 2B).

Of note, we observed the same pattern for SSTR5, thus showing that the DIA provides a reliable quantification also for this receptor. Likewise, a strong positive correlation was present both when correlating the "total" staining quantification (DIA intensity/area vs. total IRS $\rho = 0.872$, $P < .001$; Figure 2C) and the %PC component (%PC DIA vs. %PC IRS, $\rho = 0.748$, $P < .001$; Figure 2D).

### SSTs and pre-treatment

Sixty-four percent of patients ($n = 27$) received fg-SRL treatment before surgery, with a median treatment duration of 8 months (IQR 3.5-15.5 months). One of these patients was treated with fg-SRL associated with the dopamine agonist cabergoline. In order to investigate the potential impact of fg-SRL treatment on SST expression, we excluded this patient from the analysis. The patients in the pre-treated group and the naïve group were comparable in terms of age, sex, presence of microadenoma or macroadenomas, IGF-1 levels at diagnosis, and persistence of disease after trans-sphenoidal surgery (Table S1).

We observed a trend toward lower SSTR2 expression in pre-treated, compared with treatment-naïve patients, reaching statistical significance only when the receptor expression was evaluated by use of IRS ($P = .044$, Figure 3A).

A similar trend was observed for SSTR5 expression; however, contrary to what was observed for SSTR2, the difference in receptor expression between pre-treated and naïve patients was observed when using the DIA (intensity/area: $P = .018$, %PC: $P = .020$, Figure 3E and F) but not with the IRS ($P = .188$, Figure 3D).

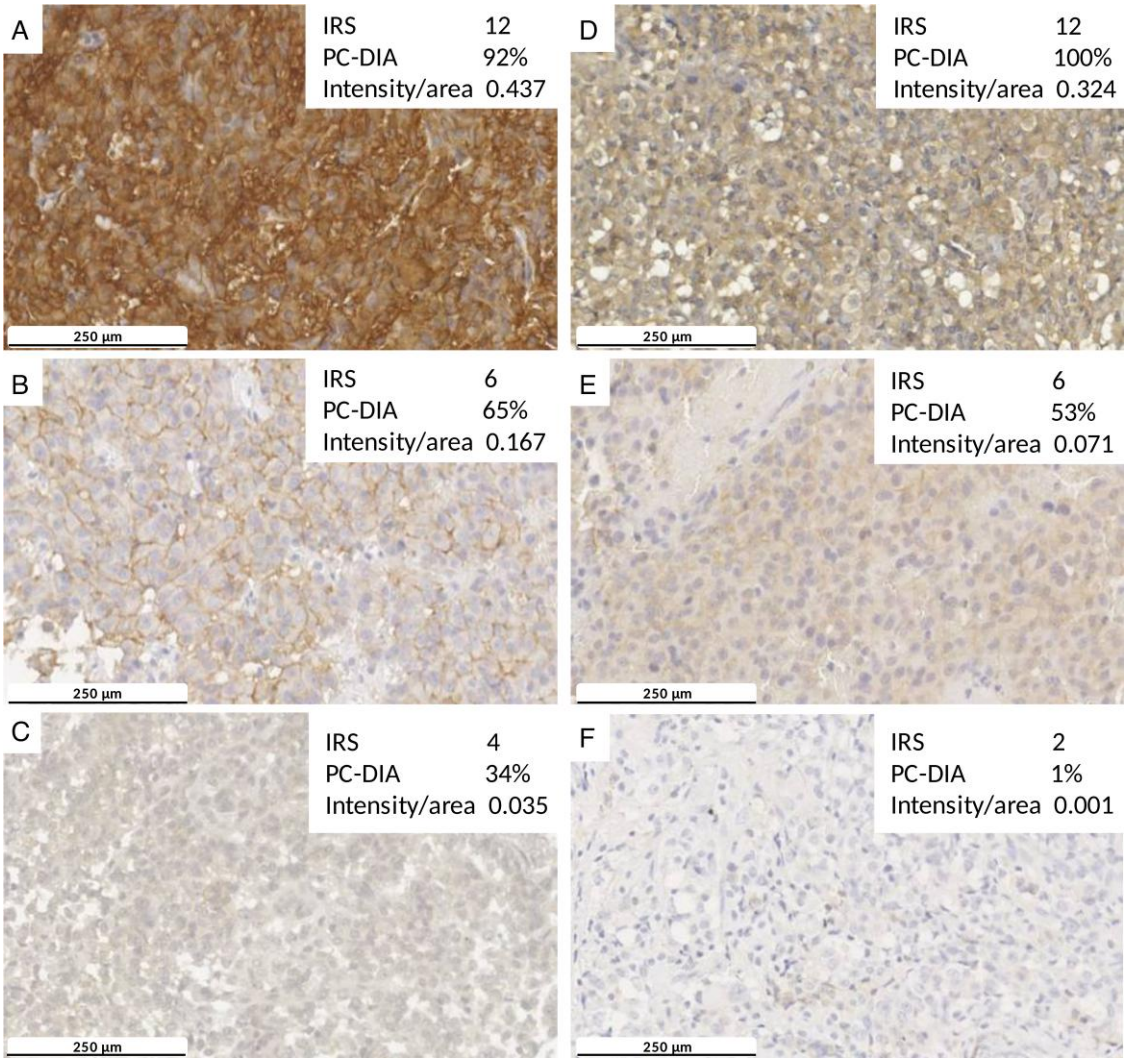


**Figure 1.** Representative images of SSTR2 (A-C) and SSTR5 (D-F) expression and corresponding analysis data in GH-secreting pituitary tumors. Photomicrographs were acquired at 20× magnification. IRS, immunoreactive score; PC-DIA, percent of positive cells calculated by the digital image analysis.

Due to the retrospective study design, we did not manage to retrieve data to evaluate the response to fg-SRLs in the preoperative setting (ie, from diagnosis to neurosurgery).

## SSTs and treatment response to adjuvant fg-SRLs

As described in the Methods section, 57% of the patients ($n = 24$) received adjuvant treatment with fg-SRLs after surgery, with data about GH and IGF-1 xULN values after treatment available on 19 patients. After 6 months of treatment, the median GH % decrease was 51.5% (IQR 28.9%-66.4%), and the median IGF-1 xULN % decrease was 37.2% (IQR 13.0%-41.5%). Thirteen out of 19 patients (68.4%) had an IGF-1 xULN % decrease >20%, while only 2/19 patients (10.5%) had an IGF-1 xULN % decrease >50%; 14 patients (63.6%) achieved IGF-1 < 1 xULN (Table 1).

Of note, the GH and IGF1 xULN % decrease were not significantly different in patients naïve to fg-SRLs compared with the pre-treated patients ($P = .266$ and 0.323, respectively).

As expected, SSTR2 expression correlated with the biochemical response to fg-SRLs. In detail, we observed a positive correlation between the IGF-1 xULN % decrease and the "total" receptor expression evaluated using both IRS ($R = 0.517$, $P = .023$, Figure 4A) and the DIA intensity/area ($R = 0.615$, $P = .007$, Figure 4B). A superimposable correlation was observed between the IGF-1 xULN % decrease and the %PC calculated by the DIA ($R = 0.615$, $P = .007$, Figure 4D) but not with the %PC component of the IRS ($\rho = 0.235$, $P = .334$, Figure 4C).

No statistically significant correlation was observed between the IGF-1 xULN % decrease and SSTR5 expression, irrespective of the quantification method used (ie, IRS and/or DIA; Figure 4E-H).

Of note, SSTR2 expression showed a strong positive correlation with GH % decrease, independent of the quantification method applied (IRS: R = 0.655, $P = .002$; DIA intensity/area: $R = 0.709$, $P < .001$; %PC-DIA: $R = 0.678$, $P = .001$), except for the %PC-IRS ($R = 0.409$, $P = .074$). No correlation was found between SSTR5 expression and GH % decrease (Figure S2).

Moreover, no correlation was observed between the SSTR2/SSTR5 ratio, calculated both with the IRS and the DIA

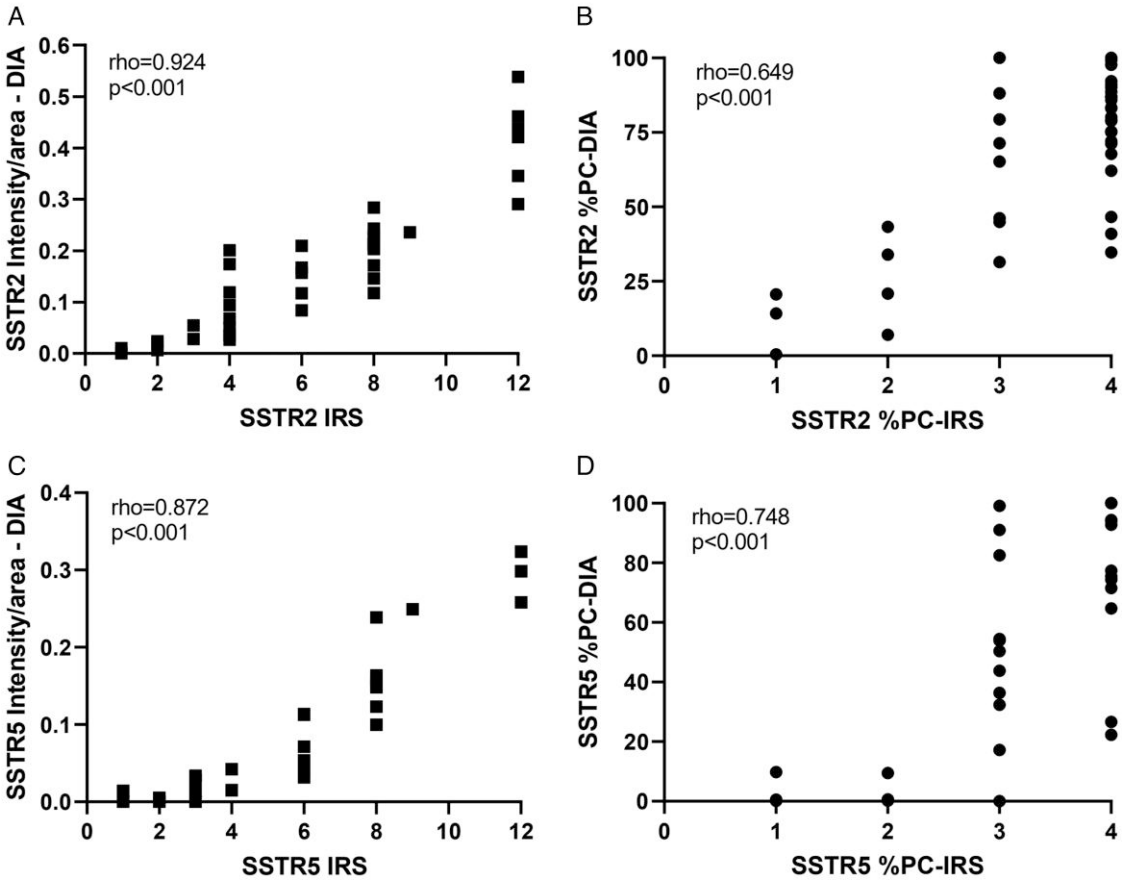


**Figure 2.** Correlation plots of DIA quantification and IRS for SSTR2 (A and B) and SSTR5 (C and D). IRS, immunoreactive score; PC-DIA, percent of positive cells calculated by the digital image analysis; PC-IRS percent of positive cells component of the IRS.

intensity/area, and the hormonal response following fg-SRL treatment (ie, IGF-1 xULN % decrease and GH % decrease, data not shown).

As described in the Methods section, based on the report by Ilie and colleagues,[5] we defined as responders (partial plus full responders) patients achieving an IGF-1 xULN % decrease >20% following fg-SRL treatment. Using this cut-off, SSTR2 expression emerged as a good predictor of fg-SRL response in the ROC curve analyses, independent of the quantification method used. In particular, the area under the curve (AUC) for the IRS was 0.763 (IC 0.501-1.000), for the DIA intensity/area it was 0.833 (IC 0.561-1.000), and for the DIA-%PC was 0.917 (IC 0.769-1.000) (Figure 5). Therefore, in our cohort, the %PC calculated by the DIA emerged as the best predictor of fg-SRL treatment, although the difference compared with the IRS did not reach statistical significance (AUC 0.917 vs 0.763, $P = .06$). No statistically significant differences were observed between IRS and DIA intensity/area ($P = .116$), or between the 2 DIA parameters (ie, intensity/area vs. %PC, $P = .606$).

We then identified the best cut-off for each method to predict fg-SRL response. These cut-offs were the following: IRS ≥5 (sensitivity 77%, specificity 83%), DIA intensity/area ≥0.106, and %PC-DIA ≥63.7% (both with a sensitivity of 92% and a specificity of 83%).

Since only 2 patients in our cohort showed an IGF-1 xULN % decrease >50%, the analysis using this cut-off has a limited value.

## Discussion

In this study, for the first time, we validated the quantification of SSTR5 expression in a series of GH-secreting pituitary tumors using a DIA pipeline, developed with a widely-available open-source software. Furthermore, we validated our DIA protocol for SSTR2 quantification in an independent cohort of patients with acromegaly and with IHC performed in a different laboratory, compared with our previous study.[9] To the best of our knowledge, only one previous study has reported a DIA of SSTR2 and SSTR5 using open-source software in another tumor type.[12] Tollefsen and colleagues evaluated SST expression in meningiomas using the software ImageJ but, in contrast to our study, no selection of the tumor area was performed (ie, ROI selection). This could lead, in case of the presence of fibrosis or artifacts, to an under- or over-estimation of the receptor expression. Moreover, with our DIA protocol, we were able to calculate the percentage of PC, since our software is able to identify cells based on hematoxylin staining of the nuclei. The choice of the specific software is influenced by their different advantages and limitations. In particular, the DIA performed with CellProfiler allowed us to define the tumoral area, have a reliable receptor expression quantification, and have an evaluation of the percentage of PC, while being easy to use with no costs (open-source program). Nevertheless, Tollefsen and colleagues reported strong correlations between manual scoring (evaluated with a staining index, similar to the IRS) and DIA quantification, comparable to those reported in

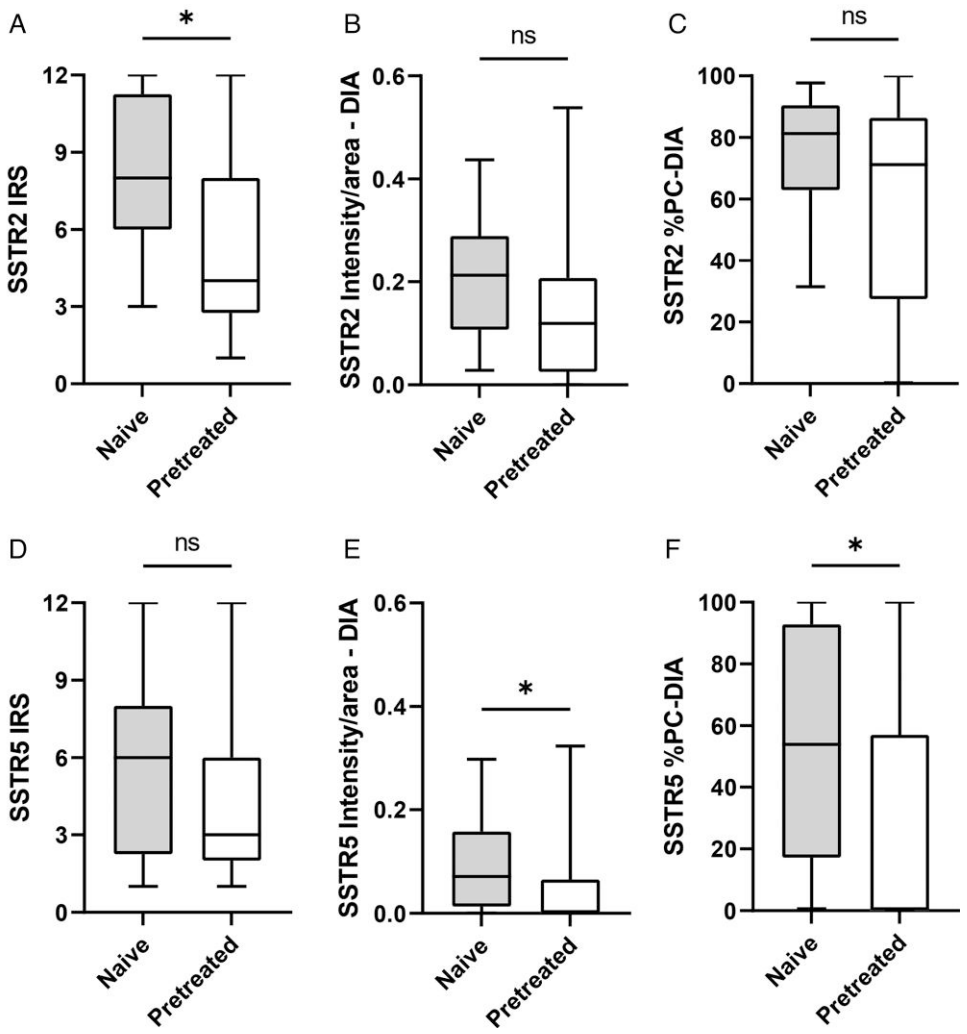


**Figure 3.** Impact of pre-treatment with fg-SRLs on SSTR2 (A-C) and SSTR5 (D-F) expression, evaluated with IRS and DIA. IRS, immunoreactive score; PC-DIA, percent of positive cells calculated by the digital image analysis. *indicates significant difference ($P < .05$).

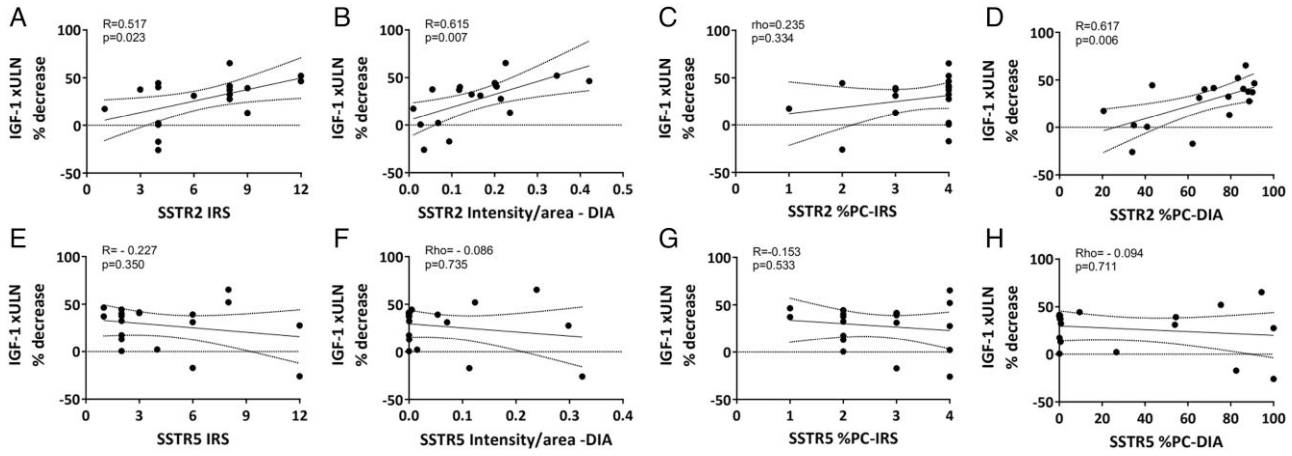


**Figure 4.** Correlation plots of SSTR2 and SSTR5 expression with the IGF1 xULN % decrease. IGF-1 xULN, IGF-1 adjusted for the upper limit of normality; IRS, immunoreactive score; PC-DIA, percent of positive cells calculated by the digital image analysis.

the present manuscript. This finding highlights the high reliability of digital quantification, irrespective of the software used.

We used the IRS as a manual quantification method to validate the DIA, as previously reported, due to its widespread use in pituitary tumors.[9] Moreover, the IRS has been recently proposed as the gold standard for routine SSTR2 expression evaluation in GH-secreting pituitary tumors.[5,6,9] In the present manuscript, we confirm the good correlation between

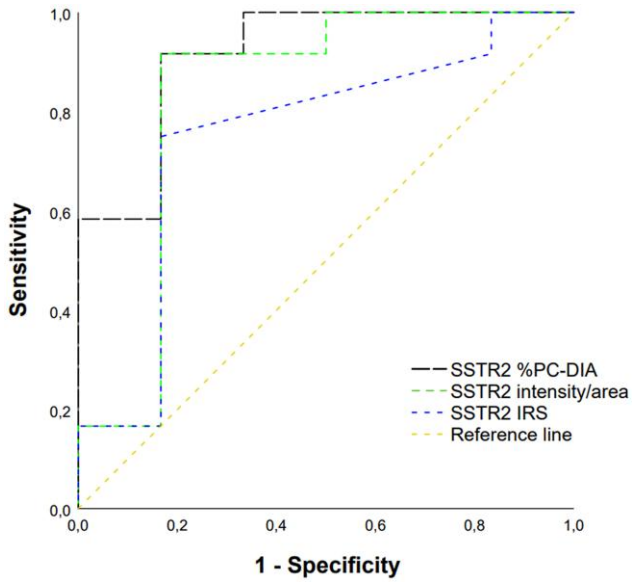


**Figure 5.** ROC curves showing predictive value of SSTR2 for fg-SRL response, using the different quantification methods. PC-DIA, percent of positive cells calculated by the digital image analysis; IRS, immunoreactive score.

DIA and IRS, both for the "total" SSTR2 expression quantification, with a correlation coefficient comparable to the one reported in our previous cohort ($\rho = 0.924$ vs. 0.922, respectively), as well as for the %PC component ($\rho = 0.694$ vs 0.876, respectively).[9] These results underline the reliability of the developed DIA protocol in different settings. Of note, in the present study the IHC was performed manually, and in a different laboratory facility than the one used to develop the original DIA protocol. In this light, as described in the Methods section, we have optimized the pipeline, based on the DAB and the hematoxylin staining intensity. However, the reproducibility of the data underlines the value of this approach and the possibility to use the DIA in different settings, by optimizing the protocol based on the specific cohort as needed. It has to be noted that it is possible to have single points of non-concordance between the DIA and the manual scoring caused by a low threshold set for the DIA which considers as negative samples that are scored as (very) weakly positive by the pathologist performing the IRS, the selection of the area analyzed by the DIA not being representative of the whole slide, or the inclusion in the analysis (both for DIA and IRS) of areas with artifacts or fibrosis. However, in the presence of heterogeneous receptor staining within the same tissue slide, the DIA is more likely to outperform the IRS, since four ROIs are analyzed, scored, and merged (the final DIA output is represented by the mean of four measures for each slide).

Despite the agreement on the use of UMB-1 clone as the preferred antibody for SSTR2 staining, the heterogeneity of the IHC staining protocols and the quantification methods used in different centers still represent an important limitation for the routine evaluation of SSTR2 in clinical practice.[7,13] The use of a standardized DIA protocol, less prone to inter-person variability, could limit this heterogeneity, leading to more comparable data between different studies in the research setting.

In the present study, we used the same DIA protocol to evaluate SSTR5 expression. This allowed us to perform a quantitative comparison between SSTR2 and SSTR5 staining, although potential confounders, such as the specific antibody affinity, have to be taken into consideration. As previously reported, we observed a higher expression of SSTR2 compared with SSTR5.[14-16] Of note, this pattern is found only in studies evaluating SST expression by IHC, whereas SSTR5 seems to be the predominant SST receptor in studies evaluating mRNA expression.[17]

In our cohort, 64% of patients were pre-treated with fg-SRLs. The impact of pre-treatment on SST expression is still debated.[14,18,19] With respect to SSTR2, a lower receptor expression in patients who received fg-SRLs before surgery compared with naïve patients has been reported in few studies.[14,19] Similarly, it was recently reported that there was a higher SSTR5 expression (at the mRNA level) in patients pre-treated with fg-SRLs, compared with treatment-naïve patients, whereas no difference between the 2 groups has been found at the protein level.[14,18] In our cohort, we observed a slight, but statistically significantly lower expression of SSTR2 in patients treated with fg-SRLs before surgery only when evaluating the staining with the IRS, whereas the DIA evaluation did not differ between the 2 groups. On the contrary, a modest but statistically significant lower SSTR5 expression in pre-treated patients was only observed when using the DIA analysis. Interestingly, a recent study investigated the difference in gene expression induced by fg-SRL treatment by use of transcriptomics. The authors found 95 differentially expressed genes, while SSTs did not come out between the significantly up or downregulated genes, suggesting the absence of a significant modulation of these genes by fg-SRL treatment.[20]

As widely demonstrated in previous studies, we show that SSTR2 expression, but not SSTR5 expression, correlates with the response to fg-SRLs.[3,5,9,14,16,21] In particular, in our study we observed a strong correlation between SSTR2 expression and the % decrease of age-adjusted IGF-1 levels, irrespective of the quantification method used. As concerns the manual scoring, we found that an SSTR2 IRS $\geq 5$ identified responder/partial responder patients with moderate sensitivity and specificity. Similar IRS cut-offs were proposed in previous studies (IRS between 5 and 7) to predict complete or partial response. However, by definition, 5 and 7 are not achievable scores for the IRS. Nevertheless, ROC curve analyses identified these numbers (computed using the Youden index) as the best cut-offs to recognize patients as responders or partial responders to fg-SRLs.[3,5,9,14] In this light, we could speculate that tumors showing an IRS $\leq 4$ would be resistant to fg-SRLs, and a proactive personalized treatment approach could be applied (using pasireotide or the GH receptor antagonist pegvisomant, based on the patient's characteristics).

We compared the ROC curves of the different quantification methods, in order to investigate a possible superiority of the DIA compared with the IRS in discriminating patients likely to respond to fg-SRLs compared with the resistant ones. A cut-off of IGF-1 xULN % decrease >20% was chosen, based on previous data from the literature,[5] as well as on the median IGF-1 xULN % decrease observed in our cohort (most of the patients included in the study had a mild/moderate disease activity after surgery, see Table 1). Interestingly, while no significant difference was observed when comparing the DIA intensity/area with the IRS, a numerically higher AUC was found for the %PC-DIA compared with the "total" IRS (AUC 0.917 vs. 0.763; $P = .06$). Therefore, we can speculate that the percentage of cells expressing the receptor, more

than the staining intensity of the PC, is crucial in determining the response to fg-SRLs. Moreover, the higher AUC observed for the DIA could also reflect its higher sensitivity compared with IRS, due to the use of a continuous scale. In particular, in our previous manuscript evaluating this DIA protocol and in the present study, a %PC >60% (63.7% and 68%, respectively) was associated with the response to fg-SRLs. A previous study from Wildemberg and colleagues also highlighted the importance of the percentage of PC in determining the response to fg-SRLs. The authors found a different cut-off (at least 25% of the cells needed to express SSTR2 in order to have a significant IGF-1 reduction), although using a categorical, manual scoring system, where only 3 categories were defined.[21] In our cohort, the evaluation of SSTR2 %PC-IRS did not correlate with the IGF-1 xULN % decrease, probably due to the relatively low sample size, whereas the %PC-DIA showed a significant positive correlation. This underscores the potential of the DIA be more sensitive and more accurate in the evaluation of IHC data.

As expected, no correlation was observed between SSTR5 expression and response to fg-SRLs.[5,14] The possible role of SSTR2/SSTR5 ratio in predicting the response to fg-SRLs is not univocal. In vitro studies showed a higher SSTR2/SSTR5 ratio in responders,[22,23] whereas a recent study as well as in the present cohort, did not confirm this finding at the protein level.[5]

Recently, some authors proposed E-cadherin protein expression as a possible alternative to SSTR2 to predict response to fg-SRLs.[18] The DIA could provide, in line with the data on SSTR, more reproducible data between studies and higher sensitivity due to the use of continuous data. This hypothesis should be tested in further studies, comparing also the performance of SSTR2 and E-cadherin as prediction factors for fg-SRLs.

We acknowledge that our study has some limitations, mainly due to the retrospective design. Not all data were available for all patients, in particular concerning the treatment outcome in the pre-treatment group, therefore limiting the possibility of assessing the predictive value of SSTRs expression in this population. Nevertheless, we believe that the sample size, taking into account the prevalence of acromegaly, is adequate for the comparison of the different staining quantification methods and for the correlation analysis between receptor expression and fg-SRL response in the adjuvant setting. Due to the nature of the data and its distribution, to our knowledge, it was not possible to evaluate the agreement between the DIA and the IRS. Nevertheless, we believe that the correlation we found between the different methods supports the reliability of the DIA.

In conclusion, the DIA performed with our protocol represents a valuable tool to reliably quantify both SSTR2 and SSTR5 using the same CellProfiler pipeline, with superimposable accuracy, in GH-secreting pituitary tumors. The DIA allows a detailed characterization of the receptor expression, unveiling the pivotal role of the SSTR2 %PC in determining the response to fg-SRLs. The method here described is easy, reproducible, and cost-effective, due to the use of an open-source program. The use of DIA can allow for more comparable results between studies in the research setting and, therefore, stronger evidence on the need for a routine evaluation of SSTR2 and SSTR5 immunostaining in clinical practice.

## Supplementary material

Supplementary material is available at *European Journal of Endocrinology* online.

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## Authors' contributions

Claudia Campana (Data curation [equal], Formal analysis [equal], Methodology [equal], Writing—original draft [equal]), Jessica Amarù (Data curation [equal], Formal analysis [equal]), Angelo Milioto (Data curation [equal], Investigation [equal]), Federica Nista (Data curation [equal], Investigation [equal]), Peter M. van Koetsveld (Methodology [equal], Software [equal]), Anand Iyer (Supervision [equal], Writing—review and editing [equal]), Marica Arvigo (Methodology [equal], Supervision [equal], Writing—review and editing [equal]), Diego Ferone (Methodology [equal], Resources [equal], Supervision [equal], Writing—review and editing [equal]), Leo J. Hofland (Methodology [equal], Software [equal], Supervision [equal], Writing—review and editing [equal]), and Federico Gatto (Methodology [equal], Resources [equal], Supervision [lead], Visualization [equal], Writing—review and editing [lead]).

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