Low beta-arrestin expression correlates with the responsiveness to long-term somatostatin analog treatment in acromegaly

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Abstract

Objective: The high expression of somatostatin receptor subtype 2 (SSTR2 also known as sst2) usually present in growth hormone (GH)-secreting adenomas is the rationale for therapy with somatostatin analogs (SSAs) in acromegaly. Although SSTR2 expression is a good predictor for biochemical response to SSA treatment, we still face tumors resistant to SSAs despite high SSTR2 expression. Recently, beta-arrestins (β-arrestins) have been highlighted as key players in the regulation of SSTR2 function.

Design: To investigate whether β-arrestins might be useful predictors of responsiveness to long-term SSA treatment in acromegaly, we retrospectively evaluated 35 patients with acromegaly who underwent adenomectomy in two referral centers in The Netherlands.

Methods: β-arrestin mRNA levels were evaluated in adenoma samples, together with SSTR2 (and SSTR5) mRNA and protein expression. Biochemical response to long-term SSA treatment (median 12 months) was assessed in 32 patients.

Results: β-arrestin 1 and 2 mRNA was significantly lower in adenoma tissues from patients who achieved insulin-like growth factor 1 normalization (P = 0.024 and P = 0.047) and complete biochemical control (P = 0.047 and P = 0.039). The SSTR2 mRNA was higher in SSA responder patients compared with the resistant ones (P = 0.026). This difference was more evident when analyzing the SSTR2/β-arrestin 1 and SSTR2/β-arrestin 2 ratio (P = 0.011 and P = 0.010). β-arrestin 1 and 2 expression showed a significant trend of higher median values from full responders, partial responders to resistant patients (P = 0.045 and P = 0.021, respectively). Interestingly, SSTR2 protein expression showed a strong inverse correlation with both β-arrestin 1 and 2 mRNA (ρ = −0.69, P = 0.011) and (ρ = −0.67, P = 0.0016).

Conclusions: Low β-arrestin expression and high SSTR2/β-arrestin ratio correlate with the responsiveness to long-term treatment with SSAs in patients with acromegaly.

Introduction

Acromegaly is a severe systemic condition characterized by elevated circulating levels of growth hormone (GH) and insulin-like growth factor 1 (IGF1), in most cases caused by a GH-secreting pituitary adenoma (GHoma) (1). Despite still being considered a rare disease, many recent reports have described a significant increase in the prevalence
of acromegaly in the general population, up to 60–100 cases/million in different geographical areas (2, 3, 4).

Based on the 2014 guidelines for the management of acromegaly, adenomectomy is the suggested first-line treatment when complete surgical cure is expected (5). However, approximately 40–60% of invasive adenomas, which represent the majority of tumors in patients with acromegaly, are unlikely to be controlled by surgery alone, and in these cases medical treatment with somatostatin analogs (SSAs) is the proposed first-line approach (5). In fact, SSAs represent the mainstay of medical therapy, due to their well-established antisecretory and antitumoral effects on GHomas (6). The usually detected high expression of somatostatin receptors (SSTRs), in particular the subtype 2 (SSTR2 also known as sst2), on GHoma cell membrane represents the molecular rationale for SSA therapy in acromegaly. However, several studies (including a recent meta-analysis) have highlighted that about half of the patients do not achieve biochemical control (GH and IGF1 levels normalization) after long-term treatment with SSAs (7). Moreover, despite the demonstration of SSTR2 protein expression as a good predictor for the biochemical response to SSA treatment in acromegaly (8, 9), we still face adenomas that are resistant to SSA therapy in spite of a high expression of this receptor subtype (10, 11). Consequently, in recent years, a number of studies focused on the investigation of different molecular determinants that potentially could affect the responsiveness to SSTR targeting (6, 12). Indeed, peculiar GHoma ‘molecular phenotypes’ (e.g. tumors harboring high SSTR5/SSTR2 ratio, high levels of SSTR5 truncated form, low AIP and ZAC1 levels, low E-cadherin or RKIP expression) have been randomly demonstrated to be associated with reduced, or even absent response to SSA treatment (13, 14, 15, 16, 17, 18).

In order to further increase the knowledge on SSTR pathophysiology, we recently initiated studies on the role of beta-arrestins (β-arrestins) and G-protein coupled receptor kinases (GRKs) in driving SSTR response to SSA treatment, via the regulation of receptor desensitization and trafficking. Briefly, after ligand-mediated receptor phosphorylation, mainly driven by GRKs (in particular GRK2), β-arrestins are recruited to the cell membrane, disrupt the link between the receptor and its related G-proteins, and afterward drive the receptor to the endocytic machinery. As such, these molecules affect the rate of receptor internalization/recycling (19, 20).

In this context, we have recently investigated the expression of β-arrestin 1 and 2 mRNA levels in different pituitary adenoma histotypes and demonstrated that low β-arrestin 1 mRNA expression correlated with improved responsiveness to both in vitro octreotide treatment and to the in vivo response to an acute octreotide administration (21).

The main aim of the present study was to investigate whether the tumor β-arrestin 1 and 2 mRNA expression correlates with the in vivo responsiveness to the long-term treatment with long-acting SSAs in a cohort of patients with acromegaly referred to two internationally recognized referral centers for pituitary diseases. Moreover, we aimed to evaluate the possible correlation between β-arrestin mRNA levels and SSTR2 receptor protein expression in GHomas.

**Subjects and methods**

**Subjects and adenoma tissues**

We restrospectively evaluated 35 patients with acromegaly (14 female; median age 44 years, range 24–70), who underwent neurosurgery for a GHoma in two referral centers for pituitary diseases in The Netherlands (Erasmus Medical Center, Rotterdam and Leiden University Medical Center). Diagnosis of acromegaly was based on clinical features, biochemical evidence of GH hypersecretion, and IGF1 levels above the age-adjusted upper limit of normality range (ULNR), as well as identification of a pituitary adenoma by MRI. Moreover, the pathology report confirmed the presence of a predominant immunoreactivity for GH in all adenoma samples analyzed.

Inclusion criteria of the study were: established diagnosis of acromegaly (based on above-mentioned criteria), neurosurgery (transsphenoidal adenomectomy) during patients’ clinical history, availability of freshly frozen adenoma tissue to evaluate β-arrestin and GRK2 mRNA expression, clinical data about long-term SSA treatment outcome and/or the availability of paraffin-embedded tissue to evaluate SSTR2 and SSTR5 expression at protein level. Exclusion criteria were adjunctive radiotherapy or other medical therapies than SSAs (e.g. dopamine agonists, pegvisomant) before or during the study period.

The main issue limiting the number of samples collected from patients meeting the (above-mentioned) criteria required to be included in the study, was the lack of enough available freshly frozen adenoma tissue to perform the molecular analysis. No preselection of the tumor samples, based on patient’s characteristics such as responsiveness to an acute octreotide test and/or presurgical SSA treatment response and/or severity of the disease was carried out in this study.
Thirty-one out of 35 patients (89%) were harboring a macroadenoma at baseline radiological evaluation.

Approval from the Medical Ethical Committee of the Erasmus Medical Center as well as informed consent to use the tumor tissues for research purposes was obtained.

Fresh adenoma tissue for β-arrestin and GRK2 mRNA detection was available for all samples, while SSTR mRNA expression was evaluated in all but one tumor sample. Moreover, for 19 tumor samples, we were able to collect paraffin-embedded tissues in order to evaluate SSTR2 and SSTR5 expression also at protein level, by immunohistochemistry (Supplementary Table 1, see section on supplementary data given at the end of this article).

GH and IGF1 values were evaluated before surgery (presurgery values, namely the last recorded biochemical values before surgery), before the initiation of long-term SSA treatment (basal values), and at the end of SSA treatment follow-up period (median 12 months, range 3–192). Long-term SSA treatment was defined as at least 3 months treatment with long-acting formulations. Information about long-term SSA treatment outcome was available in 32 out of 35 patients and was considered for the correlation analysis with β-arrestin mRNA expression and SSTR2/β-arrestin ratio.

In detail, of the 32 patients in whom the above-described analysis was carried out, 5 were treated only before surgery, 19 only after surgery, and 8 both before and after surgery (Table 1). Of these latter 8 patients, only the data from postsurgery treatment were considered for the correlation analysis with molecular parameters, since longer follow-up was available compared with presurgical treatment (Table 1 and Supplementary Table 2).

It is worth mentioning that we decided to present in this study the combined analysis of SSA pretreated and postsurgery-treated patients, since in a previous study we have demonstrated that SSA treatment does not affect β-arrestin mRNA expression (21).

Standard starting dosages and timing of administrations in patients who underwent long-term SSA treatment (20 mg i.m./4 weeks for octreotide long-acting release and 90 mg i.m./4 weeks for lanreotide autogel) were adequately titrated after 3 months, if biochemical control was not reached. Biochemical control was defined by morning (overnight fasting) plasma GH levels < 2.5 µg/L and age-corrected IGF1 levels below ULNR. Cutoff for control of GH secretion was set at GH levels < 2.5 µg/L, since all patients included in the study underwent neurosurgery before 2010 and, therefore, decisions on the clinical management of patients (e.g. decision for adjuvant SSA treatment and/or surgery) were made on the basis of the above-mentioned cutoff for single GH measurement, widely accepted at that time. Therefore, we decided to keep the same cutoff throughout the study (until the end of follow-up period).

Both GH and IGF1 concentrations were determined by use of a nonisotopic, automatic chemiluminescence immunoassay system (Immulite; Diagnostic Products Corp., Los Angeles, CA, USA).

Partial information about some patients included in this study has been previously described (21) (Supplementary Table 1 for details). Because of the retrospective design of the study, not all parameters were available for each patient.

Quantitative PCR

Quantitative PCR was performed according to a previously described method (22, 23). Briefly, to perform β-arrestin, GRK-2, and SSTR mRNA evaluation, we isolated poly A+ mRNA from adenoma tissues using Dynabeads Oligo (dT)25 (Dynal AS, Oslo, Norway). cDNA was synthesized using the poly A+ mRNA, which was eluted from the beads in H2O twice for 2 min at 65°C, using Oligo (dT)12–18 Primer (Invitrogen). Samples were analyzed on an ABI Prism 7900 Sequence Detection System (PerkinElmer) for real-time amplifications, according to manufacturer’s protocol. The primer and probe sequences, the efficiencies, and the reaction conditions that were used for the detection of β-arrestins, GRK-2, SSTRs, and the housekeeping gene hypoxanthine phosphoribosyltransferase (HPRT) have been previously described (21, 23, 24).

Immunohistochemistry

Immunostaining and subsequent immunoreactivity score (IRS) evaluation were performed as previously described (10, 25). Briefly, formalin-fixed paraffin-embedded tissues were cut, deparaffinized, and rehydrated. Tissue slides were heated in Tris–EDTA buffer for 20 min, rinsed in water, bathed in a 3% H2O2/PBS solution for 15 min, and afterward washed with Tris–HCl–Twee 0.5%. Sections were then incubated overnight at 4°C with primary antibodies for SSTR2 and SSTR5. The rabbit monoclonal anti-SSTR2 antibody (BioTrend, Köln, Germany) was used at a dilution of 1:50, whereas the rabbit monoclonal anti-SSTR5 antibody was used at dilution of 1:10 (26).

The IRS, ranging between 0 (no staining) and 12 (maximum staining) is a semi-quantitative scoring
system, which allow us to evaluate both the intensity of the staining and the percentage of positive cells in the adenoma tissue slides. IRS evaluation process has been described in detail in previous studies (10, 25).

**Statistical analysis**

Data were statistically analyzed using SPSS software version 21.0 (IBM), while graphs and figures were drawn by use of GraphPad Prism software version 5.02 (GraphPad Software). Quantitative data were presented as mean ± S.D. when data distribution was normal, otherwise median with range (minimum–maximum) was used. In this context, Kolmogorov–Smirnov test was used in order to check the normality of distribution of continuous variables. When reporting data as percentages, due to rounding they do not always add up to 100% (e.g. percentage of full responders, partial responders, and resistant patients).

### Table 1

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P: patients; –: not evaluated; n.a.: data not available; F: female; M: male; Micro: microadenoma; Macro: macroadenoma; Minor: minor tumor volume reduction, based on the neuroradiologist's report (no percentage of tumor volume reduction provided).

*Duration of SSA treatment is reported in the SSA presurgery and SSA postsurgery columns, as months (parentheses).

*Biochemical outcome after long-term SSA treatment refers to postoperative SSA treatment, except for patient number 3, 7, 12, 20, and 32, who underwent long-term SSA treatment only before surgery; *Patients underwent SSA treatment only before surgery (patient number 3, 7, 12, 20, and 32); *Tumor shrinkage refers to tumor volume reduction after presurgical SSA treatment; *Stratified response, classification of SSA responsiveness based on Colao et al. (28); 0, full responders (normalization of both GH and IGF1); 1, partial responders (significant decrease (>50%) of GH and/or IGF1 or normalization of one of the two parameters); 2, resistant (no significant decrease of GH and IGF1 with no achievement of control).
Results

Biochemical evaluation of patients and responsiveness to long-term SSA treatment

Presurgical GH and IGF1 values (presurgery values) and the last available GH and IGF1 measurements before starting long-term SSA treatment (basal values) were recorded. Presurgical GH levels were 24.2 (1.4–371) µg/L and IGF1 values were 2.6 (0.9–10.6) ULNR.

Basal GH and IGF1 values before the start of SSA were 5.3 (0.5–76.0) µg/L and 2.1 (1.15–7.7) ULNR, respectively. Basal GH and IGF1 ULNR levels were strongly and positively correlated ($\rho=0.683$, $P<0.0001$).

Long-term SSA treatment resulted in a median GH percentage decrease of $-64.3\%$ ($-94.7$ to $-412$) and a mean ($\pm$ s.d.) IGF1 decrease of $-30.0\%$ $-35.9\%$. Percent GH and IGF1 lowering were positively correlated ($\rho=0.471$, $P=0.011$).

After long-term SSA treatment, 56.7% of patients reached GH normalization (<2.5 µg/L), 40.6% normalized IGF1 levels (IGF1 ULNR ≤1), and 34.4% showed full biochemical control (GH and IGF1 normalization) at the end of follow-up period (median 12 months) (Table 2). Even taking into account that this study has been carried out in two tertiary referral centers for pituitary diseases, the outcome of long-term SSA treatment observed in our series is comparable with the efficacy data of the ‘classical’ SSAs reported in latest studies and meta-analyses (6, 7, 27).

Based upon the proposed definition of responsiveness and resistance to SSA treatment in acromegaly by Colao et al. (28), we also stratified our patients into full responders (normalization of both GH and IGF1), partial responders (significant decrease (>50%) of GH and/or IGF1 or normalization of one of the two parameters), and resistant (no significant decrease of GH and IGF1 with no achievement of biochemical control). In this context, in 2 out of 32 patients (6.3%) ‘stratification’ was not possible, due to missing GH data. Therefore, about 37% (11/30, 36.7%) were resistant to SSA treatment (Table 2), while 8/30 (26.7%) patients were classified as partial responders and 11/30 (36.7%) as full responders.

Responsiveness to SSAs was not affected by gender or tumor size. However, younger age was correlated with a significantly lower rate of IGF1 normalization ($P=0.045$).

β-arrestin, GRK2, and SSTR expression

β-arrestin and GRK2 expression was evaluated in adenoma tissues at mRNA level, while for SSTR2 and SSTR5 both...
mRNA and protein expression was investigated. As for β-arrestin and GRK2 expression, in line with recent data from our group (21), β-arrestin 2 was the most represented, while β-arrestin 1 was overall the molecule expressed at the lowest level. In detail, median (range) β-arrestin 2 mRNA expression (relative expression, normalized to hprt) was 0.668 (0.217–2.04), β-arrestin 1 levels were 0.013 (0.0–0.147), and GRK-2 levels were 0.313 (0.099–0.733).

The expression of β-arrestins and GRK2 was not affected by SSA presurgical treatment. No statistically significant difference was observed between tumor samples from SSA pretreated and naïve patients (β-arrestin 1, \( P = 0.533 \); β-arrestin 2, \( P = 0.533 \); GRK-2, \( P = 0.159 \)).

Moreover, β-arrestin 1 (\( \rho = 0.231, P = 0.197 \)), β-arrestin 2 (\( \rho = 0.184, P = 0.315 \)), GRK2 (\( \rho = -0.116, P = 0.520 \)), and also SSTR2 mRNA expression (\( \rho = -0.109, P = 0.552 \)) did not correlate with basal GH values.

SSTR mRNA expression was in line with previous data from literature (29, 30). SSTR5 was the most predominantly expressed SSTR (relative expression, normalized to hprt), with a median value of 0.206 (range 0.0–0.860), followed by SSTR2 (median 0.139, range 0.026–0.543).

The expression of SSTR2 and SSTR5 receptors was also evaluated at the protein level. The median (range) IRS was 4 (1–12) for SSTR2 and 4 (0–12) for SSTR5.

**Impact of β-arrestin expression and SSTR2/β-arrestin ratio on long-term SSA treatment**

β-arrestin 1 mRNA level was significantly lower in adenoma tissues from patients, who achieved IGF1 normalization (\( n = 32; P = 0.024 \)) and complete biochemical control (GH and IGF1 normalization; \( n = 32; P = 0.047 \)) (Fig. 1A and B). The same finding was observed for

**Figure 1**
Comparison of β-arrestin 1 and β-arrestin 2 mRNA levels in adenoma samples of patients with acromegaly that normalized or did not normalize IGF1 levels after long-term SSA treatment are depicted in panels A and C. Furthermore, differences between β-arrestin levels in adenoma from patients who achieved or did not reach full biochemical control are represented in panels B and D. Statistical significance was determined by the Mann–Whitney U test. The lower and upper bars represent the 25th and 75th percentiles (IQR), respectively. The lines across the box represent median value. The lines above and below the box represent 75th percentile plus 1.5 times IQR and the 25th percentile minus 1.5 times IQR, respectively. Black dots represent all data above or below these values. Statistically relevant differences (\( P < 0.05 \)) are reported in each graph. β-arrestin mRNA expression levels are normalized against the housekeeping gene HPRT. GH, growth hormone; HPRT, hypoxanthine phosphoribosyltransferase; IGF1, insulin-like growth factor 1; IQR, interquartile range.

**Figure 2**
SSTR2 mRNA levels in tumor samples from patients responders or resistant to long-term SSA treatment (panel A). It is worth noting that the difference between the two groups was statistically more evident when comparing the ratio between SSTR2 and β-arrestin expression (panels B and C). Statistical significance was determined by the Mann–Whitney U test. Statistically relevant differences (\( P < 0.05 \)) are reported in each graph. SSTR2 and β-arrestin mRNA expression levels are normalized against the housekeeping gene HPRT. HPRT, hypoxanthine phosphoribosyltransferase; SSA, somatostatin analog; SSTR2, somatostatin receptor subtype 2.
β-arrestin 2 mRNA expression, which was significantly lower in patients reaching IGF1 and both GH and IGF1 normalization after long-term SSA treatment ($n=32$, $P=0.047$, and $P=0.039$, respectively) (Fig. 1C and D). No statistically significant differences were observed between β-arrestin expression and GH normalization ($n=30$; β-arrestin 1, $P=0.21$; β-arrestin 2, $P=0.13$), while SSTR2 mRNA levels were significantly higher in patients, who achieved GH normalization, compared with not normalized patients ($n=29$, $P=0.017$). Interestingly, when evaluating the role of SSTR2/β-arrestin ratio, we observed that both SSTR2/β-arrestin 1 and SSTR2/β-arrestin 2 ratios were significantly higher (even more than SSTR2 mRNA alone) in GH normalized patients ($n=29$, $P=0.015$ and $n=29$, $P=0.003$, respectively). Similarly, despite we observed that SSTR2 mRNA expression was significantly higher in the SSA responder patients compared with the resistant ones ($n=29$, $P=0.026$), the difference between the two groups was statistically more evident, when analyzing SSTR2/β-arrestin 1 and SSTR2/β-arrestin 2 ratios ($n=29$, $P=0.011$ and $n=29$, $P=0.010$, respectively) (Fig. 2).

Noteworthy, both β-arrestin 1 and β-arrestin 2 mRNA expression showed a significant trend (Jonckheere–Terpstra test) across the stratified classification of SSA responsiveness, with higher median values in patients with a lower response (from full responders, partial responders, to resistant patients) ($n=30$; $T_{JT}=201$, $z=2.0$, $P=0.045$ and $T_{JT}=209$, $z=2.3$, $P=0.021$, respectively). On the contrary, SSTR2 mRNA levels did not show any statistically significant trend ($n=29$, $T_{JT}=104$, $z=–1.4$, $P=0.160$) (Fig. 3A). However, also in this case, the analysis of SSTR2/β-arrestin 1 and SSTR2/β-arrestin 2 ratios provided the most consistent trends across the three groups of patients stratified based on their responsiveness to long-term SSA treatment. Indeed, both SSTR2/β-arrestin 1 ratio ($n=29$) and SSTR2/β-arrestin 2 ratio ($n=29$) showed a strong significant trend across the three patient’s categories ($T_{JT}=71$, $z=–2.7$, $P=0.006$ and $T_{JT}=73$, $z=–2.6$, $P=0.008$, respectively) (Fig. 3B and C).

**Figure 3**

Trends of SSTR2 and SSTR2/β-arrestin ratio across the stratified classification of SSA responsiveness (panels A–C). Stratification of patients’ response into full responders, partial responders, and resistant has been performed on the basis of the proposed classification by Colao et al. (28). Statistical significance was determined by the Jonckheere–Terpstra test. Related $P$ values are reported in each panel. SSTR2 and β-arrestin mRNA expression levels are normalized against the housekeeping gene HPRT. HPRT, hypoxanthine phosphoribosyltransferase; SSA, somatostatin analog; SSTR2, somatostatin receptor subtype 2.

**Figure 4**

Correlation between SSTR2 IRS and β-arrestin 1 and 2 mRNA (panels A and B). The correlation analysis between SSTR5 IRS and β-arrestin 2 (not statistically significant) is shown in panel C. Statistical significance was determined by the Spearman rank order $\rho$ test. Related $\rho$ and $P$ values are reported in each panel. In panel D, representative examples of high and low/very low SSTR2 and SSTR5 IRS are shown (magnification, 200). A predominant membranous staining was observed in all samples. The IRS is based on the evaluation of two independent observers, who were blinded for the other observer’s score. All slides were scored identically by the two independent researchers. HPRT, hypoxanthine phosphoribosyltransferase; IRS, immunoreactivity score; SSTR2, somatostatin receptor subtype 2; SSTR5, somatostatin receptor subtype 5.
Finally, despite the possible role of GRK2 in the modulation of SSA responsiveness described in our previous study evaluating the *in vitro* and *in vivo* acute response to octreotide treatment (21), we did not find any significant correlation between GRK2 mRNA expression and the long-term responsiveness to SSA treatment in our cohort of patients with acromegaly (e.g. GRK2 and IGF1 normalization, \(P = 0.985\); GRK2 and both GH and IGF1 normalization, \(P = 0.815\); GRK2 vs stratified response, \(P = 0.894\)).

**Correlation between β-arrestin and SSTR protein expression**

As described before, we were able to perform immunohistochemistry for SSTR2 and SSTR5 in 19 adenoma samples. In line with previous data from literature, SSTR2 IRS showed a significant trend of lower median values from full responders, partial responders, to resistant patients \((n = 14, T_{1/2} = 10.5, z = -2.5, P = 0.011)\) and was significantly lower in resistant tumors compared with the (partial) responder ones \((P = 0.021, n = 14)\). Interestingly, SSTR2 and SSTR5 IRS were positively, but not significantly, correlated \((\rho = 0.394, P = 0.095, n = 19)\).

We then investigated the possible correlation between SSTR2 IRS, SSTR5 IRS, and β-arrestin mRNA expression. Interestingly, SSTR2 IRS showed a strong significant inverse correlation with both β-arrestin 1 and β-arrestin 2 mRNA expression \((n = 19, \rho = -0.69, P = 0.0011\) and \(\rho = -0.67, P = 0.0016\), respectively) (Fig. 4A and B). On the other hand, SSTR5 IRS did not show any statistically significant correlation with both β-arrestin 1 and β-arrestin 2 (Fig. 4C) mRNA expression \((n = 19, \rho = -0.35, P = 0.147\) and \(n = 19, \rho = -0.44, P = 0.057\) respectively).

**Discussion**

This is the first study demonstrating that β-arrestin mRNA expression (both β-arrestin 1 and β-arrestin 2) may affect the responsiveness to the long-term treatment with SSAs in patients with acromegaly.

Moreover, we observed that β-arrestin 1 and β-arrestin 2 mRNA had a strong inverse correlation with SSTR2 protein expression in GHomas samples.

These novel findings in patients on long-term SSA treatment strengthen our recent data, showing that β-arrestin 1 mRNA expression correlated with a better response to both *in vitro* and *in vivo* acute octreotide test in GHomas (21). However, different from what has been observed in the above-mentioned study, in the current one, we clearly demonstrate an important role of both β-arrestins, and not only of β-arrestin 1, in the modulation of long-term responsiveness to SSAs.

As already stated in the ‘Subjects and methods’ section, in this study we did not perform a formal adjustment for multiple statistical testing, since the great majority of the tests we carried out were correlated, complementary and, furthermore, their results were consistent with the starting hypotheses of the study.

We have to highlight that the measurement of β-arrestins was performed by the use of qRT-PCR since, in our opinion, nowadays is the most accurate method to discriminate between the two β-arrestin isoforms. To the best of our knowledge, there is a lack of well-validated monoclonal antibodies, suitable for immunohistochemistry, capable of identifying the two different isoforms with high specificity. This is probably due to the high homology and similarity (78 and 88%, respectively) in the amino acid sequence shared by the two β-arrestins (31, 32). Moreover, this is also confirmed by the observation that a number of studies investigating β-arrestin protein expression report the use of a polyclonal antibody that recognizes both β-arrestin isoforms, displayed in western blot experiments as two adjacent bands with a very similar molecular weight (32, 33). However, it is known that the mRNA evaluation may not always resemble the protein quantification of a given molecule. Therefore, the role of β-arrestins in driving the response to SSA treatment, to date highlighted and investigated only at mRNA level, needs to be demonstrated and confirmed at protein level as well.

A differential role of the two β-arrestin isoforms in the modulation of SSTR2 function has not been clarified in detail yet. Indeed, based on the G protein–coupled receptor (GPCR) classification made by Oakley et al. (34), which refers to the differential receptor affinity for the two β-arrestins, SSTR2 has been classified as a class B receptor, since its activation results in a robust recruitment of both β-arrestin 1 and 2, while activated SSTR5 only binds β-arrestin 2 (19). In the light of our observations and previous data from literature, we can speculate about a predominant role of β-arrestin 1 in driving SSTR2 ‘acute’ desensitization (35, 36, 37), while both β-arrestins are involved in the long-term receptor regulation, which involves internalization, resensitization, and trafficking processes (19, 38). When considering the correlation analysis between β-arrestin mRNA and SSTR2 protein expression in the present cohort, we have to take into account the
possible role of SSA pretreatment in the downregulation of SSTR2 protein expression. Although in this study we did not find a significant difference in SSTR2 protein expression between SSA pretreated and treatment-naive patients, possibly due to the low number of pretreated patients in whom protein evaluation was available (5 out of 19), we also performed the correlation analysis between β-arrestin mRNA and SSTR2 protein expression considering only the 14 treatment-naive patients. In this context, despite the relatively low number of cases included in the analysis, we found that the inverse correlation between β-arrestin mRNA expression and SSTR2 protein was still statistically significant (β-arrestin 1: \( \rho = -0.714, P=0.004 \); β-arrestin 2: \( \rho = -0.626, P=0.017 \)), thus further supporting our results.

On the contrary, SSTR5 protein expression seems not to be affected by SSA treatment (39). When analyzing the correlation between SSTR5 protein and β-arrestins, we have already mentioned that the activation of SSTR5 results in the recruitment of β-arrestin 2, and not of β-arrestin 1, as for all class A receptors. Moreover, the link between the receptor and β-arrestin 2 seems to be more transient and less tight, compared with that of class B receptors, such as SSTR2 (40). These data are in line with our findings of a lack of correlation between SSTR5 protein and both β-arrestin 1 and β-arrestin 2 mRNA.

Furthermore, we observed that the combined evaluation of both β-arrestins and SSTR2 mRNA, expressed as SSTR2/β-arrestin ratio, showed a stronger correlation with the responsiveness to SSA treatment, compared with the individual measurement of SSTR2 or β-arrestin 1 and 2 alone. Again, this finding supports the pathophysiologically rationale of β-arrestin measurements as an additional useful marker (combined with SSTR2) to predict tumor response to SSTR2 agonist treatment. Indeed, a number of studies have recently demonstrated that the responsiveness to SSA treatment depends on multiple critical factors, including obviously SSTR expression (8, 39), but also the counterregulatory processes involved in receptor desensitization, internalization, and trafficking (40, 41, 42). As already mentioned, β-arrestins play a key role in these latter processes in most GPCRs, including SSTR2. Therefore, as the data presented in this study show, it is likely that the heterogeneous expression of β-arrestins in the different GHoma tumor samples affect both SSTR2 function (signaling and desensitization processes) and its expression on cell membrane (internalization and recycling), resulting in the modulation of patient responsiveness to SSA treatment.

However, since tachyphylaxis and escape to SSA treatment are rare events in acromegaly, the steady state response to SSA treatment, which represents a pharmacologically ‘desensitized’ response (41), depends on the balance between desensitized/internalized SSTR2 and the amount of active receptor present on cell membrane. Based on these observations, the above-described balance seems to remain stable in GHomas, even in case of extended long-term treatment (years to decades). A possible explanation for this finding may be the relatively low β-arrestin (in particular β-arrestin 1) expression detected in GHomas (compared with other pituitary adenomas and tissues) and the lack of changes in the expression and/or activation state of β-arrestins (or other receptor regulators, such as GRKs and sorting proteins) during the natural history of the adenoma.

In a more clinical context, we found that about 37% of patients included in our cohort did not achieve a significant reduction in both GH and IGF1 levels after long-term SSA treatment (median 12 months). Although this study was carried out in two tertiary Centers for pituitary diseases, where, by definition, the most difficult cases are referred to, the above reported percentage is in line with the resistance rate to SSA treatment in acromegaly reported by Colao et al. (28), based on their critical review of literature.

The evidence that a consistent percentage of patients are resistant to medical treatment with the ‘classical’ SSAs (octreotide and lanreotide) raises the need for additional molecular markers to identify SSA responsiveness and pushes researchers to develop novel treatment strategies too. In this light, as already mentioned, we have demonstrated that β-arrestin evaluation could represent a valuable new molecular marker, in addition and together with SSTR2, to discriminate between responder and non-responder patients.

Moreover, the role of β-arrestins has been demonstrated to be crucial in the so-called ‘biased-agonism’ mechanism, in which specific downstream pathways of the receptor are activated by a given ligand. As β-arrestins are also able to activate specific second-messenger pathways, via activation of MAPK cascade, β-arrestin-biased agonists have been developed and tested to target a number of GPCRs (e.g. β-adrenergic receptors) (20, 43). It is worth mentioning that the novel SSA pasireotide has been demonstrated to act as a biased agonist at SSTR2 receptor, compared with both native somatostatin and octreotide (44). Interestingly, this differential receptor activation between the different ligands seems a consequence of striking differ-
ences in respect to β-arrestin recruitment. Thus, the future availability of a molecule able to act as a full agonist of SSTR2 in respect of its main second-messenger pathways (e.g. inhibition of cAMP and intracellular calcium), but ‘biased’ for β-arrestin recruitment, could represent a novel therapeutic tool to increase the rate of responder patients to SSTR2 targeting.

In conclusion, this study shows that low β-arrestin mRNA expression and high SSTR2/β-arrestin ratio correlate with the responsiveness to long-term SSA treatment. It is worth mentioning that the combined evaluation of both β-arrestins and SSTR2 results in a stronger correlation when compared with SSTR2 mRNA analysis alone. Therefore, we believe that β-arrestin expression can represent a useful additional marker, together with SSTR2 protein evaluation (and clinical predictors such as baseline biochemical parameters and patient’s response to an acute octreotide test) to predict responsiveness to long-term SSA treatment in acromegaly. The confirmation of the important role of β-arrestins also at the protein level by use of techniques routinely performed for clinical diagnosis (e.g. immunohistochemistry), will need the future availability of trustable antibodies for the evaluation of β-arrestins on paraffin-embedded tissues. When achieved, it could represent a real step forward to the identification of those patients with acromegaly likely to be good responders to adjuvant SSA treatment.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/EJE-15-0391.

Declaration of interest
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References
agons. *Journal of Clinical Endocrinology & Metabolism* 2013 **98** E1880–E1890.
39 Casar-Borota O, Heek A, Schulz S, Nesland JM, Ramm-Pettersen J, Lekka T, Aláfuzzoff I & Bollerslev J. Expression of SSTR2A, but not of SSTR1, 3, or 5 in somatotroph adenomas assessed by monoclonal antibodies was reduced by octreotide and correlated with the acute and long-term effects of octreotide.
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