RESEARCH

# Molecular profiling for acromegaly treatment: a validation study

**Manel Puig-Domingo[1,2,3,4,*], Joan Gil[2,*], Miguel Sampedro-Nuñez[4,5], Mireia Jordà[2], Susan M Webb[6], Guillermo Serra[7], Laura Pons[8], Isabel Salinas[1], Alberto Blanco[9], Montserrat Marques-Pamies[1], Elena Valassi[6], Antonio Picó[4,10,11], Araceli García-Martínez[4,10], Cristina Carrato[8], Raquel Buj[2,12], Carlos del Pozo[13], Gabriel Obiols[14], Carles Villabona[15], Rosa Cámara[16], Carmen Fajardo-Montañana[17], Clara V Alvarez[18], Ignacio Bernabéu[19]** and **Mónica Marazuela[4,5] on behalf of REMAH investigators**

[1]Department of Endocrinology and Nutrition, Germans Trias i Pujol University Hospital, Badalona, Spain
[2]Germans Trias i Pujol Research Institute (IGTP), Badalona, Spain
[3]Department of Medicine, Autonomous University of Barcelona, Barcelona, Spain
[4]Biomedical Research Networking Center in Rare Diseases (CIBERER), Institute of Health Carlos III (ISCIII), Madrid, Spain
[5]Department of Endocrinology, Hospital de la Princesa, Universidad Autónoma de Madrid, Instituto Princesa, Madrid, Spain
[6]Department of Endocrinology/Medicine, CIBERER U747, ISCIII, Research Center for Pituitary Diseases, Hospital Sant Pau, IIB-SPau, Universitat Autònoma de Barcelona, Barcelona, Spain
[7]Department of Endocrinology, Son Espases University Hospital, Palma de Mallorca, Balearic Islands, Spain
[8]Department of Pathology, Germans Trias i Pujol University Hospital, Badalona, Spain
[9]Department of Neurosurgery, Germans Trias i Pujol University Hospital, Badalona, Spain
[10]Hospital General Universitario de Alicante-Institute for Health and Biomedical Research (ISABIAL), Alicante, Spain
[11]Department of Clinical Medicine, Miguel Hernández University, Elche, Spain
[12]Department of Cellular and Molecular Physiology, Penn State College of Medicine, Hershey, Pennsylvania, USA
[13]Department of Endocrinology, Hospital Universitari Mutua Terrassa, Terrassa, Spain
[14]Department of Endocrinology, Hospital General Universitari Vall d'Hebron, Universitat Autònoma de Barcelona, Barcelona, Spain
[15]Department of Endocrinology, Hospital Universitari de Bellvitge, Barcelona, Spain
[16]Endocrinology Department, Hospital Universitario y Politécnico La Fe, Valencia, Spain
[17]Endocrinology Department, Hospital Universitario de La Ribera, Alzira, Spain
[18]Neoplasia & Endocrine Differentiation P0L5, Centro de Investigacion en Medicina Molecular y Enfermedades Cronicas (CIMUS), Instituto de Investigacion Sanitaria de Santiago (IDIS), Universidad de Santiago de Compostela (USC), Santiago de Compostela, Spain
[19]Endocrinology Division, Complejo Hospitalario Universitario de Santiago de Compostela (CHUS)-SERGAS, Santiago de Compostela, Spain

**Correspondence should be addressed to M Puig-Domingo: mpuigd@igtp.cat**

*(M Puig-Domingo and J Gil contributed equally to this work)

## Abstract

Pharmacologic treatment of acromegaly is currently based upon assay-error strategy, the first-generation somatostatin receptor ligands (SRL) being the first-line treatment. However, about 50% of patients do not respond adequately to SRL. Our objective was to evaluate the potential usefulness of different molecular markers as predictors of response to SRL. We used somatotropinoma tissue obtained after surgery from a national cohort of 100 acromegalic patients. Seventy-one patients were treated with SRL during at least 6 months under maximal therapeutic doses according to IGF1 values. We analyzed the expression of *SSTR2*, *SSTR5*, *AIP*, *CDH1* (E-cadherin), *MKI67* (Ki-67), *KLK10*, *DRD2*, *ARRB1*, *GHRL*, In1-Ghrelin, *PLAGL1* and *PEBP1* (*RKIP*) by RT-qPCR and mutations in *GNAS* gene by Sanger sequencing. The response to SRL was categorized as complete response (CR), partial (PR) or non-response (NR) if IGF1 was normal, between >2<3 SDS or >3 SDS IGF1 at 6 months of follow-up, respectively. From the 71 patients treated, there were 27 CR (38%), 18 PR (25%) and 26 NR (37%). *SSTR2,* Ki-67 and E-cadherin were associated with SRL response ($P < 0.03$, $P < 0.01$ and $P < 0.003$, respectively). E-cadherin was the best

**Key Words**

- acromegaly
- E-cadherin
- SSTR2
- Ki-67
- predictive response
- somatostatin receptor ligands (SRL)
- somatostatin analogues

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discriminator for response prediction (AUC = 0.74, $P < 0.02$, PPV of 83.7%, NPV of 72.6%), which was validated at protein level. *SSTR5* expression was higher in patients pre-treated with SRL before surgery. We conclude that somatotropinomas showed heterogeneity in the expression of genes associated with SRL response. E-cadherin was the best molecular predictor of response to SRL. Thus, the inclusion of E-cadherin in subsequent treatment-decision after surgical failure may be useful in acromegaly.

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## Introduction

Acromegaly is a rare disease, usually diagnosed late in the disease evolution, being caused in most of the cases by a pituitary adenoma (Melmed 2006). Current therapeutic modalities include surgery, pharmacologic compounds and radiotherapy when the previous two options fail (Katznelson *et al.* 2014, Melmed *et al.* 2018). The disease is severe enough to try to diagnose it as early as possible as well as to cure or control hormonal hypersecretion quickly after diagnosis. Unfortunately, most of the patients do not reach both diagnosis and disease control early (Reid *et al.* 2010, Rochette *et al.* 2016); moreover, somatotropinomas are heterogeneous tumors that may show diverse responses to the different pharmacologic options currently available. The first-generation somatostatin receptor ligands (SRL), octreotide and lanreotide, are the compounds recommended as first-line option when pharmacologic treatment is considered. When this treatment fails, which occurs in about 50% of cases (varying depending on the series from 30 to 75% (Freda 2002, Gadelha *et al.* 2017)), due mostly, but not exclusively, to the different expression levels of somatostatin receptors (SSTR) in the somatotropinoma surface (Wildemberg *et al.* 2013, Venegas-Moreno *et al.* 2018), second generation SRL or the GH receptor antagonist pegvisomant are used for controlling hormonal hypersecretion (Melmed *et al.* 2018). Current guidelines do not indicate which pharmacologic option best fits for an individual case, as treatment-decision process is based upon the trial-error approach (Chanson & Salenave 2008, Gadelha 2015). As a consequence, attainment of disease control may last considerably for a given patient after diagnosis. Additionally, response to first-generation SRL may be positive but incomplete, requiring not just the substitution by another compound but sometimes the combination with another drug, such as pegvisomant or dopamine agonists.

In the last few years, different studies (Gadelha *et al.* 2017, Gatto *et al.* 2017) have shown that the therapeutic response to first-generation SRL may be explained by the expression of the different SSTRs as well as by the downstream signaling in the somatotropinoma cells. Accordingly, the expression of *SSTR2* and *SSTR5* (Casar-Borota *et al.* 2013, Gatto *et al.* 2013, Wildemberg *et al.* 2013, Venegas-Moreno *et al.* 2018), the *SSTR2*/*SSTR5* ratio (Taboada *et al.* 2007), the expression of the long and short *DRD2* isoforms (Venegas-Moreno *et al.* 2018), aryl hydrocarbon receptor interacting protein (*AIP*) (Chahal *et al.* 2012), E-cadherin (*CDH1*) (Fougner *et al.* 2010, Kiseljak-Vassiliades *et al.* 2015*a*), Ki-67 (*MKI67*) (Kasuki *et al.* 2013), arrestin beta-1 (ARRB1) (Gatto *et al.* 2016, Coelho *et al.* 2018), ghrelin (*GHRL*), intron 1 ghrelin (In1-ghrelin) (Ibáñez-Costa *et al.* 2015), *ZAC1* (*PLAGL1*) (Chahal *et al.* 2012), Raf kinase inhibitory protein (*PEBP1* or *RKIP*) (Fougner *et al.* 2008), kallikrein 10 (*KLK10*) (Rotondo *et al.* 2015) and mutations in guanine nucleotide binding protein (G Protein), alpha stimulating activity polypeptide 1 (*GNAS* or *GSP*) mutations (Efstathiadou *et al.* 2015) have been involved in SRL response. In most of the studies, each of these molecules has been evaluated individually and their ability to predict therapeutic response has been variable. We aimed to evaluate all these potential markers to SRL response in a large series of acromegaly patients in which these compounds were used in order to identify those markers having a high predictive capacity.

## Patients and methods

### Patients

A transnational cohort consisting of 100 acromegaly patients from 26 tertiary centers from all over Spain who had undergone pituitary surgery and had tissue availability (RNA later preserved tumor sample) were included in the study. In those patients in which more than one surgery was performed, only one sample tumor per patient was analyzed. The description of the phenotypic characteristics of the cohort is presented in Table 1.

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

| Patients characteristics | |
|---|---|
| Cohort (*n*) | 100 |
| Male/female | 44/56 |
| Age | 45.5 ± 13.28 |
| Medical treatment | |
| DA treated | 12 |
| SRL presurgery | 67 |
| SRL response | |
| Non-responders | 26 |
| Partial responders | 18 |
| Complete responders | 27 |
| Comorbidities (%) | |
| Diabetes | 27 |
| HBP | 29 |
| Dyslipidemia | 27 |
| Cancer | 6 |
| Cerebrovascular accident | 3 |
| Cardiovascular incident | 13 |
| Visual alterations | 18 |
| Tumor characteristics (%) | |
| Macroadenoma | 79 |
| Extrasellar growth | 77 |
| Sinus invasion | 61 |

Follow up data after surgery regarding SRL response with a minimum follow-up of 6 months were available in 71 patients, 51 who had received SRL treatment before surgery and 20 patients who had not.

The heterogeneous cohort of patients tries to reflect the daily practice of acromegaly management. Basal molecular and clinical information for correlation analyses were available in the whole cohort. Of these 100 patients, 67 had received SRL treatment (octreotide or lanreotide) before surgery and 33 had not received treatment before surgery. Follow-up data after surgery regarding SRL response were available in 71 patients, including 51 out of 67 cases (51% females, mean age 45.3±13 years) who had received SRL treatment before surgery and 20 out of 33 patients who had not (51% females, mean age 44.6±13 years). All patients in which clinical information was available at follow-up and were treated after surgery for at least 6 months under maximal effective therapeutic (octreotide or lanreotide) doses according to IGF1 values were included in the analysis; this was possible in 71 cases. In the 29 remaining patients, 22 were cured after surgery and 7 were lost to follow-up. The IGF1 levels reported, evaluating IGF1 reduction and categorization of response to SRL, correspond only to postsurgical follow-up. Patients were categorized according to therapeutic response to SRL as complete response (CR), partial (PR) or non-responders (NR) if IGF1 was normal, between >2<3 SDS or >3 SDS IGF1 at 6 months of follow-up, respectively.

Accepting an alpha risk of 0.05 and a beta risk of 0.2 in a two-sided test, our cohort of subjects is able to recognize as statistically significant a minimum difference of 0.38 units between any pair of groups assuming that three groups exist. The common deviation is assumed to be 0.4.

The study was conducted in accordance with the ethical principles of the Declaration of Helsinki and implemented and reported in accordance with the International Conference on Harmonised Tripartite Guideline for Good Clinical Practice. The study was approved by the Germans Trias i Pujol Hospital Ethical Committee for Clinical Research (Ref.: EO-11-080, http://www.ceicgermanstrias.cat/index.html). The protocol and informed consent forms were approved by the institutional review board of the participating centers, independent ethics committee and/or research ethics board of each study site. All patients provided written informed consent to participate in the trial.

## RNA isolation and reverse transcription

Total RNA was isolated from pituitary adenomas using AllPrep DNA/RNA/miRNA Universal Kit (Qiagen). The quantity and purity of extracted RNA was quantified by measuring optical density at 260 and 280 nm using NanoDrop™ 1000 Spectrophotometer (RRID:SCR_016517, Thermo Fisher Scientific).

Integrity of the RNA was checked by agarose gel electrophoresis. To remove contaminating genomic DNA, samples were treated with RNAse-free DNAse twice, during the RNA extraction procedure following the manufacturer's protocol and before the retrotranscription using ezDNase Enzyme (Invitrogen).

Five hundred nanograms of total RNA were reverse transcribed using SuperScript IV reverse transcriptase (Invitrogen) and random hexamers in a final volume of 20 µL according to the manufacturer's protocol.

## Quantitative polymerase chain reaction

Gene expression was quantified using Taqman assays (Applied Biosystems). The genes analyzed were somastostatin receptor 2 (*SSTR2*, Hs00990356_m1), somastostatin receptor 5 (*SSTR5*, Hs00990408_s1), short dopamine receptor D2 (*DRD2*) isoform (Hs01014210_m1), long *DRD2* isoform (Hs01024460_m1), arrestin beta 1 (*ARRB1*, Hs00930516_m1), pleiomorphic adenoma gene-like 1 (*PLAGL1*, Hs00414677_m1), Raf kinase inhibitory protein (*RKIP*/*PEBP1*, Hs01110783_g1), E-cadherin (*CDH1*,

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Hs01023894_m1), Ki-67 (*MKI67*, Hs01032443_m1), ghrelin and obestatin prepropeptide (*GHRL*, Hs01074053_m1), aryl hydrocarbon receptor interacting protein (*AIP*, Hs00610222_m1) and a custom assay was ordered for intron 1 ghrelin In1-GHRL (AJ89KWC). We tested six reference genes to normalize gene expression: hypoxanthine phosphoribosyltransferase 1 (*HPRT1*, Hs99999909_m1), proteasome 26S subunit ATPase 4 (*PSMC4*, Hs00197826_m1), glucuronidase beta (*GUSB*, Hs00939627_m1), TATA-box binding protein (*TBP*, Hs00427621_m1), mitochondrial ribosomal protein L19 (*MRPL19*, Hs01040217_m1) and phosphoglycerate kinase 1 (*PGK1*, Hs00943178_g1) and selected the last three reference genes based on their stability in our samples according to Chainy software (available on: http://maplab.imppc.org/chainy/) (Mallona *et al.* 2017).

Quantitative polymerase chain reactions (qPCR) were carried out in a 7900HT Fast Real-Time PCR System (Applied Biosystems). We used TaqMan Gene Expression Master Mix (Applied Biosystems), and the amplification reactions were performed in triplicate for each sample in a final volume of 10 µL in 384-well plates. To minimize the inter-assay variation, all the genes, including the reference genes, for each sample were analyzed in the same plate. The relative quantification to reference genes was calculated according to geNorm (RRID:SCR_006763, https://genorm.cmgg.be/) algorithms (Vandesompele *et al.* 2002).

### *GNAS* sequencing

The mutations in guanine nucleotide binding protein (G protein), alpha stimulating activity polypeptide 1 (*GNAS*, also known as *GSP* oncogene) were screened by Sanger sequencing (GATC Biotech, Cologne, Germany). Samples were analyzed for mutations at codons 201 and 227 in exons 8 and 9, respectively, using cDNA and the primers 5′-CAAGCAGGCTGACTATGTGCCGA-3′ (forward) and 5′-CCACCACGAAGATGATGGCAGTC-3′ (reverse).

### Biochemical and hormonal assays

After an overnight fast, blood samples were collected from patients at baseline and at different follow-up times. Serum IGF1 was measured by two different methods and normalized for comparisons by expressing SDS values. Method 1, a two-site IRMA (Immunotech IGF1 kit; Immunotech-Beckman, Marseille, France). Expected values depending on age were: 20–30 years, 220–550 ng/mL; 30–40 years, 140–380 ng/mL; 40–50 years, 54–330 ng/mL; and 50–60 years, 94–285 ng/mL. Intra-assay CV was less than 6.3%; inter-assay CV, 6.8%; and sensitivity, 30 ng/mL. Method 2 was a non-extraction IRMA (Diagnostic Systems Laboratories, Webster, TX, USA). The theoretical sensitivity, or minimum detection limit, calculated by interpolation of the mean plus two S.D. values of 20 replicates of the 0 ng/mL IGF1 standard was 2 ng/mL. The inter-assay CV was 7.4 and 4.2, respectively, for the concentrations 32.5 and 383.8 ng/ml. The inter-assay CV was 7 and 3.9, respectively, for the mean concentration values 34.03 and 373.86 ng/mL.

To better categorize the disease status and the response to SRL and to avoid the variability over time of IGF1 measurement, results are expressed as SDS according to sex and age and percentage of decrease over basal value. Therefore, IGF1 greater than 3 SDS was considered not responsive to SRL treatment, between 2 and 3 SDS was considered a partial response to SRL and less than 2 SDS was considered a complete response to SRL treatment (Puig-Domingo *et al.* 2010).

### Immunohistochemistry

Those markers that performed better while analysed by gene expression were subsequently evaluated at protein level by immunohistochemistry. Thus, forty-six somatotropinoma tissue samples were available for immunostaining of E-cadherin, *SSTR2a*, *Ki-67* and cytokeratin CAM 5.2 as well, as CAM 5.2 has previously demonstrated to identify accurately densely granulated and sparsely granulated somatotropinomas with good identification power of responsiveness and non-responsiveness to SRL, respectively (Al-Brahim & Asa 2006, Bakhtiar *et al.* 2010). Formalin-fixed paraffin-embedded tumour samples were cut into sequential 4-µm-thick sections and stained using a fully automated Ventana BenchMark ULTRA stainer (Ventana, Tucson, AZ, USA) according to the manufacturer's instructions. Binding of peroxidase-coupled antibodies was detected using diaminobenzidine as a substrate, and the sections were counterstained with hematoxylin.

The mouse monoclonal anti-cytokeratin antibody and the mouse monoclonal anti-E-cadherin antibody (Ventana) were purchased as prediluted antibodies, with a concentration of 11 µg/dL and 0.314 µg/dL, respectively. The rabbit monoclonal anti-SSTR2a antibody (clone UMB-1, Abcam) was used at a dilution of 1:100. To analyze Ki-67, we used the rabbit monoclonal anti–Ki67 antibody 30-9 (ready-to-use formulation; Ventana). Normal appendix tissue served as the positive control for CAM 5.2 staining and mammary invasive ductal carcinoma for E-cadherin staining.

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Immunostaining for E-cadherin was scored in three intensities (0: negative, 1+: weak positivity, 2+: strong positivity) and for each intensity, the percentage of cells was determined. For the classification of the intensities, we considered 0 (negative) when there were no positivity; 1+ (weak positivity) when the adenoma cells seemed negative at low magnification (×40) but were truly positive at high magnification (×200) and we considered 2+ when the adenoma cells were clearly positive at low magnification (×40). We calculated an IHC score multiplying the percentage of cells of each intensity by the score intensity (0–200). Loss of E-cadherin was considered for IHC scores equal to 0. Partial loss of E-cadherin was considered for IHC scores below 100.

Immunostaining for *SSTR2* was scored using an H-score as performed in Franck et al. (2017). First, membrane and cytoplasmic staining intensity (0: no staining, 1+: weak positivity, 2+: moderate positivity and 3+: strong positivity) was determined for each field and then the percentage of cells at each staining intensity level was calculated. An H-score was assigned using the following formula: $(1 \times (\%\ \text{cells}\ 1+) + 2 \times (\%\ \text{cells}\ 2+) + 3 \times (\%\ \text{cells}\ 3+))$.

Ki-67 score was expressed as the percentage of the number of immunostained nuclei among the total number of nuclei of tumor cells regardless of the immunostaining intensity. The counting was performed in three randomly selected fields of the adenoma tissue section at ×400 magnification.

For the CAM 5.2 staining, the adenomas were classified in two groups: dot-type (when the pattern was exclusively dot-type which identifies accurately sparsely granulated somatotropinomas) and not-only-dot-type (when there were other patterns in addition or not to the dot-type pattern which identifies accurately densely granulated somatotropinomas).

### Statistical analysis

Descriptive results were expressed as mean ± S.D. or median and 25th to 75th percentiles, as appropriate. Unsupervised hierarchical clustering was used to investigate the potential identification of patient's response subgroups, based on their molecular expression profile. Spearman or Pearson bivariate correlations were performed for all quantitative variables and differences between groups were compared using comparison of mean tests (Student's *t*-test, Wilcoxon signed-rank test and Kruskal–Wallis and ANOVA as appropriate). A multinomial logistic regression model was used to determine the differences in each normalized gene expression between complete response and resistant patients. The model was adjusted by age, gender and SRL pre-surgical treatment. Receiver operating characteristic (ROC) curve analyses were performed to assess the classification power of each logistic regression model. Samples from all groups within an experiment were processed at the same time. The *P* values were two-sided, and statistical significance was considered when $P<0.05$. All statistical analyses were performed using STATA (StataCorp LLC, College Station, Texas, USA, RRID:SCR_012763) and R version 3.3.2 (R Project for Statistical Computing, RRID:SCR_001905). Unsupervised hierarchical clustering was performed using the R package pheatmap (Pretty Heatmaps, https://CRAN.R-project.org/package=pheatmap). The graphical representation was done using package ggplot 2 (RRID:SCR_014601, Whickham https://CRAN.R-project.org/package=ggplot2) and the *P* values were added using ggpubr package ('ggplot2' Based Publication Ready Plots, https://CRAN.R-project.org/package=ggpubr). Finally, the ROC curves were plotted using pROC package (Display and Analyze ROC Curves, https://CRAN.R-project.org/package=pROC).

## Results

### Clinical variables according to biomarkers expression

In the whole cohort ($n=100$), we analyzed the expression of 12 genes previously reported to be involved in SRL response, including *SSTR2*, *SSTR5*, *AIP*, E-cadherin, Ki-67, *KLK10*, *DRD2*, *ARRB1*, *GHRL*, In1-Ghrelin, *PLAGL1* and *RKIP*. Tumor size was related to *SSTR2* (Pearson's $r=0.25$, $P=0.01$) and showed a negative association with *DRD2* (short *DR2D* isoform Pearson's $r=-0.29$, $P<0.01$, and long *DRD2* isoform Pearson's $r=-0.37$, $P<0.001$) and E-cadherin (Pearson's $r=-0.28$, $P<0.01$). Extrasellar extension was also related to long *DRD2* isoform ($P=0.01$) and Ki-67 ($P=0.04$). Moreover, visual alteration was negatively related to *DRD2* ($P=0.01$ for both isoforms) and E-cadherin ($P=0.02$) (Fig. 1). We also found a negative correlation between IGF1 levels at diagnosis and expression of *ARRB1* (Pearson's $r=-0.31$, $P=0.002$), *KLK10* (Pearson's $r=-0.23$, $P=0.02$) and E-cadherin (Pearson's $r=-0.29$, $P=0.003$). Furthermore, we analyzed the correlation of the expression of each marker with IGF1 index at diagnosis and IGF1 % decrease after SRL treatment, and E-cadherin was the only marker that showed significant correlations with the three IGF1-related measurements (Supplementary Table 1, see section on supplementary materials given at the end of this article), while Ki-67 has

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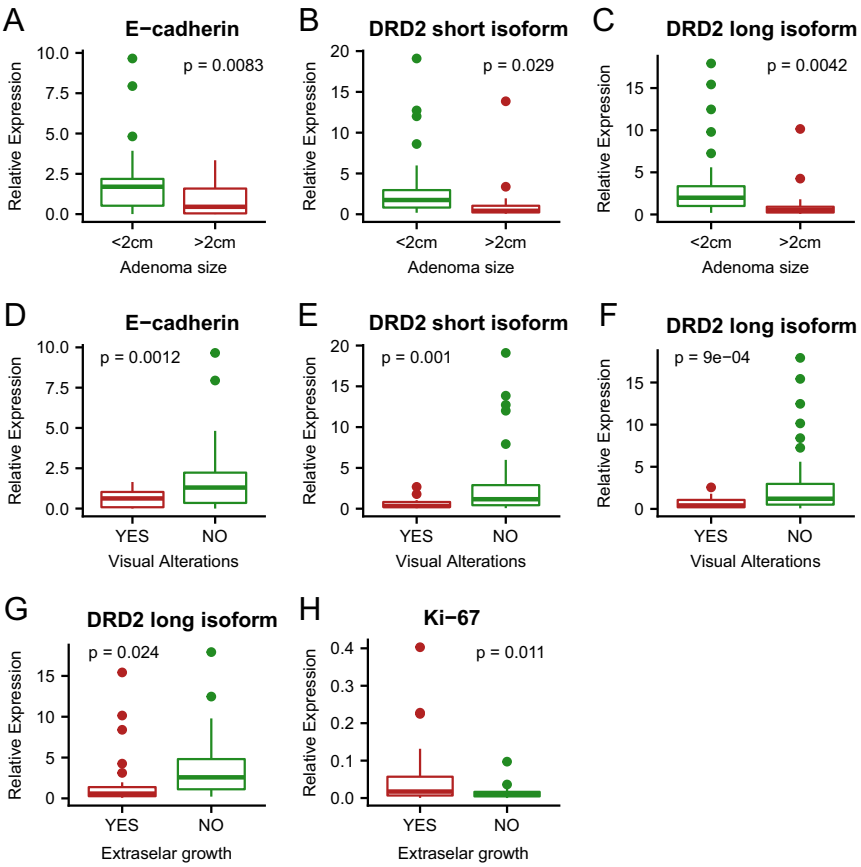


**Figure 1**
Boxplot showing gene expression according to tumor characteristics. Relative expression in tumors smaller and larger than 2 cm (an arbitrary threshold that separates our cohort in two equivalents subsets) (A, B and C), in tumors causing visual alterations before the surgery (D, E and F) and in tumors with or without extrasellar extension (G and H). A full color version of this figure is available at https://doi.org/10.1530/ERC-18-0565.

the strongest correlation with IGF1 % decrease (Pearson's $r=-0.357$, $P=0.002$).

According to SRL biochemical categorized response analyzed in 71 patients, 27 patients (38%) were CR, 18 (25%) PR and 26 (37%) were considered NR. In 20 of these 71 cases, treatment with SRL was only given after surgical procedure, while the rest received SRL therapy before and after surgery. When an unsupervised hierarchical clustering analysis of the expression of the studied genes was performed in all 71 cases, we found that clustering was not related to or influenced by either the overall SRL response or the SRL treatment given before or after surgery (Fig. 2). This indicates that, as a group, acromegaly patients treated with SRL do not present a specific pattern of expression in relation to a given response to SRL and, thus, confirming the heterogeneous nature of somatotropinomas.

### *GNAS* mutation analysis

*GNAS* mutations were studied in a subset of 50 patients and we found mutations in 33%, c.601C>T being the most frequent (Fig. 3). SRL response was not significantly different in those patients presenting *GNAS* mutations; mutated cases were found in 29% of the CR group, 38% of PR and 36% of NR. Neither did we find that clinical variables were related to mutational status regarding comorbidities, tumor size and age among the patients in which the analysis was performed, nor any association with the expression of the different analyzed markers with *GNAS* mutations (Fig. 2).

### Influence of SRL treatment given before or after surgery in the expression of molecular markers

Molecular markers expression was compared between patients who had received SRL treatment before surgery ($n=67$) and those that had not received it ($n=33$). We found that those in which pre-surgical treatment was performed showed higher expression levels of *RKIP* and *SSTR5* ($P=0.006$ and 0.017, respectively) than those not pre-treated (Fig. 4). Interestingly, the expression of the *SSTR5* in the pre-treated patients was not different according to the SRL response ($0.46\pm0.61$, $1.41\pm2.39$ and $0.51\pm0.39$, *SSTR5* expression for CR, PR and NR, respectively, $P=0.087$), suggesting that the mechanism regulating *SSTR5* expression upon SRL treatment is different from that reducing GH secretion. By contrast, *SSTR2* expression was not affected by pre-surgical

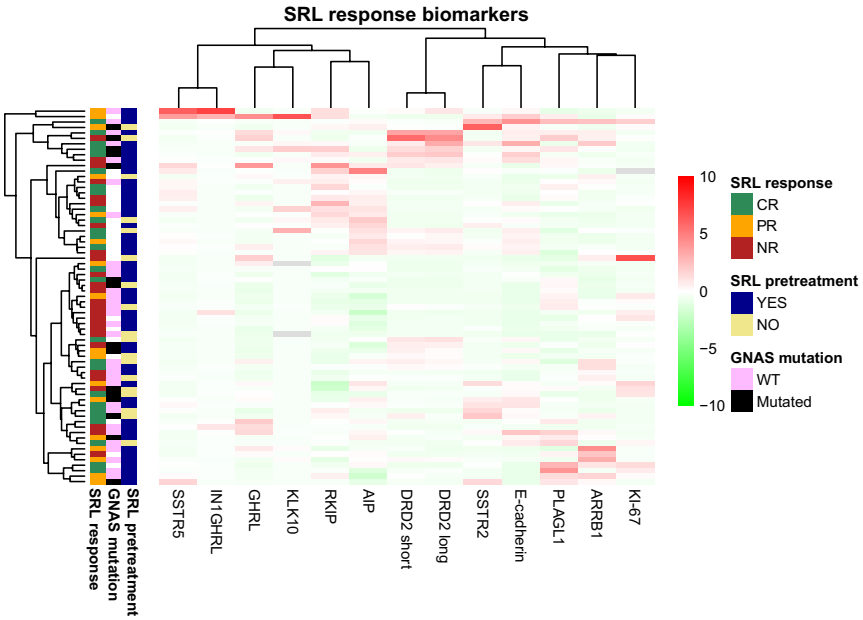


**Figure 2**
Dendrogram and unsupervised hierarchical clustering heat map of the expression of analyzed SRL response biomarkers using Ward's minimum variance method and Minkowski distance. For every patient, *GNAS* mutation, SRL treatment before surgery and SRL response category are shown if available ($n = 71$).

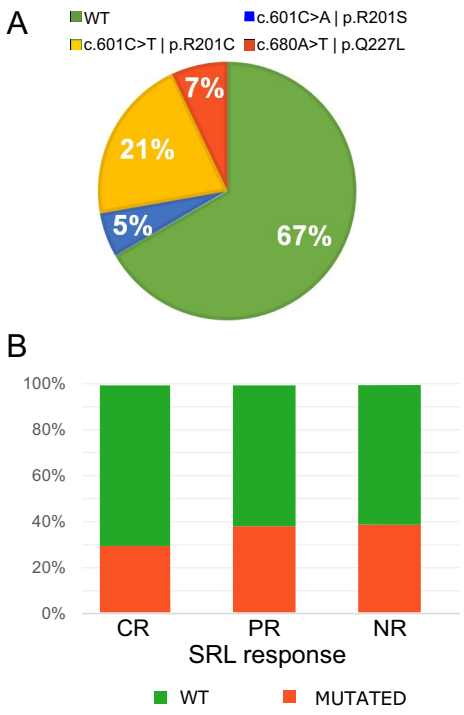


**Figure 3**
Results from mutational analysis of *GNAS* gene ($n = 50$). (A) Percentage of the different mutations found in our cohort. (B) Proportion of patients carrying *GNAS* mutations grouped according to therapeutic response to SRL in complete responders (CR), partial responders (PR) and non-responders (NR). A full color version of this figure is available at https://doi.org/10.1530/ERC-18-0565.

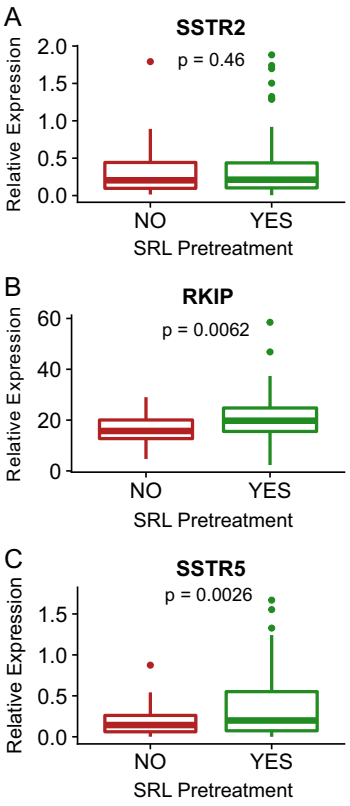


**Figure 4**
Relative expression of *SSTR2* (A), *RKIP* (B) and *SSTR5* (C) in tumors receiving SRL or not receiving SRL before surgery ($n = 100$). A full color version of this figure is available at https://doi.org/10.1530/ERC-18-0565.

treatment ($P$=0.46) at mRNA level. We validated this result by *SSTR2a* immunohistochemistry (IHC) ($P$=0.28).

### Predictive response to SRL according to molecular markers expression

Neither *SSTR5* nor *SSTR5/SSTR2* ratio, *ARRB1*, *PLAGL1*, *GHRL*, In-1-Ghrelin and *RKIP* showed any statistically different expression among the three therapeutic response categories when the 71 cases were analyzed as a whole. *AIP* showed a trend toward significance when extreme phenotypes were compared (CR vs NR) with a $P$=0.054 (Supplementary Table 1).

However, E-cadherin, *SSTR2* and Ki-67 expression were associated with response to SRL ($P$=0.006, $P$=0.068 – near significance – and $P$=0.03, respectively) (Fig. 5). Higher expression of E-cadherin and *SSTR2* was observed in CR group when compared to NR ($P$<0.003 and $P$<0.03, respectively). The opposite pattern was observed for Ki-67, as NR showed higher levels ($P$<0.001). Interestingly, E-cadherin and Ki-67 showed expression differences in a stepwise manner. E-cadherin was the marker that presented more differences between the three different categories of therapeutic response, showing a tendency between PR and NR ($P$<0.1). E-cadherin presented 2.41-fold change between CR and NR and 1.52 when PR were compared to NR.

In addition, categorical analyses for each normalized gene expression in quintiles were performed to evaluate any nonlinearity in estimated effects. Interestingly, *SSTR2* did not show any further risk increase over the second quintile. Similarly, E-cadherin expression levels did not increase the risk above the third quintile. This finding indicates the nonlinearity of gene expression for these two variables, suggesting that SRL response is related to a specific expression level conferring a permissive effect regarding therapeutic response closer to a categorical behavior of these biomarkers rather than a dose-response effect.

When multinomial logistic regression was constructed for extreme phenotypes (NR and CR), *SSTR2* showed an AUC-ROC curve of 0.68, for a cut-off of 0.3, with a sensitivity of 61.5%, specificity of 69.2%, positive predictive value of 66.0% and negative predictive value of 62.6%; the OR for sensitivity toward response to SRL treatment was 3.729 (IC 97.5: 1.242–21.619; $P$=0.06, non-significant). In contrast, ROC curve for E-cadherin showed an AUC of 0.74 and a sensitivity of 65.4%, specificity of 88%, positive predictive value of 83.7% and a negative predictive value of 72.6%. The effect sensitivity to SRL expressed as OR was 1.9319 (IC 97.25: 1.207–3.52; $P$<0.02). When Ki-67 was analyzed by the multinomial logistic model, no significant results were obtained ($P$=0.14). When the ROC curve was constructed combining both the expression of *SSTR2* and E-cadherin together, no additional predictive power was obtained from the one observed for E-cadherin alone ($P$=0.824) (Fig. 6).

In addition, gene expression correlations were explored to assess their possible relationships (Fig. 7). Interestingly, E-cadherin and *SSTR2* had a moderate-strong positive correlation of $r$=0.539 ($P$<0.00001). Other correlations – either positive or negative – were also found between different biomarkers, indicating a multiplicity of functional relationship between them.

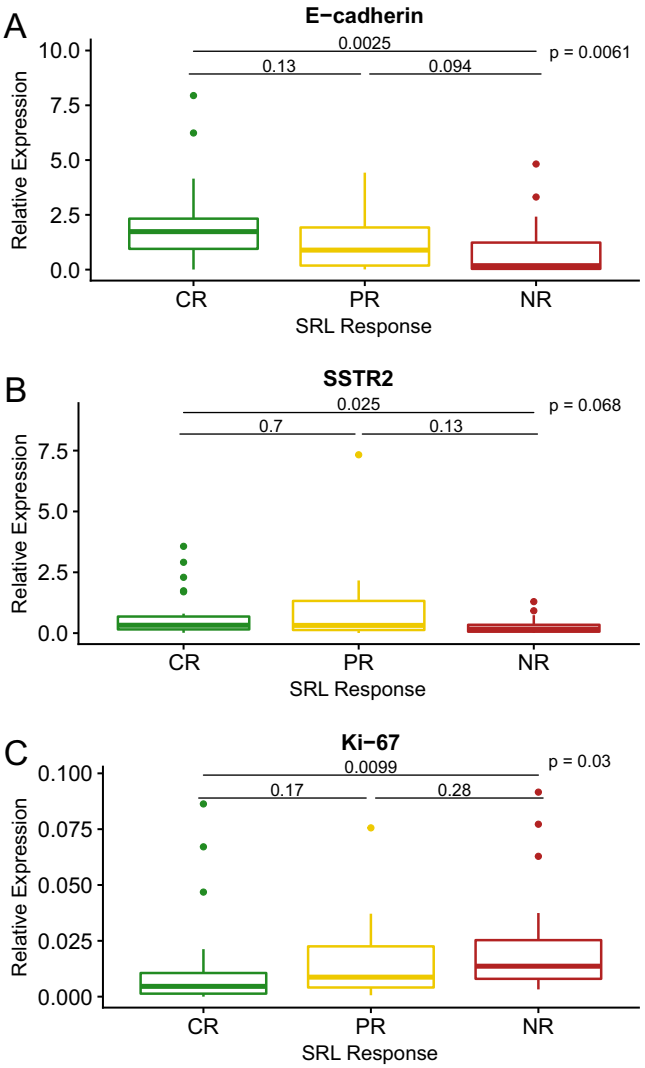


**Figure 5**
Relative expression of SSTR2 (A), Ki-67 (B) and E-cadherin (C) in complete responders (CR), partial responders (PR) and non-responders (NR) ($n$ = 71). A full color version of this figure is available at https://doi.org/10.1530/ERC-18-0565.

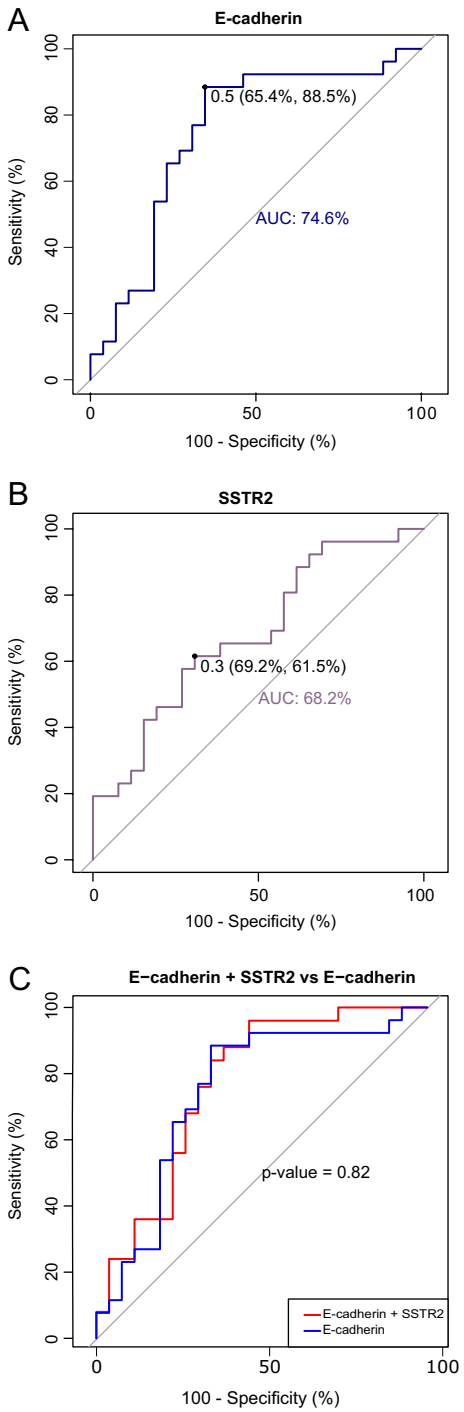


**Figure 6**
ROC curves calculated with extreme phenotypes, complete responders (CR) ($n = 27$) and non-responders (NR) ($n = 26$) to SRL for E-cadherin (A) and *SSTR2* (B). Comparison of the ROC curve obtained with E-cadherin expression alone or in combination with *SSTR2* expression (C). A full color version of this figure is available at https://doi.org/10.1530/ERC-18-0565.

## Validation of E-cadherin expression by immunohistochemistry

We analyzed the protein amount of E-cadherin, SSTR2a and Ki-67 in 47 samples by IHC. E-cadherin H-score correlated with E-cadherin mRNA expression (Pearson's $r=0.4$, $P<0.003$), and likewise E-cadherin H-score showed significant differences in SRL response stratification between CR and NR ($P=0.019$) (Fig. 8A). Interestingly, E-cadherin loss by IHC defined as non-staining was found in PR and NR but not in CR (Fig. 8B). This behavior did not occur with partial loss which was found in both CR and NR. When multinomial logistic regression was constructed for extreme phenotypes (NR and CR), E-cadherin H-score showed an AUC-ROC curve of 0.79, for a cut-off of 30, with a sensitivity of 53.8%, specificity of 100%, positive predictive value of 100% and negative predictive value of 81.3% (Fig. 8C). These findings suggest that a completely negative IHC for E-cadherin may discard a complete biochemical control of IGF1 levels using only first-generation SRL.

*SSTR2* H-score also showed a correlation with *SSTR2* mRNA (Pearson's $r=0.46$, $P<0.01$). However, the highest *SSTR2* H-scores were found in the PR instead of the CR patients (Supplementary Fig. 1A). Furthermore, when multinomial logistic regression was constructed for CR vs NR and PR vs NR comparisons, *SSTR2* showed an AUC-ROC curve of 0.62 (sensitivity of 50% and specificity of 77.8%) and an AUC-ROC curve of 0.70 (sensitivity of 60.2% and specificity of 76.2%), respectively, but neither of them were significant ($P=0.41$ and $P=0.19$, respectively).

Ki-67 IHC did not show any significant difference between the groups (Supplementary Fig. 1B). Moreover, the correlation between mRNA and protein was not significant (Pearson's $r=0.21$, $P=0.144$). We think that the superior performance of the qPCR in comparison to IHC could be explained by the low levels of Ki-67 on these adenomas that make the levels difficult to quantify.

## Influence of histological pattern on SRL response and E-cadherin expression

Finally, we analyzed the cytokeratin CAM 5.2 by IHC, as SRL response has been linked to histological subtypes (Kiseljak-Vassiliades *et al.* 2015*b*) and, particularly, CAM 5.2 immunostaining (Bakhtiar *et al.* 2010). Only 15 (32%) out of 47 samples presented a dot-type pattern. However, we observed that CR patients did not present dot-type tumors. Moreover, dot-type immunostaining for CAM 5.2 was negatively related to E-cadherin expression (Fig. 9). Altogether, these results suggest a link between

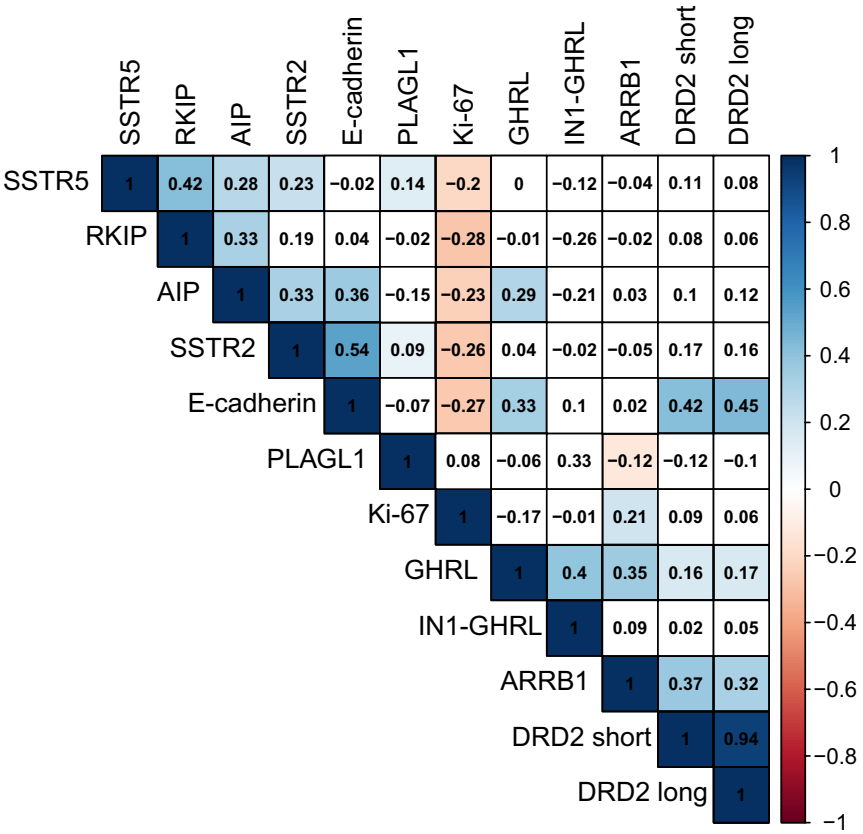


**Figure 7**
Spearman's correlation matrix among the genes studied ($n = 100$). Genes are ordered according to hierarchical clustering using complete linkage method. Spearman's correlation coefficients are shown in the matrix; the intensity of color reflects the correlation magnitude.

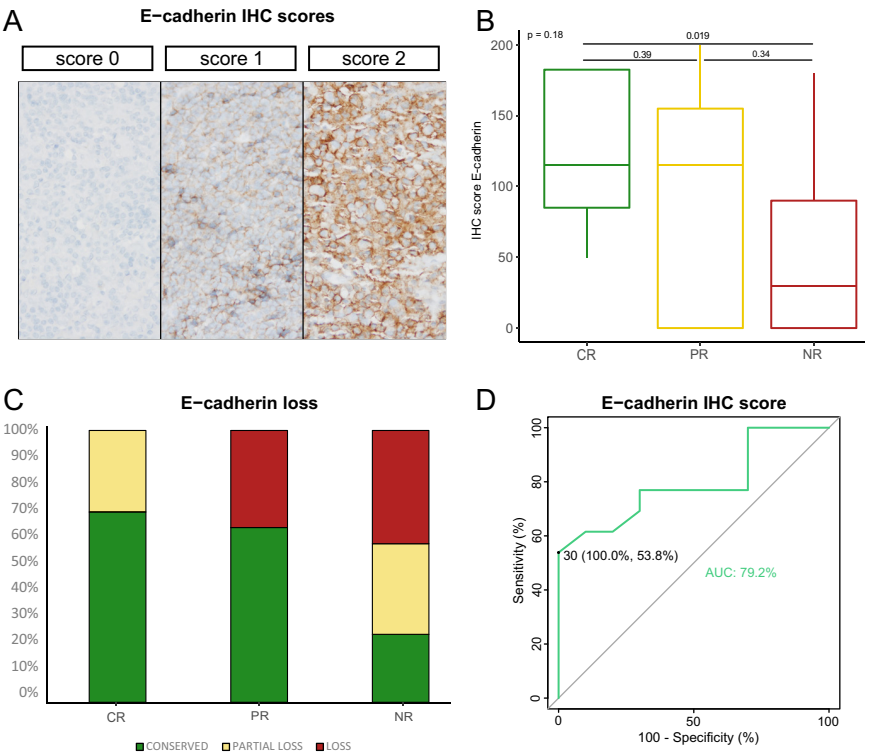


**Figure 8**
Representative images of E-cadherin immunohistochemical scores in somatotropinomas (200×) (A). E-cadherin IHC score in complete responders (CR), partial responders (PR) and non-responders (NR) ($n = 47$) (B). E-cadherin IHC categorized in loss, partial loss and conserved in CR, PR and NR (C). ROC curve calculated with extreme phenotypes, complete responders (CR) ($n = 13$) and non-responders (NR) ($n = 14$), to SRL for E-cadherin IHC (D).

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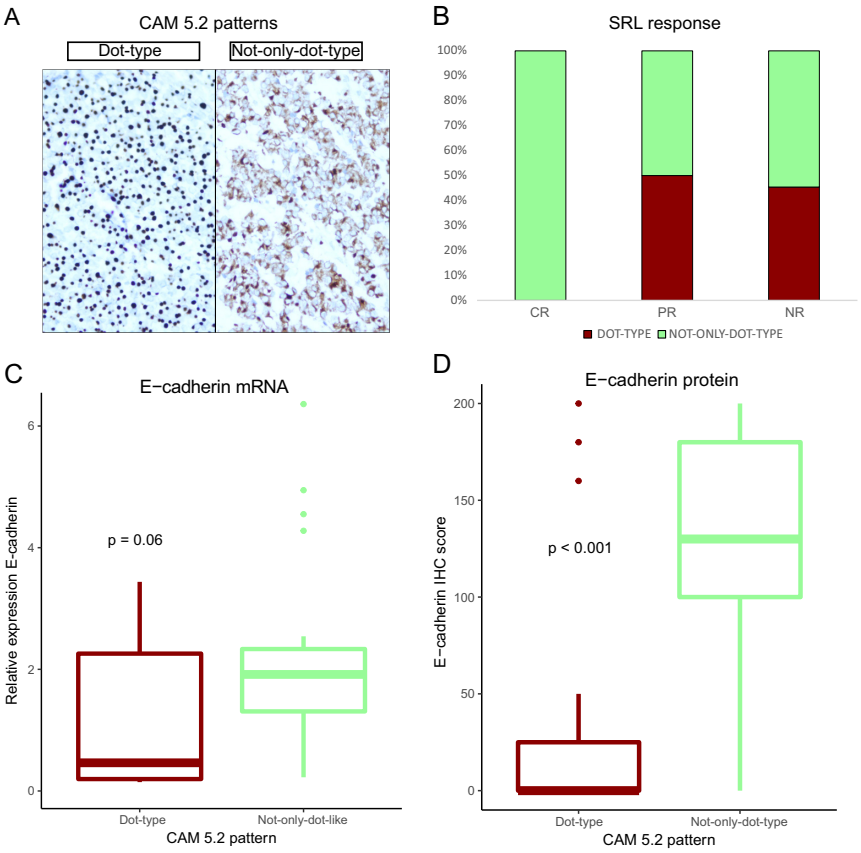


**Figure 9**
Representative images of cytokeratin CAM 5.2 immunohistochemical patterns in somatotropinomas (200×) (A). Proportion of tumors with dot-type pattern and not-only-dot-type pattern according to therapeutic response to SRL (B). Relative expression of E-cadherin (C) and E-cadherin IHC score (D) in dot-type CAM 5.2 pattern and not-only-dot-type.

the histological pattern, E-cadherin expression and SRL response in somatotropinomas.

## Discussion

Acromegaly, a rare disease caused mostly by somatotropic tumors located in the pituitary gland, requires a prompt diagnosis in order to amend what often happens currently, where patients are only diagnosed after suspicion is aroused by the visibility of long-running phenotypic anatomical changes. If this was not already an important unresolved challenge, a second and equally necessary one is the need for an effective treatment for each patient to be delivered in a timely way. Different approaches have been taken to resolve this second issue, but they have not yet been introduced into clinical guidelines (Puig Domingo 2015, Kasuki *et al.* 2018). As somatotropinomas are heterogeneous tumors from a pathologic point of view, it is not unexpected that their response to first-line pharmacologic treatment, namely first-generation SRL, will not be homogeneous either. If certain biomarkers, such as T2 weighted signal from the MRI, seem to identify reasonably different response groups of patients (Puig-Domingo *et al.* 2010, Kiseljak-Vassiliades *et al.* 2015*a*), the information obtained from evaluation of somatotropinoma tissue samples after surgical treatment has raised high expectations, as nowadays the SRL signaling pathways are relatively well known. Therefore, different studies have been performed and published reporting that different receptors or molecules involved in SRL signaling may identify specific responses to these compounds (Gadelha *et al.* 2013). As far as we know, at least a dozen different biomarkers have been tested individually and have apparently shown interesting information potentially useful for developing a pharmacological treatment in those patients not cured by surgery. These molecules have never been considered altogether in the same patient and in a large series of patients in order to establish their true clinical usefulness for their possible incorporation into clinical guidelines as biomarkers of prediction of pharmacologic response.

In our study, we aimed to evaluate the mRNA expression of a combined panel composed of almost all SRL response biomarkers published in the last decade, for verification of previous results and definition of their predictive power, either individually or in specific combinations; those showing the best predictive

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performance were validated at protein level. We found that among all the biomarkers studied, E-cadherin, *SSTR2* and Ki-67 showed potential usefulness for incorporation into clinical practice. E-cadherin expression was the best predictor between the SRL response categories. None of the other evaluated biomarkers showed statistical differences among the different response categories, although some of them showed a trend toward statistical significance, in particular *AIP* ($P=0.06$). The *SSTR2*/*SSTR5* ratio was not different among response categories and nor was *PLAGL1* (*ZAC1*), a molecule which participates in the downstream pathway of *SSTR2*, in close relation to *AIP* (Chahal *et al.* 2012). Discrepancies with previous reports could be partially explained because of the methodology used and the population studied. We analyzed the gene expression of the panels of markers, while other studies measured the markers by IHC as in the case of *AIP* (Chahal *et al.* 2012) and *KLK10* (Rotondo *et al.* 2015) or by Western blot as in the case of *RKIP* (Fougner *et al.* 2008). In the case of *SSTR2*, the concordance between RNA levels and IHC staining has been previously confirmed (Wildemberg *et al.* 2012), as we also found. However, in contrast to previous published results, we did not find a strong correlation between *SSTR2* IHC and SRL response (Casar-Borota *et al.* 2013, Gatto *et al.* 2013, Wildemberg *et al.* 2013). This discrepancy could be explained by different reasons: (1) previous reports used a more homogeneous cohort (our patients were treated in different hospitals, some of them with octreotide while others with lanreotide); (2) response categories were different between studies and (3) scoring IHC is a subjective quantification of the protein that can vary between pathologists. The same reasons could also explain why other studies did not find a correlation between *SSTR2* IHC and SRL response (Gonzalez *et al.* 2014). Regarding the discordance of *SSTR2/SSTR5* ratio in our study with the work by Taboada (Taboada *et al.* 2007), it could be due to the fact that we used probe-base qPCR (Taqman technology) to measure gene expression, while the latter designed the primers and used intercalating dye-based qPCR which is less specific.

Of particular interest is the fact that the ROC curve analyses of E-cadherin and *SSTR2* or their combination showed similar results, although E-cadherin presented better predictive power (either positive (84%) or negative (73%) for gene expression and even better for protein expression, positive predictive value of 100% and negative predictive value of 81.3%), and moreover, the combination of E-cadherin and *SSTR2* was not superior than the one showed by E-cadherin alone. This indicates that if one single marker is to be chosen for incorporation into a decision-making therapeutic algorithm, E-cadherin might be the first one to be included to clinical guidelines.

Our study clearly exemplifies the biological heterogeneity of somatotropinomas, which by extension is also reflected in the response to SRL, the first-line pharmacological treatment of acromegaly recognized nowadays by clinical guidelines. Until now, all the studies investigating the biological behavior of somatotropinomas have built up a lot of information with high value for understanding somatotropinoma biology. Unfortunately, the expression of all the biomarkers identified so far is so wide and the variability among groups of responders and non-responders so high that it leads to an important degree of overlapping among SRL response categories, which does not allow the definition of specific cut-off values that could be currently applied to clinical practice. In this regard, E-cadherin expression is able to partially resist this overlapping effect among groups, although in some particular patients it may also fail because the overall predictive power – either positive or negative – is around 75% when gene expression is considered. The predictive value of E-cadherin levels was validated at protein level, which is of paramount importance from a clinical point of view, as IHC is easily implementable in the clinical routine.

Why is E-cadherin a better predictive biomarker than the rest when it would be more expected to find better results for the somatostatin receptor family? This issue requires further studies, although some remarkable information has already been generated by some studies, mostly coming from Bollerslev's group. (Fougner *et al.* 2010, Lekva *et al.* 2012, 2013). E-cadherin is, among others, a biomarker of epithelial-mesenchymal transition (EMT), a biological process that seems also to be operative for somatotropinomas, at least in part, and may have implications for SRL response. As a matter of fact, the more advanced the EMT, the less responsive the tumor may be to SRL. This may explain in some way the biological heterogeneity shown in our cohort in which no specific expression pattern of the different markers evaluated present a strong concordance. The progressive loss of response to SRL seems to involve a concerted loss of E-cadherin and *SSTR2* expression together with a gain in Ki-67, and thus the tumor losses its classic GH-secreting phenotype with a higher sparsely granulated pathologic pattern, according to cytokeratin CAM 5.2 staining (Fougner *et al.* 2012). Our results also validated that dot-type CAM 5.2 immunostaining correlates with poor response to SRL and E-cadherin loss in somatotropinomas (Bakhtiar *et al.* 2010).

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Another interesting finding of our study is that *SSTR5* expression was higher in those cases in which pre-surgical treatment with SRL was performed when compared to non-pre-treated patients. These patients were not different in terms of size of the tumor or other clinical variables, thus it is intriguing to understand this finding and it may be even postulated if SRL may have induced changes in the expression of *SSTR5*, a question that has previously been invocated for *SSTR2* (Franck *et al.* 2017, Liu *et al.* 2017). We did not find changes for *SSTR2* in our series in cases in which pre-surgical treatment with SRL was performed. The in-deep explanation of our finding requires additional *in vitro* and *in vivo* experiments for its confirmation, but if it would be so, it would open new potential therapeutic options, as the combined and sequential treatment with first-generation SRL followed by pasireotide may be a new possibility which has never been previously tested.

Our study has some weaknesses, as it has a retrospective design in which not all the patients had all the complete information for full analyses and that not all the markers described have been included in the molecular evaluation, such as the measurement of SST truncated receptors (Luque *et al.* 2015). On the other hand, the strength of our study is that a relatively large cohort with no special selection bias from real clinical practice was included.

Thus, in summary, we conclude that E-cadherin expression is different between responder, partial responder and resistant patients to first-generation SRL and that it may be considered as a potential biomarker for predictive response to SRL, to be included in therapeutic algorithm in acromegaly patients after surgical failure in order to perform a more personalized and predictive treatment of acromegaly.

**Supplementary materials**

This is linked to the online version of the paper at https://doi.org/10.1530/ERC-18-0565.

**Declaration of interest**

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