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Clinical Research Article

Integrative Clinical, Radiological, and Molecular Analysis for Predicting Remission and Recurrence of Cushing Disease

Paloma Moreno-Moreno,[1,2,3] Alejandro Ibáñez-Costa,[1,2,4,5] Eva Venegas-Moreno,[6] Antonio C. Fuentes-Fayos,[1,2,4,5] María R. Alhambra-Expósito,[1,2,3] Carmen Fajardo-Montañana,[7] Araceli García-Martínez,[1,2,4] Elena Dios,[6] Mari C. Vázquez-Borrego,[1,2,4,5] Pablo Remón-Ruiz,[6] Rosa Cámara,[8] Cristina Lamas,[9] José Carlos Padillo-Cuenca,[1,2,3] Juan Solivera,[10] David A. Cano,[6] Manuel D. Gahete,[1,2,4,5] Aura D. Herrera-Martínez,[1,2,3] Antonio Picó,[11] Alfonso Soto-Moreno,[6] María Ángeles Gálvez-Moreno,[1,2,3] Justo P. Castaño,[1,2,4,5] and Raúl M. Luque[1,2,4,5]

[1]Maimonides Biomedical Research Institute of Cordoba (IMIBIC), 14004 Cordoba, Spain
[2]Reina Sofia University Hospital (HURS), 14004 Cordoba, Spain
[3]Service of Endocrinology and Nutrition, IMIBIC, HURS, 14004 Cordoba, Spain
[4]Department of Cell Biology, Physiology and Immunology, University of Cordoba, 14004 Cordoba, Spain
[5]CIBER Physiopathology of Obesity and Nutrition (CIBERobn), 14004 Cordoba, Spain
[6]Unidad de Gestión de Endocrinología y Nutrición. Instituto de Biomedicina de Sevilla (IBiS), Hospital Universitario Virgen del Rocío/CSIC/Universidad de Sevilla, 41013 Sevilla, Spain
[7]Department of Endocrinology, Hospital Universitario de La Ribera, Alzira, 46600, Valencia, Spain
[8]Department of Endocrinology and Nutrition, Polytechnic University Hospital La Fe, 46026, Valencia, Spain
[9]Department of Endocrinology and Nutrition, Albacete University Hospital, 02006, Albacete, Spain
[10]Service of Neurosurgery, HURS, 14004 Cordoba, Spain
[11]Department of Endocrinology and Nutrition, Alicante General University Hospital. Institute for Health and Biomedical Research (ISABIAL). University Miguel Hernandez, CIBER Rare Diseases, 03010, Alicante, Spain

Correspondence: R. M. Luque, PhD, IMIBIC Building, Av. Menéndez Pidal s/n. 14004-Córdoba, Spain. Email: raul.luque@uco.es; or J. P. Castaño, PhD, IMIBIC Building, Av. Menéndez Pidal s/n. 14004-Córdoba, Spain. Email: justo@uco.es; or A. D. Herrera-Martínez, MD, PhD, IMIBIC Buliding, Av. Menéndez Pidal s/n. 14004-Córdoba, Spain. Email: aura.herrera.sspa@juntadeandalucia.es.

Abstract

Context: Adrenocorticotropin (ACTH)-secreting pituitary tumors (ACTHomas) are associated with severe comorbidities and increased mortality. Current treatments mainly focus on remission and prevention of persistent disease and recurrence. However, there are still no useful biomarkers to accurately predict the clinical outcome after surgery, long-term remission, or disease relapse.

Objectives: This work aimed to identify clinical, biochemical, and molecular markers for predicting long-term clinical outcome and remission in ACTHomas.

Methods: A retrospective multicenter study was performed with 60 ACTHomas patients diagnosed between 2004 and 2018 with at least 2 years' follow-up. Clinical/biochemical variables were evaluated yearly. Molecular expression profile of the somatostatin/ghrelin/dopamine regulatory systems components and of key pituitary factors and proliferation markers were evaluated in tumor samples after the first surgery.

Results: Clinical variables including tumor size, time until diagnosis/first surgery, serum prolactin, and postsurgery cortisol levels were associated with tumor remission and relapsed disease. The molecular markers analyzed were distinctly expressed in ACTHomas, with some components (ie, *SSTR1*, *CRHR1*, and *MKI67*) showing instructive associations with recurrence and/or remission. Notably, an integrative model including selected clinical variables (tumor size/postsurgery serum cortisol), and molecular markers (*SSTR1/CRHR1*) can accurately predict the clinical evolution and remission of patients with ACTHomas, generating a receiver operating characteristic curve with an area under the curve of 1 ($P < .001$).

Conclusion: This study demonstrates that the combination of a set of clinical and molecular biomarkers in ACTHomas is able to accurately predict the clinical evolution and remission of patients. Consequently, the postsurgery molecular profile represents a valuable tool for clinical evaluation and follow-up of patients with ACTHomas.

Key Words: ACTH-secreting pituitary tumors, Cushing disease, biochemical variables, molecular markers, predictive biomarkers, remission, relapsed disease

Abbreviations: ACTH, adrenocorticotropin; ACTHoma, adrenocorticotropin-secreting pituitary tumor; AUC, area under the curve; CD, Cushing disease; DST, dexamethasone suppression test; LNSC, late-night salivary cortisol; mRNA, messenger RNA; OR, odds ratio; qPCR, quantitative real-time polymerase chain reaction; ROC, receiver operating characteristic; TSH, thyrotropin; UFC, 24-hour urinary free cortisol.

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Cushing disease (CD) is the most frequent cause of endogenous hypercortisolism. It originates with an adrenocorticotropin (ACTH)-secreting pituitary tumor (ACTHoma) (1). The incidence of CD, which ranges from 0.7 to 2.4 cases per million inhabitants/year, is more frequent between the third and fourth decade of life and is 8 times more common in women (1). In this sense, a relevant clinical problem of patients with CD is their considerable morbidity and increased mortality. Uncontrolled hypercortisolemia is associated with metabolic, cardiovascular, cognitive, and psychological alterations, and in consequence with increased mortality (2). Normalization of cortisol levels leads to substantial improvement in these parameters and decreased mortality (3, 4). However, growing evidence indicates that the risk of developing certain morbidities, in particular cardiovascular diseases, persists for several years after remission (4, 5). In this context, control of hypercortisolism and management of comorbidities in a multidisciplinary setting, together with long-term follow-up, are essential for optimal recovery in CD (2, 6).

In general, duration of hypercortisolism in CD appears to be inversely related to the reversibility of different complications, which, in turn, can increase mortality. This indicates that once the diagnosis of CD has been established, the production of cortisol should be normalized as soon as possible (7). Transsphenoidal surgery remains the first-line therapy, but persistence and recurrence of CD is substantial after surgery. Indeed, about 1% of patients with CD do not achieve remission after transsphenoidal pituitary surgery, and many patients recur annually after achieving surgical remission, leading to a cumulative recurrence rate that ranges from 8% to 66% during long-term follow-up (8-10).

Several epidemiologic variables, biochemical values, and functional tests have been evaluated for predicting remission and clinical evolution of these patients with contradicting results (7, 9). In addition, although it has been suggested that some genetic mutations might be associated with recurrence rate (ie, patients with a *USP8* mutation presented with a higher recurrence rate) (11), to the best of our knowledge, no molecular markers have been identified to date for predicting long-term clinical outcomes and remission in ACTHomas after transsphenoidal surgery. In this scenario, it has been previously shown that some components of the somatostatin, ghrelin, and dopamine regulatory systems could play relevant functional roles in ACTHomas, which can entail clinical implications (12-16). In fact, in other pituitary tumors, the expression levels of some elements of these regulatory systems have been associated with tumor size, behavior prediction, and response to treatment with first-generation somatostatin analogues or dopamine agonists (14, 17-20).

Based on the aforementioned information, we aimed to analyze the clinical behavior of a well-characterized cohort of patients with ACTHomas (N = 60) with a minimum follow-up of 2 years, and evaluated individually and collectively several clinical, biochemical, and molecular markers (including the expression of somatostatin, ghrelin and dopamine system components) for predicting long-term clinical outcomes and remission in these patients.

Materials and Methods

Patients

Clinical and molecular variables of 60 patients harboring ACTHomas from different hospitals in Spain were analyzed in the present study (mean age at diagnosis, 40 years [range, 15-78 years]; 88% women). All techniques carried out in this study were conducted in accordance with the ethical standards of the Declaration of Helsinki, the World Medical Association, and with the approval of the ethics committees from all hospitals involved in the study. Informed consent from each patient was obtained. Specifically, patients with ACTHomas who underwent surgery from 2004 to 2018 and with a minimum follow-up of 2 years were included (follow-up time 24-152 months after surgery). Demographic, clinical, radiological, and biochemical characteristics of the patients were collected to analyze potential associations with molecular data (discussed later) and are summarized in Tables 1 and 2. Clinical response and long-term follow-up in patients with ACTHomas were estimated by remission after surgery and the presence of recurrence, respectively. Remission was defined as normalization of 24-hour urinary free cortisol (UFC), late-night salivary cortisol (LNSC), and overnight 1-mg dexamethasone suppression test (DST) in patients who did not present central adrenal failure after surgery (cutoffs for this test are < 5 µg/dL, and are usually performed immediately after surgery or within 1 month). Recurrence after successful pituitary surgery was defined as the reappearance of clinical and biochemical features of hypercortisolism after initial remission (upper levels UFC [1.6 × upper level of normal]) and/or upper levels LNSC (cutoff = 0.27 µg/dL or 7.5 mmol/L) and/or lack of inhibition of plasma cortisol after overnight 1-mg DST (cutoff > 1.8 µg/dL) (21, 22). Cortisol levels were analyzed after 1 mg of dexamethasone 1 month after surgery in all patients who did not present with hypocortisolism after surgery.

Patients were treated according to the available clinical guidelines (23-25). Surgery was performed in all patients. Standardized protocols were followed in the different institutions participating in this study to obtain tumor samples immediately after the surgery, keeping them in phosphate-buffered saline for immediate examination and processing by an experienced pathologist. Specifically, the pathologist preserved a fragment for anatomopathological analyses and another fragment was rapidly transferred and stored in RNA-later reagent (a solution used for RNA stabilization and storage that protects the integrity of RNA in unfrozen tissue samples). Samples were immediately shipped to the laboratory in Cordoba wherein RNA extraction was immediately performed (≤ 24 hours), and the integrity of the RNA analyzed using a Bioanalyzer. RNA was stored frozen at – 80 °C until the reverse transcription and quantitative real-time polymerase chain reaction (qPCR) analyses, as previously reported (26, 27). To ensure the pituitary tumor tissues were not contaminated by normal pituitary tissues, the phenotype of the pituitary samples collected was always confirmed by 3 separate methods, as previously described (27): histological examination by an expert anatomopathologist; through a molecular screening by qPCR (including expression of all pituitary hormone types); and, whenever possible, by testing the hormonal phenotype using single-cell secretion. Therefore, in this study we have confirmed that all the samples analyzed were ACTHoma tissues.

RNA Isolation, Reverse Transcription, and Quantitative Real-time Polymerase Chain Reaction

RNA extraction followed by reverse transcription and qPCR were performed for each sample, as previously described

Table 1. Baseline demographic, clinical, radiological, and biochemical characteristics of patients with adrenocorticotropin-secreting pituitary tumors (n = 60)

Demographics, Sex (M/F)	7 (11.7%)/53 (88.3%)	Comorbidities	
Age at diagnosis, y	40 ± 19 (15-78)	Arterial hypertension	30 (50%)
BMI	31.22 ± 12.75 (20.58-50.77)	Dyslipidemia	13 (21.7%)
Cyclic Cushing disease	4 (6.7%)	Type 2 diabetes	17 (28.3%)
Signs and symptoms		Osteoporosis	12 (44.4%)
Central obesity	42 (85.7%)	Atypical fracture	5 (8.5%)
Facial plethora	39 (88.6%)	Infections	2 (3.4%)
Cutaneous atrophy	20 (74.1%)	Cardiovascular disease	4 (6.7%)
Full-moon face	39 (88.6%)	Autoimmune disease	8 (13.8%)
Cervical lipomatosis	33 (78.6%)	Gastric ulcer	3 (5.1%)
Supraclavicular fat	29 (78.4%)	Time from symptoms onset to diagnosis, mo	24 ± 35 (1-120)
Hirsutism	27 (79.4%)	**Radiological variables**	
Muscular atrophy	26 (78.8%)	Tumor size, mm	8 ± 10 (3-35)
Amenorrhea	11 (50%)	Macroadenoma/Microadenoma	26 (43.3%)/34 (56.7%)
Red abdominal stretch marks	27 (60%)	**Biochemical variables**	
Capillary fragility	16 (59.3%)	Baseline serum cortisol after 1 mg dexamethasone, µg/dL	12.64 ± 14.34 (0.34-41)
Acne	12 (70.6%)	Presurgery baseline cortisol, µg/dL	23 ± 8.2 (7.9-71.90)
Lower-limb edema	7 (36.8%)	Presurgery midnight serum cortisol, µg/dL	12.60 ± 9.52 (3.55-74)
Headache	7 (12.7%)	Presurgery LNSC, µg/dL	0.66 ± 1.36 (0.02-30)
Asthenia	28 (87.5%)	Presurgery 24-h urinary cortisol, µg/24 h	316 ± 380.88 (21.90-3729.15)
Visual defects	3 (5.5%)	Presurgery serum ACTH, pg/mL	74.48 ± 64.80 (12-281)
Emotional lability	19 (82.6%)		
Depression	11 (25%)		
Psychosis	2 (3.3%)		

Abbreviations: ACTH, adrenocorticotropin; BMI, body mass index; F, female; M, male.

(28). Specifically, expression levels (absolute mRNA copy number/50 ng of sample) of different key regulatory components of the hypothalamic-pituitary-corticotropic axis, including receptors (somatostatin [*SSTR1*, *SSTR2*, *SSTR3*, *SSTR5*, and truncated splicing variants, SST_5TMD5 and SST_5TMD4], dopamine [*DRD1*, *DRD2* total and *DRD2* long isoform, *DRD4*, and *DRD5*], ghrelin [*GHSR1a* and truncated *GHSR1b* variant], corticotropin-releasing hormone [*CRHR1*] and arginine vasopressin receptor 1b [*AVPR1b*]), hormones/enzymes (pro-opiomelanocortin [*POMC*; precursor of ACTH], ghrelin, In1-ghrelin variant, and ghrelin O-acyl transferase enzyme [*GOAT*]), and proliferation markers (pituitary tumor transforming gene [*PTTG1*] and *MKI67*) were measured using previously validated primers (28). We evaluated the stability of the expression of 3 reference genes (beta actin, hypoxanthine-guanine phosphoribosyltransferase and glyceraldehyde-3-phosphate dehydrogenase [*ACTB*, *HPRT1*, and *GAPDH*, respectively]) in all samples using RefFinder, a comprehensive tool that integrates the currently available major computational programs (29), and found *HPRT1* to be the most stable. Taking this into account, the expression values of the genes of interest were normalized to *HPRT1* levels.

Determination of Biochemical Variables

Measurement of cortisol, ACTH, prolactin, and thyrotropin (TSH) levels was performed in the laboratory services of the different hospitals involved using different assays and following the manufacturers' instructions, as previously reported (14, 27, 30-34). It should be noted that the biochemical magnitudes measured and included in this manuscript (cortisol, ACTH, prolactin, and TSH) lack bias attributable to interassay variability (intralaboratory/interlaboratory) inside/between the different hospitals since all the assays were performed under the same quality control program (ie, an internal quality control program and an external quality control program according to the indications of the International Federation of Clinical Chemistry and the American Association for Clinical Chemistry).

Statistical Analysis

Mann-Whitney *U* tests were used to evaluate clinical-molecular associations within ACTHoma samples. The chi-square test was used to compare categorical data. Kruskal-Wallis and analyses of variance were used for multiple comparisons. Binomial univariate and multivariable logistic regression analyses were performed to assess the risk factors for long-term outcome of CD patients. Receiver operating characteristic (ROC) curves were performed for evaluation of the discriminant accuracy of binomial logistic regression models between cured and noncured CD patients. Statistical analyses were performed using SPSS v20, and GraphPad Prism v7. Graphs and tables include data expressed as median ± interquartile range. Moreover, minimum to maximum range is indicated in the tables to outline the distribution of each parameter.

Table 2. Baseline clinical characteristics of patients with adrenocorticotropin-secreting pituitary tumors. Comparison between groups based on remission after first surgery and recurrence of disease

Clinical characteristics	Total (N = 60)	Remission after surgery (n = 45)	No remission after surgery (n = 15)	*P*	Recurrence (n = 12)	Nonrecurrence (n = 33)	*P*
Sex (M/F)	7 (11.7%)/53 (88.3%)	2 (4.4%)/43 (95.6%)	5 (33.3%)/10 (66.7%)	**.008**	0/12 (100%)	2 (6.1%)/31 (93.9%)	≥ .999
Age at diagnosis, y	40 ± 19 (15-78)	40 ± 17 (15-78)	44 ± 25 (24-68)	.463	36 ± 21 (15-65)	40 ± 23 (17-78)	.310
BMI	31.22 ± 12.75 (20.58-50.77)	31.05 ± 10.66 (20.58-50.77)	32.64 ± 16.30 (22.67-49)	.671	28.93 ± 9.81 (23.09–39.96)	31.22 ± 11.55 (20.58-50.77)	.445
Cyclic Cushing disease	4 (6.7%)	4 (8.9%)	0	.564	0	21 (91.3 %)	.561
Signs and symptoms							
Central obesity	42 (85.7%)	30 (81.1%)	12 (100 %)	.171	9 (81.8%)	21 (80.8%)	≥ .999
Facial plethora	39 (88.6%)	32 (94.1%)	7 (70 %)	.069	11 (100%)	3 (75%)	≥ .999
Cutaneous atrophy	20 (74.1%)	14 (73.7%)	6 (75%)	≥ .999	8 (88.9%)	6 (60%)	0.303
Full-moon face	39 (88.6%)	31 (91.2%)	8 (80%)	.317	11 (100%)	20 (87%)	0.535
Cervical lipomatosis	33 (78.6%)	27 (84.4 %)	6 (60%)	.181	8 (80%)	16 (86.4%)	.637
Supraclavicular fat	29 (78.4%)	22 (81.5 %)	7 (70%)	.655	8 (88.9%)	14 (77.8%)	.636
Hirsutism	27 (79.4%)	21 (87.5%)	6 (60%)	.157	7 (100%)	14 (82.4%)	.530
Muscular atrophy	26 (78.8%)	19 (86.4%)	7 (63.6%)	.186	6 (100%)	13 (81.3%)	.532
Amenorrhea	11 (50%)	9 (60%)	2 (28.6%)	.361	3 (75%)	6 (54.5%)	.604
Red abdominal stretch marks	27 (60%)	21 (61.8%)	6 (54.5%)	.732	8 (88.9%)	13 (52%)	.107
Capillary fragility	16 (59.3%)	14 (73.7%)	2 (25%)	**.033**	7 (100%)	7 (58.3%)	.106
Acne	12 (70.6%)	9 (81.8%)	3 (50%)	.28	4 (100%)	5 (71.4%)	.491
Lower limb edema	7 (36.8 %)	4 (33.3%)	3 (42.9%)	≥ .999	3 (60%)	1 (14.3%)	.222
Headache	7 (12.7%)	3 (7.3%)	4 (28.6%)	.061	0	3 (10.3%)	.543
Asthenia	28 (87.5%)	21 (87.5%)	7 (87.5%)	≥ .999	5 (83.3%)	16 (88.9%)	≥ .999
Visual defects	3 (5.5%)	1 (2.4%)	2 (14.3%)	.156	0	1 (3.4 %)	≥ .999
Emotional lability	19 (82.6%)	17 (94.4%)	2 (40%)	**.021**	6 (100%)	11 (91.7%)	≥ .999
Depression	11 (25%)	10 (30.3%)	1 (9.1%)	.241	1 (14.4%)	9 (34.6%)	0.397
Psychosis	2 (3.3%)	2 (4.4%)	0	≥ .999	0	2 (6.1%)	≥ .999
Comorbidities							
Arterial hypertension	30 (50%)	19 (42.2%)	11 (73.3%)	.072	4 (33.3%)	15 (45.5%)	.517
Dyslipidemia	13 (21.7%)	8 (17.8%)	5 (3.33%)	.279	3 (25%)	5 (15.2%)	.661
Type 2 diabetes	17 (28.3%)	10 (22.2%)	7 (46.7%)	.064	2 (16.7%)	8 (24.2%)	.226
Osteoporosis	12 (44.4%)	11 (47.8%)	1 (25%)	.605	1 (14.3%)	10 (62.5%)	.069
Atypical fracture	5 (8.5%)	5 (11.4%)	0	.315	1 (9.1%)	4 (12.1%)	≥ .999
Infections	2 (3.4%)	1 (2.2%)	1 (7.1%)	.421	0	1 (3%)	≥ .999
Cardiovascular disease	4 (6.7%)	2 (4.4%)	2 (13.2%)	.258	0	3 (9.1%)	≥ .999
Autoimmune disease	8 (13.8%)	5 (11.4%)	3 (21.4%)	.385	1 (8.3%)	4 (12.5%)	≥ .999
Gastric ulcer	3 (5.1%)	2 (4.4%)	1 (7.1%)	.564	0	2 (6.1%)	≥ .999
Time from symptoms onset to diagnosis, mo	24 ± 35 (1-120)	24 ± 36 (1-120)	18 ± 41 (1-96)	.292	18.50 ± 49 (1-72)	36 ± 35 (6-120)	.463
Time from diagnosis to surgery, mo	162 ± 199 (21-780)	150 ± 219 (31-780)	182 ± 184.25 (21-339)	0.984	150 ± 189.5 (31-352)	178 ± 259 (40-780)	**.012**

Abbreviations: ACTH, adrenocorticotropin; BMI, body mass index; F, female; M, male.
Significant differences are highlighted in bold.

Proportions were expressed as percentages. In all analyses, *P* values less than .05 were considered statistically significant.

Results

Patient Population

Sixty patients with ACTHomas were included in this study and followed-up for a minimum of 2 years, 88.3% being female. Only 4patients presented with cyclic CD. Demographic, clinical, radiological, and biochemical characteristics of the patients are included in Table 1. Most cases presented with central obesity, facial plethora, full-moon face, asthenia, and emotional lability (> 80%). Hirsutism and muscular atrophy were also noteworthy (79.4% and 78.8%, respectively). The most prevalent comorbidity was hypertension (50%). Thirty four out of 60 patients (56.7%) had microadenomas (median size ± SD: macroadenomas [17 ± 8.5 mm], microadenomas [6 ± 1.6 mm]).

Expression Profile of Key Components of Hypothalamic-Pituitary-Corticotrope Axis in Adrenocorticotropin-secreting Pituitary Tumors

The analysis of the expression of somatostatin receptors (including *SSTR5* truncated variants) (35, 36) revealed that *SSTR5* is the dominant receptor subtype expressed in ACTHoma samples, followed by *SSTR1* >> *SSTR2* > *SSTR 3* > SST_5TMD5 > SST_5TMD4 (Fig. 1A). The most expressed receptor of the dopamine system was *DRD2*, followed by *DRD4* >> *DRD5* > *DRD1* (Fig. 1B). In the case of the ghrelin system, the dominant components of this system were the receptors (*GHRS1b* ≥ *GHSR1a*), followed by *GOAT* enzyme ≥ *In1-ghrelin* variant > native *ghrelin* (Fig. 1C). As expected, *POMC*, *AVPR1b*, and *CRHR1* were highly expressed in ACTHoma samples (Fig. 1D). We also found that the expression levels of the proliferation marker *PTTG1* were significantly higher than *MKI67* levels (see Fig. 1D). Specific data about mean, median values, and copy number of all these components are presented in Supplementary Table 1 (37).

Correlation Analysis Between Clinical, Biochemical, and Molecular Variables in Patients With Adrenocorticotropin-secreting Pituitary Tumors

We found that some key clinical variables in CD were correlated with other biochemical and molecular variables in the cohort of patients with ACTHomas (Fig. 2A). Specifically, age was directly correlated with LNSC and serum cortisol after the 8 mg DST, inversely correlated with baseline insulin-like growth factor 1, and with the expression levels of *SSTR5*, SST_5TMD4, *DRD2T*, and *DRD2L* (see Fig. 2A).

Baseline serum cortisol was directly correlated with the expression levels of *POMC* and GHSR1b, whereas it was inversely correlated with the expression of *SSTR1*, *SSTR2*, *SSTR3*, *DRD1*, *DRD2T*, *DRD2L*, and *DRD5*. Similarly, baseline serum ACTH was inversely correlated with the expression of *SSTR1*, *SSTR2*, *SSTR3*, *DRD1*, *DRD2T*, *DRD2L*, *DRD4*, *DRD5*, and *GHSR1a* (see Fig. 2A). Moreover, late-night serum and salivary cortisol were directly correlated the expression of *AVPR1b* (see Fig. 2A).

The expression of *POMC* was inversely correlated with *SSTR1* and *DRD1*, and directly correlated with *SSTR5*, *ghrelin*, *GHSR1a*, *GHSR1b*, *AVPR1b*, *CRHR1*, and *PTTG1* (Fig. 2B). *AVPR1b* expression was also directly correlated with *PTTG1* and *CRHR1* (see Fig. 2B). *CRHR1* expression was directly correlated with *POMC*, *SSTR5*, *DRD4*, *GHSR1a*, *GHSR1b*, and *AVPR1b*, and inversely correlated with *SSTR1* (see Fig. 2B). Moreover, the proliferation markers *PTTG1* and *MKI67* were directly correlated with them and with other molecular markers (see Fig. 2B). Specifically, *PTTG1* was directly correlated with *POMC*, *GHSR1a*, *AVPR1B*, and *CRHR1*, while *MKI67* was directly correlated with *In1-ghrelin* and *GHSR1b* (see Fig. 2B).

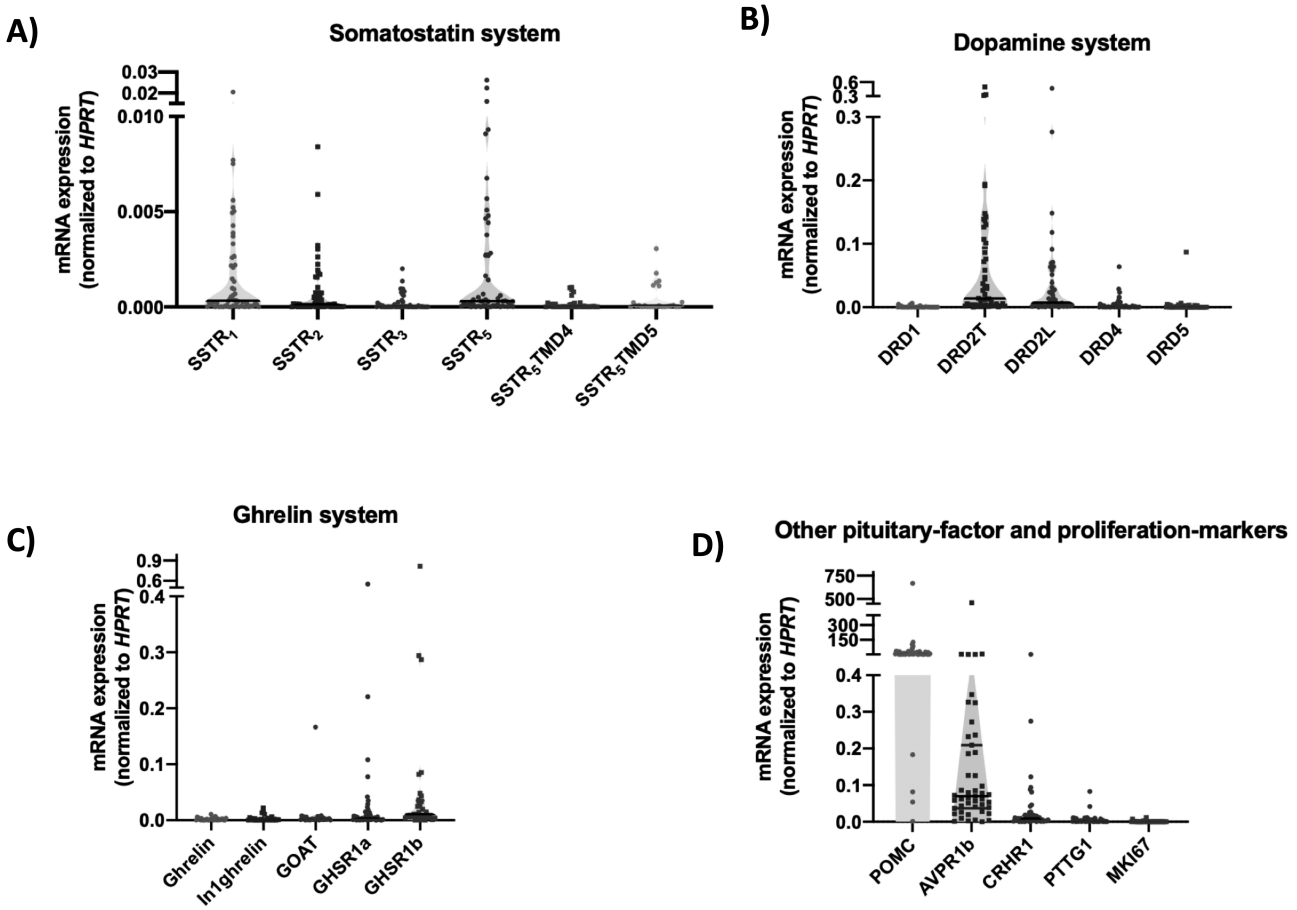

Figure 1. Molecular expression of key genes belonging to the hypothalamic-pituitary-corticotropic axis in adrenocorticotropin (ACTH)-secreting pituitary tumors. Molecular profile (determined by quantitative real-time polymerase chain reaction) of the components of the A, somatostatin; B, dopamine; and C, ghrelin systems, and D, of other key pituitary-factors and proliferation markers. Data represent median (interquartile range) of the absolute expression levels (copy number) of each transcript adjusted by the expression levels of a housekeeping gene (*HPRT1*).

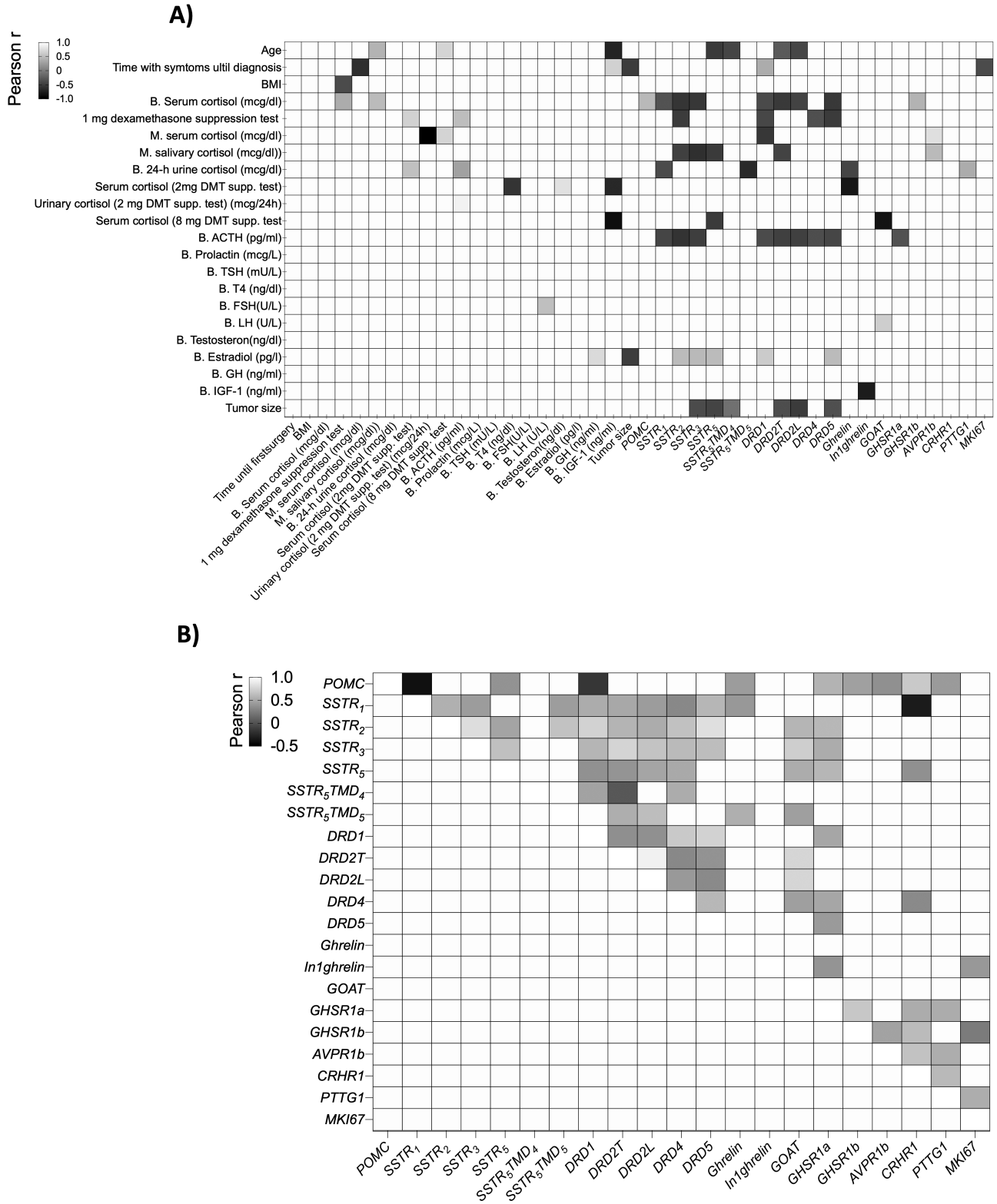

Figure 2. Clinical and molecular correlations in adrenocorticotropin (ACTH)-secreting pituitary tumor patients. A, Correlations between clinical, biochemical, and molecular variables. B, Correlations between the expression levels of all the molecular components measured in ACTH-secreting pituitary tumors. The grayscale corresponds to the Pearson *r* value obtained after statistical analysis. Dark gray means "inverse correlations" and light gray means "direct correlations."

Tumor Size, Hormone Levels, and Time Until Treatment Were Associated With Clinical Response and Long-term Follow-up of Patients With Adrenocorticotropin-secreting Pituitary Tumors

As previously mentioned, clinical response and long-term follow-up in patients with ACTHomas were estimated by remission after surgery and the presence of recurrence, respectively. In this cohort, remission after the first surgery was reached in 45 patients out of 60. Of those 45 patients with remission, the presence of relapsed disease was reported in 12 patients (Table 3).

Patients who reached remission after first surgery presented with lower tumor size, higher baseline serum prolactin, and

lower postsurgery baseline serum cortisol (Fig. 3A). Moreover, cured patients also had lower baseline serum cortisol and 24-hour urinary cortisol levels, as well as 1-mg DST result 1 month after surgery (see Fig. 3A). In addition, cured patients presented with increased incidence of capillary fragility and emotional lability, but the incidence of comorbidities was similar in both patient groups (Tables 2 and 3).

Patients who developed recurrence presented with lower LNSC at diagnosis, lower serum TSH levels, and shorter time from diagnosis to first surgery (Fig. 3B). Baseline signs, symptoms, and comorbidities were similar in patients with or without recurrence (Table 2 and 3).

Finally, patients who were cured at the end of follow-up presented with lower tumor size, longer time with symptoms until diagnosis, longer time until first surgery, higher LNSC, but lower postsurgery cortisol and lower time until recurrence (Fig. 1C).

Expression Levels of Some Components of the Somatostatin System and *MKI67* Were Associated With Remission After First Surgery in Adrenocorticotropin-secreting Pituitary Tumors

Cured patients after the first surgery had lower expression levels of *SSTR1* compared to noncured patients (Fig. 4A). The expression levels of the other somatostatin receptor subtypes, or of the dopamine and ghrelin system components, were not associated with remission in this cohort (Fig. 4A-4C). Interestingly, increased expression of *CRHR1* and *MKI67* were observed in cured patients (Fig. 4D). Finally, we found that the expression level of *SSTR5* was increased in microadenomas compared with macroadenomas (Supplementary Fig. 1) (38).

SSTR1 Expression Levels Are Associated With Disease Recurrence in Adrenocorticotropin-secreting Pituitary Tumors

Expression levels of *SSTR1* were lower in patients who showed recurrence during follow-up (Fig. 5A). The expression levels of the other molecular components analyzed were not associated with recurrence in this study (Fig. 5A-5D).

A Combination of Clinical and Molecular Parameters Accurately Predicts Patient Remission After First Surgery

To further explore the capacity of the clinical and molecular parameters analyzed in the prediction of the outcome of CD patients, binomial logistic regression analyses were implemented. First, no clinical or molecular parameters were significantly associated with the prediction of patient recurrence (Supplementary Tables 2 and 3) (39, 40). In contrast, several molecular and clinical parameters were significantly associated with the remission of the patients (Supplementary Tables 4 and 5) (41, 42). Indeed, tumor size (odds ratio [OR]: 1.124), basal serum prolactin (OR: 0.762), postsurgery serum and urinary cortisol (OR: 1.199 and 1.20, respectively), and postsurgery 1 mg DST (OR: 1.741) were significantly associated with patient remission ($P < .05$ in all cases; see Supplementary Table 4) (41). Similarly, expression of *SSTR1* (OR: 3.052) and *CRHR1* (OR: 0.175) was significantly associated with CD patient remission ($P < .05$ in both cases; see Supplementary Table 5) (42).

Interestingly, adjusted binomial logistic regression analyses revealed that the combination of clinical and/or molecular parameters could finely discriminate patients in remission after the first surgery (Fig. 6). In particular, a clinical model including the 2 clinical variables most significantly associated with remission (ie, tumor size and postsurgery serum cortisol) generated a ROC curve with an AUC of 0.888 (model 1; $P < .001$; see Fig. 6), while the molecular model generated with the expression of *SSTR1* and *CRHR1* resulted in a ROC curve with an AUC of 0.913 (model 2; $P < .001$; see Fig. 6). Remarkably, a clinical-molecular model combining these 2 clinical and 2 molecular parameters generated an impeccable ROC curve with an AUC of 1 (model 3; $P < .001$; see Fig. 6) for the prediction of clinical evolution and remission in patients with ACTHomas.

Discussion

Transsphenoidal pituitary surgery is the cornerstone of treatment for most patients with CD (25). However, a relevant proportion of CD patients do not achieve remission after pituitary surgery, leading to high recurrence rates on long-term follow-up (9, 10). In this context, prediction of postsurgical remission and/or recurrence in CD is a challenging goal, especially because of variable rates of remission and high risk of recurrence (43, 44). Surgical remission rates depend on tumor size and location, neurosurgeon skill and experience, and biochemical criteria used to assess remission (25). Long-term recurrence is variable in the literature, ranging from 8% to 66% (8). Several series with extended follow-up periods (≤ 20 years) report recurrence rates of even 36%, with a mean time to recurrence of 15 to 50 months (45, 46). However, preoperative clinical variables, including age, sex, disease duration, and severity of clinical signs and symptoms, have not aided in consistently identifying patients at higher risk for recurrence (1, 47).

In this study, a well-characterized multicentric cohort of CD patients (N = 60) served to identify clinical, biochemical, radiological, and molecular variables for predicting long-term clinical outcome and remission. Specifically, the expression levels of several key components of the hypothalamic-pituitary-corticotropic axis were comprehensively evaluated and their associations with different key clinical, biochemical, and radiological parameters were explored. Finally, logistic regression models based on clinical and molecular data were constructed to predict long-term outcome of CD patients.

In the present cohort, tumor size was associated with remission after the first surgery and also at the end of the follow-up. This is consistent with a large series of 230 patients with ACTHomas, wherein tumor size (microadenoma) was considered only predictor of positive outcome in these patients (48). In addition, higher tumor size (macroadenomas), invasion into surrounding structures, and aggressive histological factors have been correlated with higher recurrence rates, whereas smaller tumor size and greater neurosurgical experience of the primary surgeon have been associated with lower rates of recurrence (1, 49, 50). However, a recent meta-analysis demonstrated that tumor size and macroscopic invasion were not reliable predictors of recurrence (9).

To the best of our knowledge, the biochemical severity of hypercortisolism has not been consistently studied in relation to postoperative outcomes. In our cohort, serum ACTH levels were not correlated with remission or disease recurrence. However, higher preoperative ACTH levels have been associated with lower biochemical remission rates (8). In addition, higher serum ACTH levels correlate with increased incidence

Table 3. Baseline radiological and biochemical characteristics of patients with adrenocorticotropin-secreting pituitary tumors. Comparison between groups based on remission after first surgery and recurrence of disease

	Total (N = 60)	Remission after sugery (n = 45)	Nonremission after sugery (n = 15)	*P*	Recurrence (n = 12)	Nonrecurrence (n = 33)	*P*
Radiological variables							
Tumor size, mm	8 ± 10 (3-35)	8 ± 37.5 (3-35)	25 ± 17 (5-35)	**.006**	7 ± 6.50 (4-17)	6.5 ± 4.75 (3-35)	.831
Macroadenoma/ Microadenoma	26 (43.3 %)/34 (56.7%)	10 (27.8 %)/26 (72.2%)	9 (81.8 %)/2 (18.2%)	**.002**	3 (30%)/7 (70%)	7 (26.9%)/19 (73.1%)	≥ .999
Biochemical variables							
Baseline serum cortisol after 1 mg dexamethasone, µg/dL	12.64 ± 14.34 (0.34-41)	13.30 ± 14.70 (0.34-41)	11.82 ± 13.10 (4-27.60)	.670	18.50 ± 14.10 (0.34-41)	12.30 ± 14.85 (0.36-41)	.149
Presurgery baseline cortisol, µg/dL	23 ± 8.2 (7.9-71.90)	23.05 ± 7.43 10.40-71.90)	21.30 ± 14.21 (7.90-41)	.561	23 ± 6.89 (10.87-71.90)	23.10 ± 9.53 (10.40-41)	.851
Presurgery midnight serum cortisol, µg/dL	12.60 ± 9.52 (3.55-74)	12.80 ± 9.52 (3.55-23)	12.40 ± 50.70 (8.30-74)	.522	11.10 ± 15.40 (3.55-74)	14.20 ± 11.10 (3.55-23)	.877
Presurgery LNSC, µg/dL	0.66 ± 1.36 (0.02-30)	0.80 ± 1.53 (0.02-30)	0.80 ± 1.53 (0.42-1.75)	.394	0.20 ± 0.11 (0.12-0.27)	0.85 ± 1.74 (0.02-30)	**.046**
Pre-surgery 24-h urinary cortisol, µg/24 h	316 ± 380.88 (21.90-3729.15)	313 ± 380.56 (21.90-3729.15)	277.42 ± 190.41 (32.40-597.60)	.075	452 ± 400.85 (161-1403.85)	317.45 ± 426.40 (21.90-3729.15)	.482
Presurgery serum ACTH, pg/mL	74.48 ± 64.80 (12-281)	78.75 ± 69 (12-281)	47.40 ± 68.80 (22-265.10)	.461	77.24 ± 71.85 (31.80-281)	78.75 ± 67.45 (12-179)	.813

Abbreviation: ACTH, adrenocorticotropin.

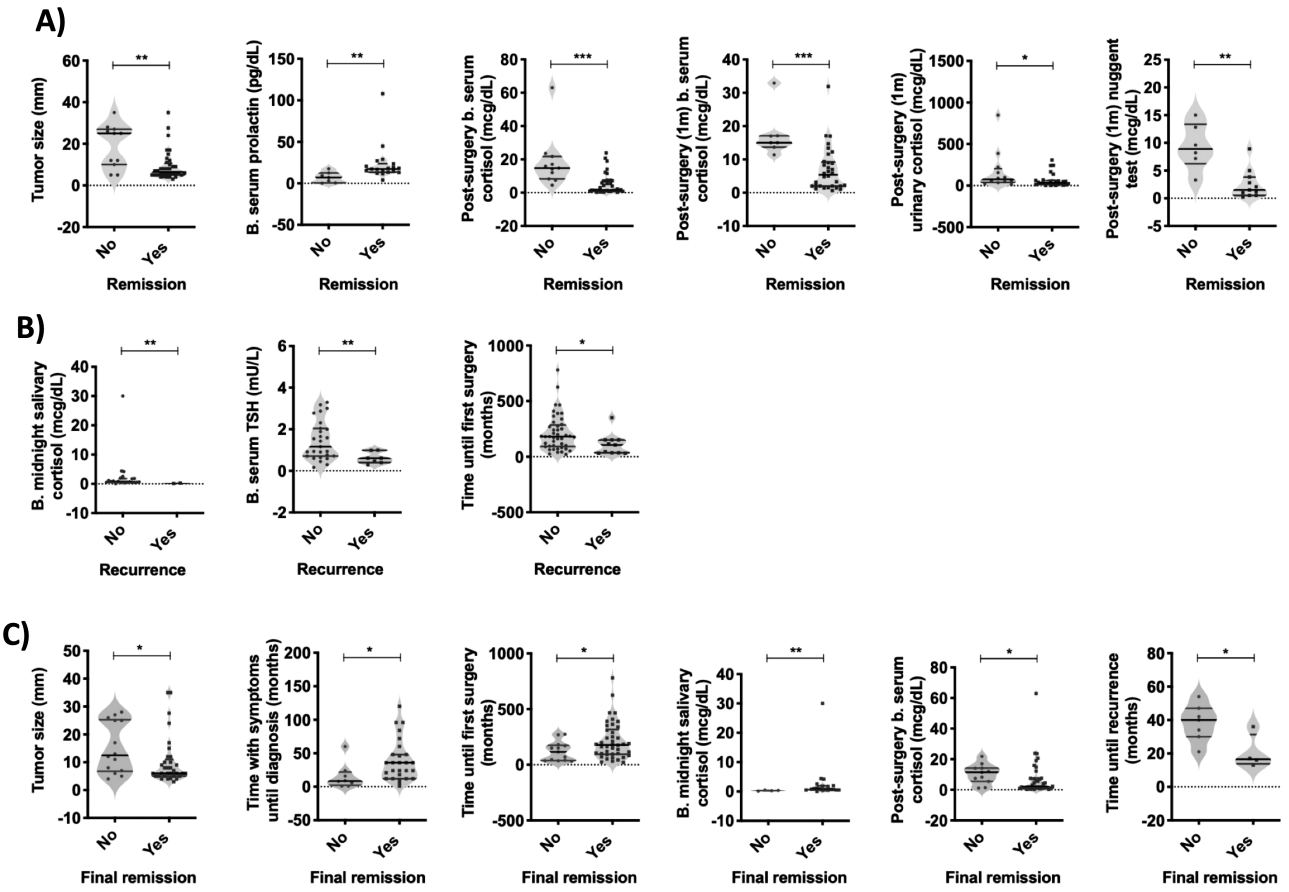

Figure 3. Clinical and biochemical associations for predicting A, remission after first surgery; B, recurrence; and C, remission after final follow-up, in patients with adrenocorticotropin (ACTH)-secreting pituitary tumors. Data represent median (interquartile range) of each parameter analyzed. Asterisks (**P* < .05, ***P* < .01; ****P* < .001) indicate statistically significant differences across groups.

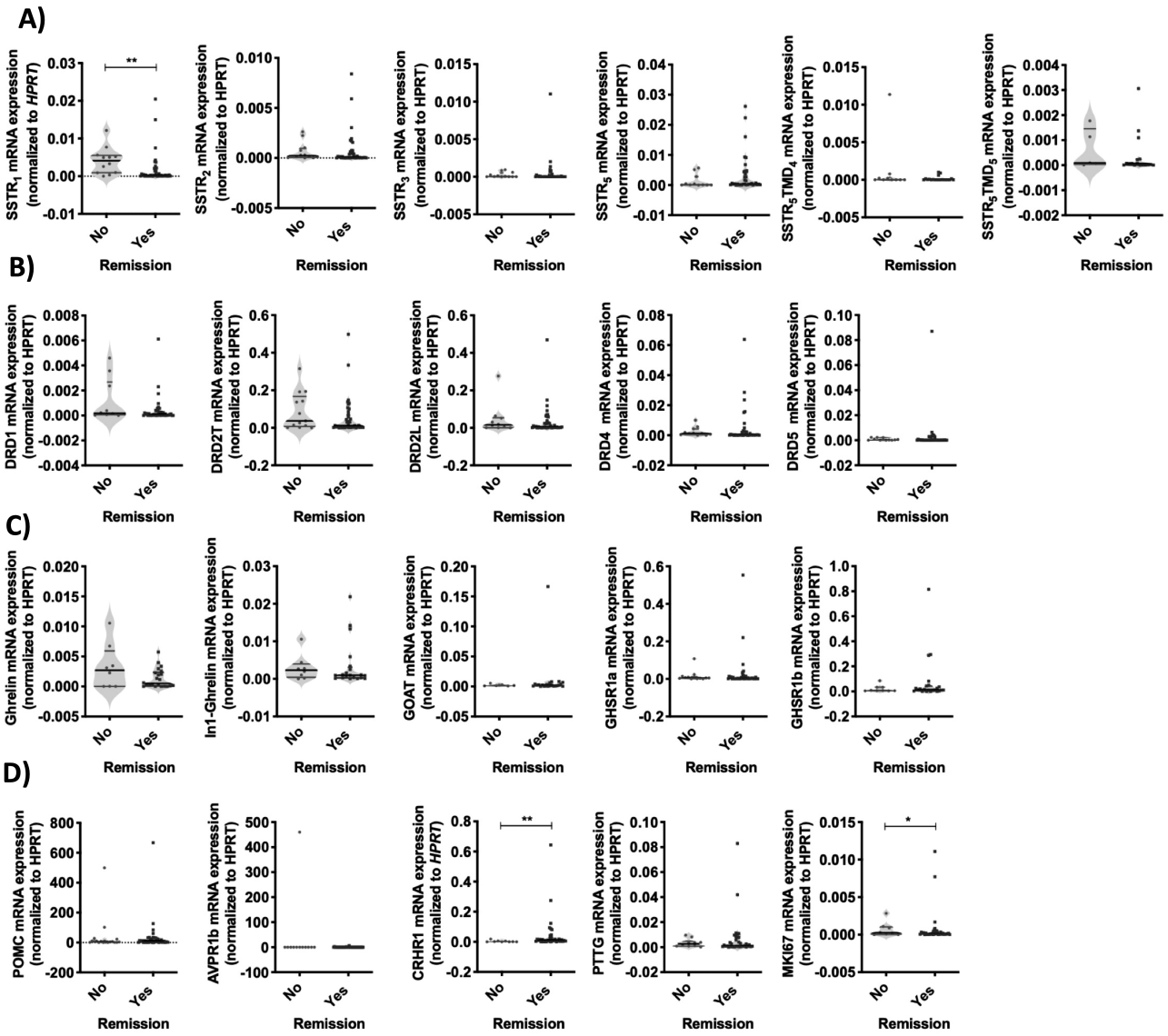

Figure 4. Molecular expression in adrenocorticotropin (ACTH)-secreting pituitary tumors of patients with and without remission. Molecular profile (determined by quantitative real-time polymerase chain reaction) of the components of the A, somatostatin; B, dopamine; and C, ghrelin systems, and D, of other key pituitary-factors and proliferation markers. Data represent median (interquartile range) of the absolute expression levels (copy number) of each transcript adjusted by the expression levels of a housekeeping gene (*HPRT1*). Asterisks (* $P < .05$, ** $P < .01$) indicate statistically significant differences across groups.

of CD recurrence, whereas low serum ACTH levels correlate with sustained disease remission (51, 52). Specifically, several studies consider subnormal serum ACTH values as a less sensitive marker for sustained disease remission, but they are considered as a more specific marker for long-term disease remission than subnormal serum cortisol (45, 52). Nevertheless, a cutoff value of ACTH that may predict long-term recurrence has not been determined and the data presented herein, together with previous literature, challenge the utility of biochemical severity of hypercortisolism as a predictor of postoperative outcomes.

In contrast, as observed in our cohort, a longer duration of postoperative hypocortisolism has been described as an important predictor of sustained remission (46, 53). Specifically, several studies suggest that the duration of postoperative corticoadrenal insufficiency is inversely correlated with the risk of recurrence (8). Specifically, lower recurrence rates have been observed in patients with subnormal postoperative cortisol levels compared with patients with normal or supranormal levels (54). Based on these studies, a consensus statement recommends immediate reevaluation of patients with persistent serum cortisol levels greater than 5 µg/dL, and careful observation of those patients with cortisol levels between 2 and 5 µg/dL (55). Nevertheless, it is important to emphasize that disease recurrence does still occur in approximately 10% of patients with low (< 2 µg/dL) or even undetectable postoperative serum cortisol levels (56). There is no clear cutoff value that excludes the risk of recurrence, and some patients with elevated postoperative serum cortisol also achieve long-term remission (1, 45). Current recommendations indicate that UFC excretion measurements should be used only when serum cortisol levels are equivocal. In this case, UFC levels lower than 20 µg/24 h are suggestive of surgical remission,

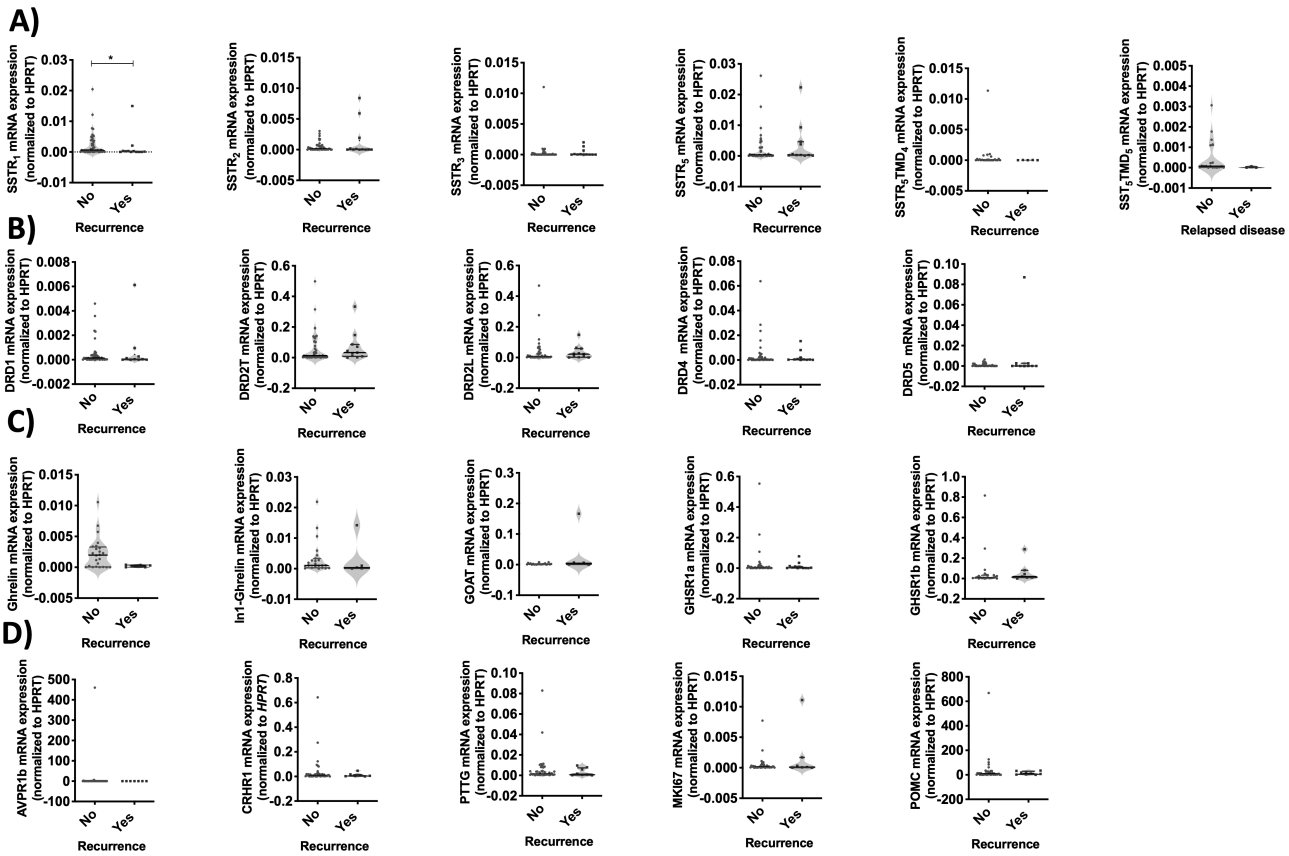

Figure 5. Molecular expression in adrenocorticotropin (ACTH)-secreting pituitary tumors of patients with and without recurrence. Molecular profile (determined by quantitative real-time polymerase chain reaction) of the components of the A, somatostatin; B, dopamine; and C, ghrelin systems, and D, of other key pituitary factors and proliferation markers. Data represent median (interquartile range) of the absolute expression levels (copy number) of each transcript adjusted by the expression levels of a housekeeping gene (*HPRT1*). Asterisk (* $P < .05$) indicate statistically significant differences across groups.

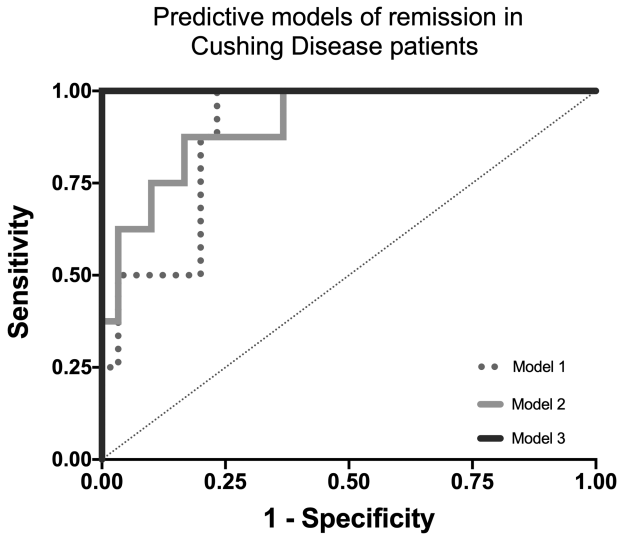

Figure 6. Receiver operating characteristic (ROC) curve analysis of different clinical-molecular models to predict remission in patients with adrenocorticotropin (ACTH)-secreting pituitary tumors. Clinical models include the 2 clinical variables most significantly associated with remission (model 1: tumor size and postsurgery serum cortisol; model 2: the molecular expression of *SSTR1* and *CRHR1*; or model 3: the combination of the previous clinical variables and molecular markers).

normal UFC levels are equivocal, and elevated values suggest remaining tumor (8, 45). The LNSC has not been sufficiently validated as a useful predictor of long-term recurrence (1). In our cohort, in contrast, only postoperative morning serum cortisol was associated with remission after first surgery, as well as at the end of follow-up (1, 44), reinforcing the prognostic potential of this marker.

Among clinical parameters, tumor size and postsurgery serum cortisol exhibited the most statistically significant associations with remission of CD patients after the first surgery (AUC = 0.888; $P < .001$). Although the data presented herein were not able to predict recurrence, machine-learning models have been used for predicting CD recurrence after 1 year of surgery (57). In this study, postoperative morning serum cortisol nadir, postoperative, and preoperative morning ACTH levels were significantly associated with recurrence (57). Furthermore, after using a random forest algorithm, age, postoperative serum cortisol, and postoperative ACTH have been considered as the best predictive variables for recurrence of CD during the first year after surgery (57). In another recent study that also used machine learning, it was suggested that sex, age, tumor detection at magnetic resonance imaging, size smaller than 10 mm, Hardy-Wilson grade, and histological confirmation of ACTH secretion, preoperative/postoperative hypopituitarism, and presurgical medical treatment might have a role in predicting long-term remission (58). Therefore, the data presented herein, together with previous evidence, suggest it may be possible to define mathematical models based on clinical parameters (especially tumor size and postsurgical serum cortisol) capable of predicting long-term outcome of CD patients, although more studies will be necessary to confirm this hypothesis.

In terms of the molecular expression of key genes belonging to the hypothalamic-pituitary-corticotropic axis in ACTHomas, it has been previously described that *SSTR5* is one of the predominant somatostatin receptors expressed in these tumors, whereas expression levels of *SSTR2* are lower because of cortisol modulation (59-61) A similar expression pattern was observed in our cohort and represents a basis for molecular-directed therapies in CD (61). Interestingly, our data revealed that *SSTR1* is notably expressed in ACTHomas, its expression being even higher than that of *SSTR2*. Remarkably, we observed that decreased *SSTR1* expression levels were associated with remission after the first surgery. This finding might be clinically relevant in that *SSTR1* expression has been previously described in other hormone-related tumors and associated with cell proliferation and aggressiveness (62-65). These data invite the suggestion that pharmacologic treatments specifically targeting *SSTR1* might be a promising option to treat patients with ACTHomas, providing a relevant clinical conclusion, which should be tested for their use in humans.

Regarding the dopamine system, *DRD2* was the most expressed dopamine receptor in our patients, in line with previously data (66). In this context, some authors have highlighted that the expression of *DRD2* is correlated with the clinical response to dopamine agonists of some pituitary tumor types (67). However, our study showed no association between the expression of DRDs and clinical outcome, which is consistent with recent findings in nonfunctioning pituitary tumors (68). In addition, it is also established that ghrelin stimulates ACTH and cortisol secretion under normal conditions, and this regulation is accompanied by negative glucocorticoid feedback. This stimulatory effect persists in patients with CD (69, 70), and even in patients with ectopic ACTH-secreting tumors, in which an ACTH hyperresponsiveness to ghrelin has been described (70). In this context, the expression levels of some components of the ghrelin system were also evaluated in our cohort. Specifically, ghrelin and ghrelin-receptors (*GHSR1a* and *GHSR1b*) were correlated with *POMC*, which is in line with other publications indicating that ghrelin excites *POMC* neurons through an unidentified mechanism and stimulates ACTH secretion in ACTHomas (31, 71). In this context, previous publications have described an increased expression of the splicing variant In1-ghrelin in pituitary tumors, wherein it is associated with aggressive features, and its silencing was accompanied by reduced cell proliferation in pituitary primary cultures (31). In our cohort, the expression of In1-ghrelin variant was directly correlated with *MKI67* in ACTHomas, suggesting an association with proliferation markers. Moreover, the *GOAT* enzyme was strongly correlated with the expression of key somatostatin and dopamine receptors (eg, *SSTR2*, *SSTR5*, and *DRD2*) as has been reported in other neuroendocrine tumors (72). Finally, the truncated receptor *GHSR1b* also demonstrated significant correlations including with *CRHR1* and *PTTG1*, which should be further investigated.

In recent years, systematical evaluation of *MKI67* in pituitary tumors has been a matter of debate (73). Several clinical studies have described the association between *MKI67* and clinical features in pituitary tumors, including invasion characteristics, tumor size, risk of recurrence, and growth rate, but despite this, results are contradictory (74). In ACTHomas, *MKI67* seems to be increased in tumors larger than 1 cm and related to lower long-term tumor remission rate (75-77). Another study has suggested that Ki67 levels greater than 4% (evaluated by immunohistochemistry) and ACTH serum values above 40 ng/mL 1 month after surgery were associated with the absence of biochemical control in these patients (78). In our cohort, *MKi67* expression was not associated with remission, recurrence, or tumor size, but it was correlated with expression of In1-ghrelin and *GHSR1b*, 2 splicing variants that have been previously reported to be overexpressed in different pituitary tumors, including ACTHomas, and play a pathophysiological role in this pituitary tumor type (31). Interestingly, we found that *PTTG1* expression levels were significantly higher than *MKI67* in ACTHomas. *PTTG1* has been associated with increased invasion capacity of pituitary tumors (79), and recently, it has been associated with invasiveness, age, and female sex in nonfunctioning pituitary tumors (80). In our cohort of CD patients, *PTTG1* was correlated with the expression *of POMC*, *AVPR1b*, and *CRHR1*. This correlation might be pathophysiologically relevant because we have demonstrated that *AVPR1b* is overexpressed in ACTHomas, wherein it might exert an important functional role since its levels are directly correlated with elevated plasma ACTH levels in CD patients and might be a responsible factor for the direct desmopressin stimulatory effects in CD patients (27).

Finally, binomial logarithmic regression studies further supported the overall alterations and key associations/correlations observed in our cohort of CD patients. Although we could not identify any robust singular clinical or molecular parameter that was significantly associated with the prediction of the recurrence of CD patients, our analyses identified some molecular (ie, *SSTR1* and *CRHR1*) and clinical (ie, tumor size, basal serum prolactin, postsurgery serum and urinary cortisol, and postsurgery 1 mg DST) parameters that were significantly associated with patient remission. Most important, we demonstrated that a model combining the 2 clinical variables that were found to be the most significantly associated with remission (ie, tumor size and postsurgery serum cortisol; prediction model 1 in Fig. 6) together with the molecular markers identified (ie, *SSTR1* and *CRHR1*; prediction model 2 in Fig. 6) could better improve the predictive capacity of both individual models in that the ROC curve analysis based on tumor size, postoperative serum cortisol, and *SSTR1* and *CRHR1* expression levels could perfectly discriminate between patients in remission and nonremission patients after the first surgery (predictive model 3: ROC curve with AUC = 1). To the best of our knowledge, this is the first study showing a clinical-molecular–based algorithm capable of identifying patients with CD at different risks of being in remission or not after their first surgery, a prediction that has been a challenging goal to date for clinicians managing patients with CD. At this point, it is interesting to mention that a recent systematic review including a relevant number of CD patients from 5 different cohorts revealed that the probability of remission was found to be higher in patients with the somatic *USP8*-mutated allele vs patients with the *USP8*–wild-type allele (11). Therefore, it would be interesting to perform in the future multicenter analyses to evaluate whether the analysis of the mutational status of *USP8* together with the molecular and clinical parameters identified in this study (ie, expression of *SSTR1*/*CRHR1*, tumor size, and postoperative serum cortisol) could accurately predict the remission of patients with CD after first surgery. Nevertheless, we should also mention that a limitation of our study might be that mRNA levels may

not always directly translate into functional protein levels; however, several studies analyzing the correspondence between mRNA and protein state, using transcriptomic and proteomic technologies, have revealed that the abundance of an mRNA is often an excellent proxy for the presence of a protein (81, 82).

Altogether, our results suggest that the molecular analysis of ACTHomas could represent a valuable, complementary tool for improving the understanding of the pathophysiology of ACTHomas. Future studies should be conducted in larger cohorts and with patients of different ethnicities to appropriately corroborate our main conclusions. Nevertheless, this study reveals that the expression of 2 molecular determinants, *SSTR1* and *CRHR1*, also help to predict, together with some key clinical parameters, tumor behavior of patients with CD.

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Disclosures

The authors have nothing to disclose.

Data Availability

Some or all data generated or analyzed during this study are included in this published article or in the data repositories listed in "References."

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