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Original Research

Integrative clinical, hormonal, and molecular data associate with invasiveness in acromegaly: REMAH study

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Abstract

Introduction: Growth hormone (GH)-secreting pituitary tumors (GHomas) are the most common acromegaly cause. At diagnosis, most of them are macroadenomas, and up to 56% display cavernous sinus invasion. Biomarker assessment associated with tumor growth and invasion is important to optimize their management.

Objectives: The study aims to identify clinical/hormonal/molecular biomarkers associated with tumor size and invasiveness in GHomas and to analyze the influence of pre-treatment with somatostatin analogs (SSAs) or dopamine agonists (DAs) in key molecular biomarker expression.

Methods: Clinical/analytical/radiological variables were evaluated in 192 patients from the REMAH study (ambispective multicenter post-surgery study of the Spanish Society of Endocrinology and Nutrition). The expression of somatostatin/ghrelin/dopamine system components and key pituitary/proliferation markers was evaluated in GHomas after the first surgery. Univariate/multivariate regression studies were performed to identify association between variables.

Results: Eighty percent of patients harbor macroadenomas (63.8% with extrasellar growth). Associations between larger and more invasive GHomas with younger age, visual abnormalities, higher IGF1 levels, extrasellar/suprasellar growth, and/or cavernous sinus invasion were found. Higher *GH1* and lower *PRL/POMC/CGA/AVPR1B/DRD2T/DRD2L* expression levels ($P < .05$) were associated with tumor invasiveness. Least Absolute Shrinkage and Selection Operator's penalized regression identified combinations of clinical and molecular features with areas under the curve between 0.67 and 0.82. Pre-operative therapy with DA or SSAs did not alter the expression of any of the markers analyzed except for DRD1/AVPR1B (up-regulated with DA) and *FSHB/CRHR1* (down-regulated with SSAs).

Conclusions: A specific combination of clinical/analytical/molecular variables was found to be associated with tumor invasiveness and growth capacity in GHomas. Pre-treatment with first-line drugs for acromegaly did not significantly modify the expression of the most relevant biomarkers in our association model. These findings provide valuable insights for risk stratification and personalized management of GHomas.

Keywords: pituitary tumor, curation, combined molecular and clinical biomarkers, transsphenoidal surgery, REMAH study

Significance

This study identifies crucial biomarkers associated with the size and invasiveness of growth hormone-secreting pituitary tumors (GHomas), providing a comprehensive understanding of their molecular characteristics. Interestingly, younger age, visual abnormalities, higher GH1, and lower PRL/POMC/CGA/AVPR1B/DRD2T/DRD2L expression levels were associated with tumor invasiveness. Pre-operative therapy with dopamine agonist or somatostatin analogs did not alter the expression of any of the markers analyzed except for DRD1/AVPR1B (up-regulated with dopamine agonist) and FSHB/CRHR1 (down-regulated with somatostatin analogs). Finally, Least Absolute Shrinkage and Selection Operator's (LASSO) penalized regression identified combinations of clinical and molecular features with areas under the curve between 0.67 and 0.82. These findings offer significant advancements in risk stratification to improve diagnostics and therapeutic interventions in GHomas.

Introduction

Growth hormone (GH)-secreting pituitary tumors (GHomas) are the most common cause of acromegaly. Acromegaly is characterized by an excess of GH and consequently of insulin-like growth factor 1 (IGF-1) and is associated with several health complications and increased mortality.[1] Clinical conditions derived from acromegaly include hypertension, diabetes, sleep apnea, goiter, colonic polyps, and skeletal deformities, as well as metabolic dysfunction including insulin resistance, diabetes mellitus, and cardiovascular-related diseases.[2] Additionally, visual disturbances and headache related to mass effects are observed as one of the most concerning features in these patients.[3] At diagnosis, macroadenomas, eg, tumors ≥1 cm, may be present in 75%-85% of patients with GHomas, whereas cavernous sinus invasion is found in 25%-56% of macroadenomas.[4-6] Patients with tumor invasion have significantly poorer disease outcomes.[4]

Treatment of patients with acromegaly aims to normalize elevated GH/IGF-1 levels, in order to improve the disease-related symptoms and complications,[7,8] to reduce tumor mass and the local effects as well as the related mortality.[8,9] Surgery is considered the treatment of choice in GHomas since it is associated with long-term biochemical and tumor control in about 60% of patients.[10-12] Pituitary surgery is necessarily conservative, to maintain as much as possible the residual pituitary function. In this sense, surgical outcome has been related to different factors including pre-operative serum GH and IGF-1 levels, tumor size, and invasion and also to the skill of the neurosurgeon when accessing invasive pituitary macroadenomas.[13] Indeed, tumor size and tumor invasion are not only determinants of surgical cure but also of resistance to pharmacological treatments and/or early/multiple recurrences after surgery.[14,15] Importantly, there are no reliable pathological predictors of tumor behavior.[14] Thus, it is relevant to identify biomarkers associated with pituitary neoplasms that exhibit clinically invasive behavior. In this regard, the importance of an accurate assessment of the tumor proliferative capacity by mitotic count and/or Ki-67

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index for evaluating pituitary tumor clinical behavior has been highlighted.[16] Other factors such as magnetic resonance imaging (MRI) tumor signal, GH value after acute octreotide test, granular adenoma pattern in immunohistochemistry, somatostatin receptor phenotype, *AIP* and *GNAS* mutations, RAF kinase activity, E-cadherin and beta-arrestin-1, and *RET/GDNF* signaling, among others,[17-19] have also been evaluated as biomarkers of GHoma behavior, and some authors suggest their use as prognostic and predictive markers in clinical predictive models.[20,21] However, to the best of our knowledge, there is not a reliable specific, updated, and integrative clinical and molecular algorithm that can predict tumor behavior in acromegalic patients.

The Spanish Molecular Registry of Pituitary Adenomas (REMAH) is a nationwide study that was organized in 6 nodes by recording patients' data and collecting pituitary tumors from patients who underwent surgery across the major hospitals in Spain.[22] Specifically, all Spanish public hospitals followed similar clinical management of the acromegalic patients in terms of diagnosis, treatment, tumor sample collection, and follow-up, in order to (1) analyze the clinical behavior after a long follow-up of our well-characterized cohort of acromegalic patients ($n = 192$) and (2) apply a multivariate clinical–hormonal–molecular algorithm that could predict the invasion capacityof GHomas. Additionally, we explored the putative effect of pre-surgical treatment with dopamine agonists (DAs) or somatostatin analogs (SSAs) on the molecular profile of these tumors to better understand their possible effect on tumor behavior.

Methods

Patients and tumor sample processing

Clinical, analytical, and radiological variables from 192 patients with GHomas enrolled in the REMAH study, a nationwide project conducted by the working group on Neuroendocrinology of the Spanish Society of Endocrinology and Nutrition, were analyzed in the present study. All techniques carried out in this study were conducted in accordance with the ethical standards of the Helsinki Declaration, of the World Medical Association, and with the approval of the Ethics Committees from all the hospitals involved in the study. Informed consent from each patient was obtained. The specific characteristics of this registry have been previously described.[22] Briefly, participating centers were organized into 6 nodes from all over Spain, each one coordinated by a basic researcher with molecular experience, and a clinical researcher experienced in the care of patients with pituitary diseases who underwent surgery. Node distribution and location was as follows: Andalusia (Córdoba), Community of Madrid (Madrid), Valencian Community (Alicante), Galicia (Santiago de Compostela), Catalonia (Barcelona), and Basque Country (Bilbao).

Standardized protocols were followed in the different institutions participating in this study to obtain tumor samples immediately after the surgery, which were rapidly included in phosphate-buffered saline for immediate exam and processing by an experienced pathologist following standardized diagnosis criteria. Then, the pathologist preserved a fragment for anatomopathological analyses, and another small 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 transported to the laboratory of the different nodes wherein RNA extraction was immediately performed, and integrity of RNA was analyzed using a Bioanalyzer. RNA was stored frozen at −80 °C until the retro-transcription and quantitative real time polymerase chain reaction (qPCR) analyses (see below).

Furthermore, a database was used to collect standardized information from the medical history of all patients (demographic, biochemical, and radiological characteristics of the patients with GHomas from the REMAH cohort are summarized in Tables 1 and 2). Patients were treated according to the available clinical guidelines, and surgery was the treatment of choice in all patients.[7] Ninety-three patients received pre-surgical treatment (with DAs [$n = 15$] or with first-generation SSAs [$n = 78$]; 73% of patients that were treated with DAs were receiving combined treatment with SSAs). Specifically, DA treatment had a median duration of 9.6 months, while SSAs had a median duration of 7.4 months. Baseline characteristics of naïve and pre-treated patients were comparable (Tables 1 and 2). In this context, the initial molecular analysis of this cohort of patients was performed and included naïve ($n = 99$) and pre-treated ($n = 93$) patients. None of the 192 cases were syndromic cases.

Measurement of GH and IGF-1 levels was performed in the laboratory services of the different hospitals involved using different assays and following the manufacturer's instructions. It

Table 1. Clinical characteristics according to the medical pre-treatment.

	Total (192)	Dopamine agonists (15)	Somatostatin analogs (78)	P^a	P^b
General characteristics					
Female sex, % (*n*)	54.2 (104/192)	53.3 (8/15)	55.1 (43/78)	.51	.53
Age (years), mean ± SD	45.5 ± 13.8	42.8 ± 15.5	43.9 ± 14.5	.69	.13
Body mass index (kg/m^2), mean ± SD	28.9 ± 5.4	27.6 ± 4.8	29.02 ± 5.4	.50	.52
Radiological parameters					
Macroadenoma (≥10 mm)	80.7 (134/166)	92.9 (13/14)	85.1 (57/67)	.22	.16
Tumor size (mm), mean ± SD	19.7 ± 11.5	19.8 ± 10.1	19.79 ± 11.4	.64	.77
Extrasellar growth, % (*n*)	63.8 (102/160)	71.4 (10/14)	68.7 (46/67)	.43	.15
Suprasellar growth, % (*n*)	47.9 (70/146)	46.2 (6/13)	55 (33/60)	.49	.17
Infrasellar growth, % (*n*)	23.3 (24/103)	11.1 (1/9)	29.3 (12/41)	.35	.17
Sinus invasion, % (*n*)	27.6 (35/127)	27.3 (3/11)	31.5 (17/54)	.62	.47

P^a reflects the comparison between the use and non-use of dopamine agonists.
P^b reflects the comparison between the use and non-use of somatostatin analogs.

Table 2. Biochemical characteristics of patients with growth hormone-secreting pituitary tumors treated with medical therapies before surgery.

Biochemical parameters	Total	Dopamine agonists			Somatostatin analogs		
		No use	Use	P^a	No use	Use	P^b
Fasting glucose (mg/dL)	106	106	99	.03	106	106	.74
HbA1c (%)	6.1	6	7.0	.40	6	6.2	.69
Cholesterol (mg/dL)	199	195	211	.83	187	199	.74
LDL (mg/dL)	125	120	29	.17	131.5	112	.09
HDL (mg/dL)	52	52	140	.09	52	53	.75
GH (ng/mL)	12.9	11.4	18.3	.36	10.2	16.1	.28
IGF-1 (ng/mL)	806	780	799	.40	806	873	.23
Prolactin (mIU/mL)	205.6	200	61.5	.18	255.5	189.4	.43
ACTH (pg/mL)	28.8	31.2	25.5	.85	33.3	27.6	.88
Basal cortisol (uU/dL)	12.9	12.9	7.0	.05	15.0	11.2	.02
LH (mIU/mL)	3.4	3.5	2.6	.09	3.7	2.9	.10
FSH (mIU/mL)	5.4	5.4	5	.29	5.7	5	.07
TSH (uIU/mL)	1.3	1.3	1.1	.53	1.4	1.2	.99
Free thyroxin (ng/L)	1.1	1.1	1.0	.87	1.2	1.1	.89

P^a reflects the comparison between the use and non-use of dopamine agonists.
P^b Reflects the comparison between the use and non-use of somatostatin analogs.

should be noted that the biochemical magnitudes measured and included in this manuscript lack bias attributable to inter-assay variability (intra-laboratory/inter-laboratory) 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 [IFCC] and the American Association for Clinical Chemistry [AACC]). Pituitary resonance imagings were conducted at each hospital using state-of-the-art resonance imaging equipment. Invasiveness was defined by radiologic data on the images obtained by MRI and using Knosp classification[23]; Knosp categories 3 and 4 were considered as invasive tumors.

Immuno-histochemical analysis

Immuno-histochemical analysis of pituitary tissue was performed in each center as follows. Procedure began with tissue fixation, sectioning, and subsequent deparaffinization and rehydration steps. Antigen retrieval was performed to enhance protein accessibility while blocking non-specific binding sites and was followed with the application of a primary antibody specific to pituitary hormones (eg, ACTH, GH, TSH) and/or transcription factors (eg, PIT-1). Subsequently, a secondary antibody was used for detection. This analysis was performed at each hospital as part of routine pathological examinations, and data were retrieved retrospectively from these analyses.

RNA isolation, reverse transcription, and qPCR

RNA extraction followed by reverse transcription and qPCR was performed in each sample, as previously described.[22] Specifically, expression levels (absolute mRNA copy number/50 ng of sample) of a set of critical genes previously selected for their involved in the pathophysiology of all types of pituitary tumors and their potential diagnostic value and the possible clinical usefulness of the information they provide were evaluated; pituitary hormones (*GH1*, prolactin [*PRL*], pro-opiomelanocortin [*POMC*], luteinizing hormone [*LHB*], follicle-stimulating hormone [*FSHB*], thyroid-stimulating hormone [*TSHB*], free alpha-subunit glycoprotein hormones [*CGA*]), different key receptors for major factors regulating the function of all pituitary cell types (somatostatin [*SSTR1*, *SSTR2*, *SSTR3*, *SSTR5*], dopamine receptors [*DRD1*, *DRD4*, *DRD5*] including the total pool of DRD2 receptors [DRD2T] and long isoform [DRD2L], gonadotropin-releasing hormone receptor [*GNRHR*], GH-releasing hormone receptor [*GHRHR*], corticotropin-releasing hormone receptor [*CRHR1*], native ghrelin receptor [*GHSR1A*], arginine vasopressin receptor 1b [*AVPR1B*]), and key proliferation markers (pituitary tumor transforming gene [*PTTG1*] and *MKI67*) were measured using previously validated primers.[22] The stability of 3 control genes (*ACTB*, *HPRT1*, and *GAPDH*) was evaluated as previously reported,[22] being *HPRT1* the most stable according to a comprehensive tool which integrates the currently available major computational programs.[24] Therefore, *HPRT1* was used to adjust the expression of all the genes analyzed. Copy number values adjusted by *HPRT1* were logarithmic transformed in base 2, and missing values were replaced with the minimum value of the data distribution. The STTR2/SSTR5 ratio was determined by quantifying the amount of these receptor subtypes present in pituitary tumor tissue. After molecular analysis, a calculated variable was generated as follows: SSTR2 ÷ SSTR5. Detailed information about the primers used can be found in Table S1.

Statistical analysis

Univariate and multivariate analysis were performed. For the univariate analysis, Mann–Whitney *U* tests were used to evaluate clinical–molecular associations within GHoma samples. Chi-squared test was used to compare categorical data. In this section, statistical analyses were performed using SPSS statistical v20 and GraphPad Prism v7 and R v4.3. Quantitative variables are expressed as mean ± standard error of mean (SEM) (Gaussian distribution) or median and interquartile range (IQR) (non-Gaussian distribution) and categorical variables as absolute and frequencies (*n*) and percentage (%). Spearman's rho correlation was calculated between continuous variables. A correlation map was plotted as a visualization tool. This correlation was generated by first preparing a Spearman's correlation matrix, and this was ordered by hierarchical clustering using Euclidean distance to group similar variables. Euclidean analysis quantifies the distances between data points representing different variables in

a multidimensional space, and shorter distance implies similar behavior of molecular variable. Furthermore, a univariant comparison was done for the estimation of the relationships between qualitative variables and IGF-1 values using the median as cutoff point.

Additionally, we evaluated the molecular expression of GHomas and its association with tumor behavior. We performed a multivariate analysis based on a logistic LASSO regression analysis using glmnet package of software R.[25,26] LASSO regression models are less reliable when the number of parameters is larger than sample size and when there are high levels of multicollinearity. To solve this, it sets some of the coefficients exactly equal to 0, returning a list of relevant associated biomarkers included in the model. This approach is a regression technique which penalizes the model with a regularization parameter, which controls the sparsity, and is selected by cross-validation (CV) techniques. This parameter shrinks coefficients toward 0 and ignores the 0 coefficient terms in the model.[26] Particularly, the LASSO penalization coefficient was tuned through 10-fold CV, and the variable selection was based on the area under the curve (AUC) values in our study. Due to possible missing values in the database, multiple imputation was used to estimate the missing values in the predictors using chained equations approach in order to fit a LASSO regression model. Different clinical variables and hormonal values were selected for each response model. Furthermore, the receiver operating characteristic (ROC) curves and area under the ROC curve were used to determine the predictive capacity of the following models: (i) clinical model (performed only with clinical variables); (ii) molecular model (included molecular and immuno-histochemical [IHC] variables); and (iii) full model, which included both, clinical and molecular predictors. Importantly, for each model, a new variable selection process was performed. We evaluated the differences between the ROC curves performing De-Long tests using MedCalc v13. Area under the curve and 95% confidence intervals are presented as well. In all analyses, *P*-values <. 05 were considered statistically significant.

Results

Patient population

A total of 192 patients with GHomas from the REMAH cohort were included in the study. Clinical, biochemical, hormonal, and radiological findings are summarized in (Tables 1 and 2). Specifically, 54.2% of the patients were women. Radiological findings showed that 81% of the tumors were macroadenomas (mean size of 19.67 ± 11.5 mm), and almost 64% of patients had extrasellar invasion (48% suprasellar invasion, 28% cavernous sinus invasion, and 23% sphenoidal sinus invasion). Interestingly, almost 50% of the evaluated patients had cardiovascular complications.

Relationships between clinical, hormonal, and radiological findings

Male patients had higher baseline IGF-1 levels (Figure 1A). Macroadenomas and extrasellar invasion were more frequent in younger patients (Figure 1B and C, respectively). The presence of larger tumors was associated with higher circulating IGF-1 levels as well as higher GH expression levels in the tumor sample (Figure 1D and E, respectively). Larger tumors were also accompanied by a higher frequency of cavernous sinus invasion and by a higher incidence of visual defects as expected (Figure 1F and G, respectively). Interestingly, age showed

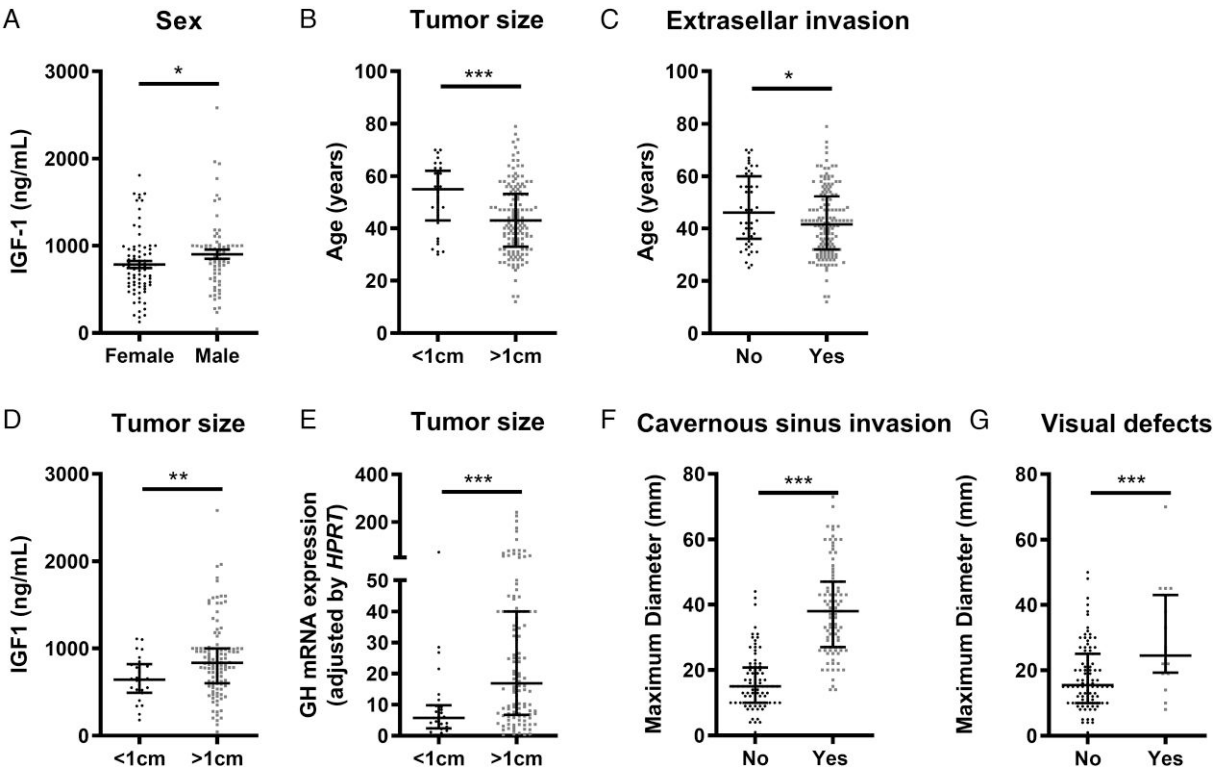


Figure 1. Univariate exploratory analysis of clinical, biochemical, molecular, and radiological features in GHomas. (A) IGF-1 (ng/mL) by sex. (B) Age (years) by tumor size. (C) Age (years) by extrasellar invasion. (D) IGF-1 (ng/mL) by tumor size. (E) GH mRNA expression (adjusted by HPRT) by tumor size. (F) Maximum diameter (mm) by Carvenous sinus invasion. (G) Maximum diameter (mm) by visual defects.Asteriks (**P* < .05, ***P* < .01, ****P* < .001).

Table 3. Analysis of clinical, biochemical, and radiological features according to insulin-like growth factor 1 initial value (median as cutoff of the total population).

	Low IGF-1 patients	High IGF-1 patients	*P*-value
General characteristics			
Female	64.9	46.3	.04
Age (years)	47.7 + 13.8	41.6 + 12.9	.006
BMI (kg/m²)	29.0 + 5.1	29.3 + 6.0	.78
Tumor			
Macroadenoma, %	74.0	89.7	.02
Tumor size (mm)	19.1 ± 12.0	20.0 + 11.0	.24
Comorbidities			
Arterial hypertension, %	30.8	35.5	.71
Hypercholesterolemia, %	23.6	24.6	1.00
Type 2 diabetes, %	23.8	24.6	1.00
Cancer, %	3.3	0.0	.50
Cardiovascular disease, %	3.3	6.8	.44
Apnea, %	15.9	14.0	1.00
Goiter, %	13.3	20.4	.42
Colonic polyps, %	5.1	13.3	.28
Radiological parameters			
Extrasellar growth, %	67.7	63.2	.72
Suprasellar growth, %	53.2	45.2	.47
Infrasellar growth, %	16.3	25.0	.43
Sinus invasion, %	30.0	22.9	.50

positive correlations with the expression levels of *FSHB* (Spearman correlation = 0.19, $P = .019$), *GNRHR* (Spearman correlation = 0.19, $P = .01$), *SSTR1* (Spearman correlation = 0.17, $P = .019$), *CRHR* (Spearman correlation = 0.22, $P = .005$), *POMC* (Spearman correlation = 0.2, $P = .020$), and *AVPR1B* (Spearman correlation = 0.21, $P = .009$).

Furthermore, the study evaluated the impact of previous medication. Patients who did not use DAs exhibited higher fasting glucose (Table 2). Those without SSAs or DAs showed lower basal cortisol. No other treatment-related differences were identified among patients (Tables 1 and 2). Additionally, in the analysis based on baseline IGF-1 values (Table 3), it was found that high IGF-1 values correlated with a younger age at diagnosis and a higher prevalence of macroadenomas (*P*-value < .05). No other statistically significant relationships were observed.

Correlation of clinical/hormonal data with molecular findings

Serum GH values were positively correlated with *SSTR2* expression while negatively associated with *GNRHR*, *CRHR*, and *AVPR1B* expression (Figure 2A). In contrast, IGF-1 levels were positively correlated with *GHSR* isoform 1a expression (Figure 2B). A heatmap summarizing the molecular correlations observed in our cohort is depicted in Figure 2C. Briefly, 2 clusters of genes were observed, the first including those genes that correlated positively (red color) between themselves (the big group in the center: *POMC*, *CRHR1*, *AVPR1B*, *DRD2T*, *TSHB*, *LHB*, *FSHB*, *PRL*, and *PTTG1*) and the second smaller group in the lower-right corner (*GHRHR*, *GHSR* isoform 1a, *SSTR5*, *SSTR2*, and *DRD5*). Other small groups are formed including the rest of genes paired that tended to have negative correlations (blue) on analysis (hierarchical clustering performed by Euclidean distance analysis).

Combination of clinical, analytical, radiological, and molecular features enhances characterization of tumor size and invasiveness in GHomas

In order to assess the association of tumor size (classified as macro- or microadenoma), with different biomarkers, a multivariate analysis was performed including baseline clinical and biochemical parameters (age and serum levels of HDL, GH, LH, and IGF-1) and also some key molecular parameters (*PRL*, *TSHB*, *SSTR3*, *GNRHR*, *CRHR1*, and *AVPR1B*) that were selected using the LASSO regression model for the construction of predictive models. Using this approach, the AUC was 82% indicating that most of the determinant factors associated with the recognition of micro- and macroadenomas (AUC 0.828; 95% CI 0.75-0.99) were included in the model. The combined model (clinical/biochemical and molecular parameters) was significantly more robust than the clinical/biochemical and the molecular models separately ($P < .05$; $P < .01$ respectively, Figure 3). The variables included in each model are presented in Table S2 with their estimated coefficients.

Regarding tumor invasiveness, the molecular profile showed that those tumors with higher extrasellar invasion had lower expression levels of *POMC*, *AVPR1B*, *DRD2T*, and *DRD2L* isoform (Table 4) and higher expression levels of *MKI67* (Table 4).

In order to assess the tumor extrasellar invasion capacity, a combination of clinical parameters, IHC staining, and mRNA expression data was evaluated using the LASSO regression model. This combined model had higher efficacy for extrasellar invasion capacity recognition compared with the model that used clinical/biochemical parameters alone (AUC 0.81 [95% CI 0.74-0.87] *vs* 0.73 [95% CI 0.68-0.82]) or models that used molecular parameters alone (AUC 0.67, 95% CI 0.59-0.75; $P < .01$, respectively). The evaluated variables in each model with their estimated coefficients are presented in Table S3. In contrast, the combined model did not outperform the suprasellar or infrasellar invasion prediction compared with the clinical or molecular models. The variables included in each model are presented in Tables S4 and S5, respectively. Finally, the combined model (molecular and clinical/biochemical parameters) was more strongly associated with cavernous sinus invasion and outperformed the clinical/biochemical model. The specific variables in this final combined model are presented in Table S6.

Pre-surgical treatment with agonists in GHomas

In our cohort, pre-treatment with DAs did not significantly alter the molecular expression of the key markers analyzed with the exception of *DRD1* and *AVPR1B* which were up-regulated (Figure 4A). In the case of pre-treatment with somatostatin analogs, we also did not observe any major significant alteration in the expression of the majority of the key molecular markers analyzed with the exception of *FSHB* and *CRHR1* expression which were down-regulated while *GHSR1A* expression was up-regulated (Figure 4B).

Discussion

In the present study, we comprehensively evaluated the association between tumor size and invasiveness with different clinical/biochemical features and with the expression levels of key hypothalamic factors/pituitary hormones and receptors, as well as several proliferation-related genes in a large series of

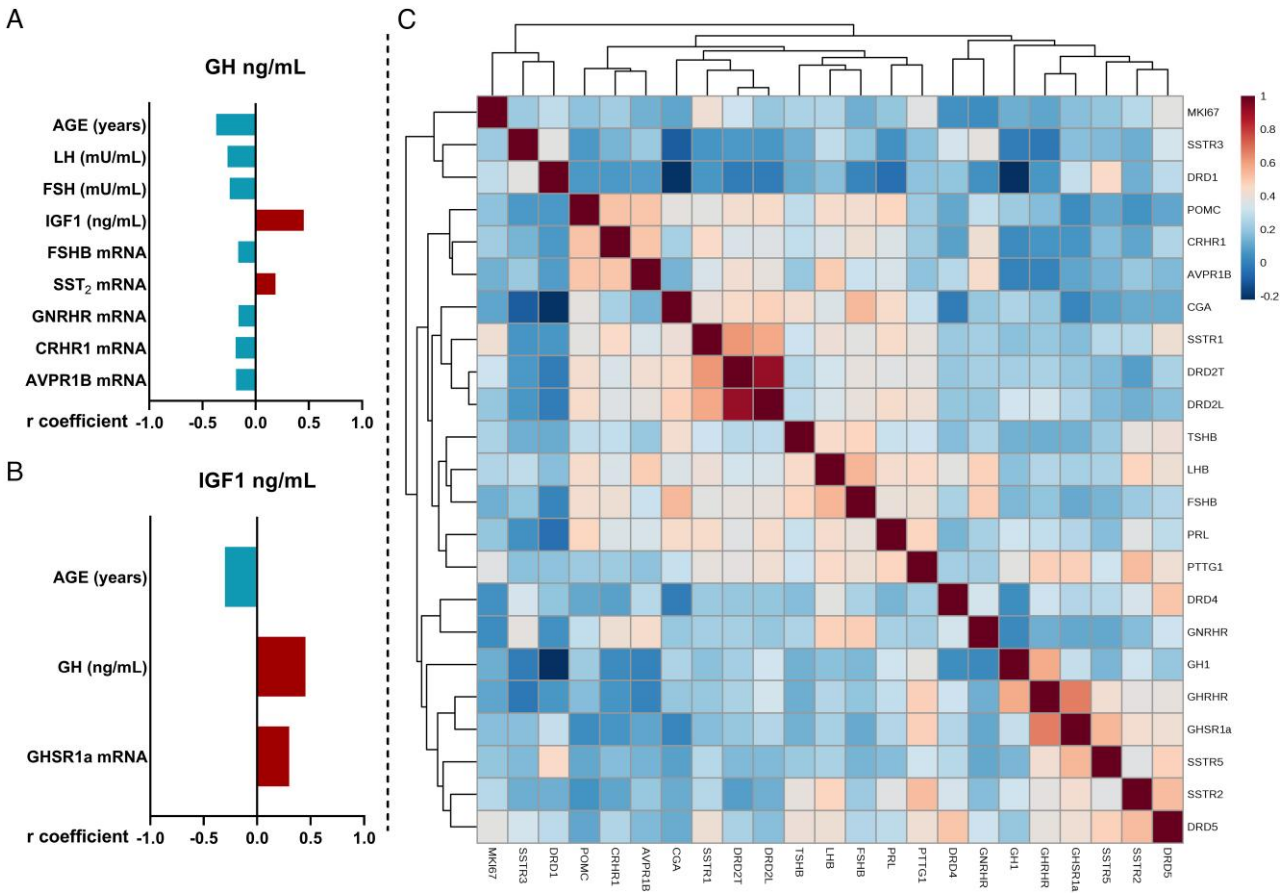
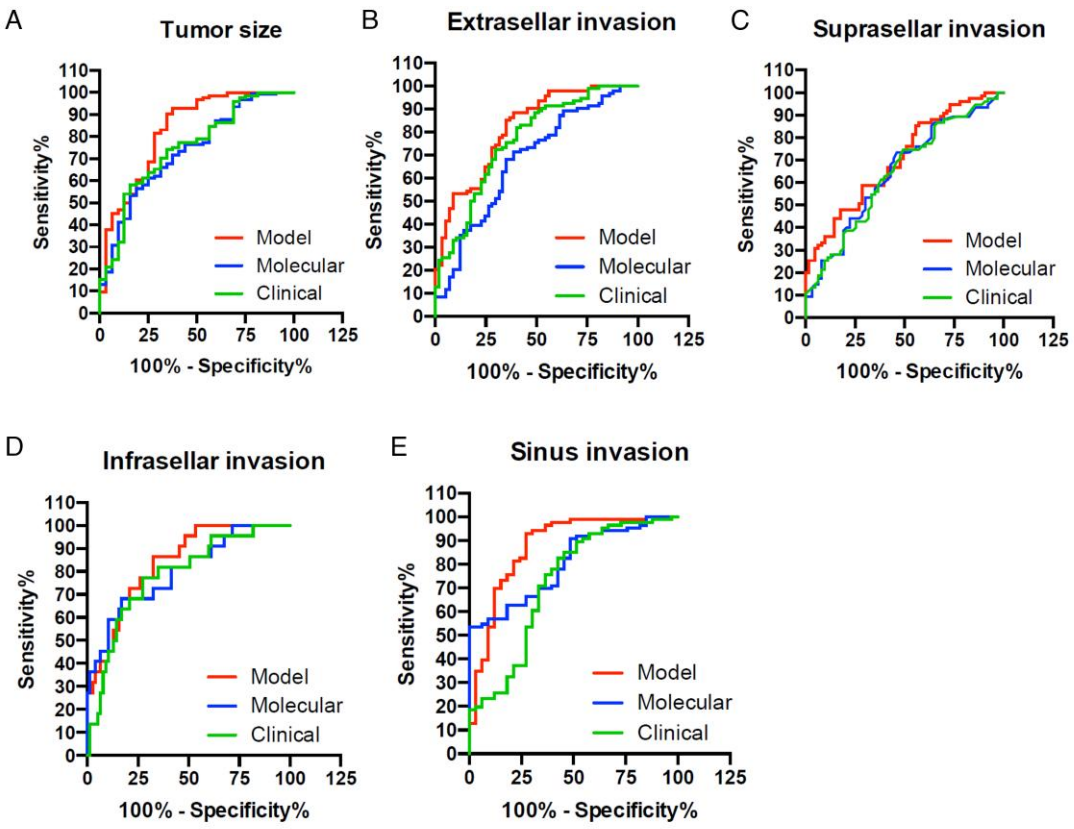


Figure 2. Clinical and molecular correlations with GH and IGF-1 in GH-secreting pituitary tumors (panels A and B, respectively). Only significant correlations ($P < .05$) are presented. Correlation map of molecular biomarkers in GH-secreting pituitary tumors (panel C). Variables were ordered with brackets representing Euclidean analysis. Legend: Euclidean analysis was performed to evaluate the relationships between various clinical and biochemical markers in pituitary tumor tissue. It quantifies the distances between data points representing different variables in a multidimensional space.

GHomas from the REMAH cohort. This study represents the largest Spanish cohort of GHomas performed so far, wherein clinical, radiological, histo-pathological, and molecular information was combinedly analyzed.

Specifically, in our cohort of 192 acromegalic patients, younger individuals presented larger and more invasive tumors as well as higher GH/IGF-1 levels, as previously described.[27-29] Previous studies have reported that female patients have more invasive and larger GHomas than male patients[29,30]; however, this observation was not confirmed in our cohort, as has been also observed in other series.[31] In addition, some molecular components of the somatostatin receptor pathway have been also associated with invasive features and prognosis, ie, somatostatin receptor subtypes 2 and 5, and the *SSTR2/SSTR5* ratio, among others.[1,32-34] In this context, the *SSTR2/SSTR5* ratio tended to correlate with baseline serum GH levels in our study ($P = .08$, data not shown). Additionally, the expression of *SSTR2* was also correlated with GH serum levels, but not with IGF-1 values. Importantly, the expression of *SSTR2* was found systematically associated with invasion in all the multivariate analyses performed in our cohort except for suprasellar invasion. This suggests a relevant role of this receptor in tumor invasiveness. This finding is remarkable since current evidence suggests that the expression of *SSTR2*, both at mRNA and protein levels, could help to identify SSA responsive cases in GHomas, as this treatment is currently the pharmacologic tool most frequently used in clinical practice of acromegaly.[33,35-38] Conversely, the multivariate analysis revealed that the expression of $SSTR_5$ was significantly associated with cavernous sinus invasion, the most challenging situation for invasive cases. In this sense, some studies have described *SSTR5* as a poor predictor of clinical response in GHomas.[33]

Our data also suggest that other SST receptors might be patho-physiologically interesting in GHomas. Specifically, *SSTR1* and *SSTR3* were found to be significantly associated with invasiveness in the non-supervised model. The patho-physiological importance of *SSTR1* in the inhibitory actions of endogenous somatostatin and somatostatin agonists in GH-secreting cells has been previously demonstrated, suggesting that analogs with affinity for *SSTR1* may be useful to control hormone hypersecretion and to reduce neoplastic growth of pituitary tumors.[2,39,40] In addition, the role of *SSTR3* has not yet being clearly defined on GHomas, but some reports have indicated that *SSTR3* is highly expressed in GHomas, and that could be associated with their invasiveness capacity.[36,41,42] In this regard, some reports have also suggested the important role of *SSTR3* on tumor shrinkage during treatment with SSAs.[36,43] In fact, a positive correlation between the percentage of tumor reduction and *SSTR3* expression has been demonstrated,[36] and the constitutive activation of *SSTR3* is associated with the suppression of GH synthesis.[44]



Areas under the curve (AUC), 95%confidence intervals of the clinical, molecular an full prediction models							
Clinical variable for predictic	**Model**	**AUC**	**SE**	**95% CI**	**p[a]**	**p[b]**	**p[c]**
A. Tumor size	Clinical	0.749	0.049	0.674 to 0.815			
	Molecular	0.736	0.049	0.659 to 0.803	**0.0423**	**0.6689**	**0.0083**
	Combined	0.828	0.045	0.759 to 0.884			
B. Extrasellar invasion	Clinical	0.762	0.041	0.686 to 0.827			
	Molecular	0.676	0.046	0.596 to 0.750	0.1897	0.1349	**0.0021**
	Combined	0.815	0.036	0.744 to 0.874			
C. Suprasellar invasion	Clinical	0.644	0.047	0.558 to 0.723			
	Molecular	0.649	0.047	0.564 to 0.729	0.1392	0.6933	0.1783
	Combined	0.706	0.044	0.622 to 0.780			
D. Infrasellar invasion	Clinical	0.782	0.055	0.688 to 0.859			
	Molecular	0.799	0.056	0.706 to 0.873	0.3088	0.8427	0.5325
	Combined	0.838	0.044	0.750 to 0.904			
E. Cavernous sinus invasion	Clinical	0.719	0.057	0.629 to 0.798			
	Molecular	0.803	0.041	0.720 to 0.870	**0.0014**	0.2360	0.2426
	Combined	0.873	0.041	0.799 to 0.927			

p[a] Reflects the comparison between the clinical and the full model

p[b] Reflects the comparison between the clinical and molecular model

p[c] Reflects the comparison between the molecular and the full model

Figure 3. ROC curves after multivariate analysis for predicting (A) tumor size, (B) extrasellar invasion, (C) suprasellar invasion, (D) infrasellar invasion, and (E) cavernous sinus invasion. Red lines represent the full model, blue lines the molecular model, and green lines the clinical/biochemical model. Tables show AUC and 95% confidence intervals of the clinical, molecular, and combined prediction models.

The expression of dopamine system receptors (*DRD*s) has also been explored in our cohort, as other previous works.[35,45,46] Importantly, the expression of *DRD5* has been reported as dominant in non-functioning pituitary tumors,[47] and we found that the expression of this receptor was a significantly associated factor in the multivariate analysis for extrasellar, sphenoid, and cavernous sinus invasion. These data are consistent with a previous report indicating *DRD5* expression levels were associated with extrasellar and/or suprasellar extension in GHomas.[42] Other series have described *DRD2*

Table 4. Molecular biomarkers according to extrasellar invasion.

mRNA expression	Total Median (p25-p75)	No extrasellar invasion Median (p25-p75)	Extrasellar invasion Median (p25-p75)	*P*- value
POMC	0.86 (0.16-5.58)	2.35 (0.39-34.11)	0.53 (0.13-2.11)	.022
AVPR1b	0.009 (0.001-0.056)	0.023 (0.004-0.137)	0.006 (0.001-0.039)	.009
DRD2T	0.68 (0.23-2.88)	1.41 (0.50-3.29)	0.47 (0.19-1.94)	.008
DR2L	0.43 (0.10-1.29)	0.70 (0.23-1.89)	0.22 (0.06-1.06)	.002
Ki67	0.008 (0.001-0.036)	0.006 (0.001-0.017)	0.010 (0.001-0.066)	.049

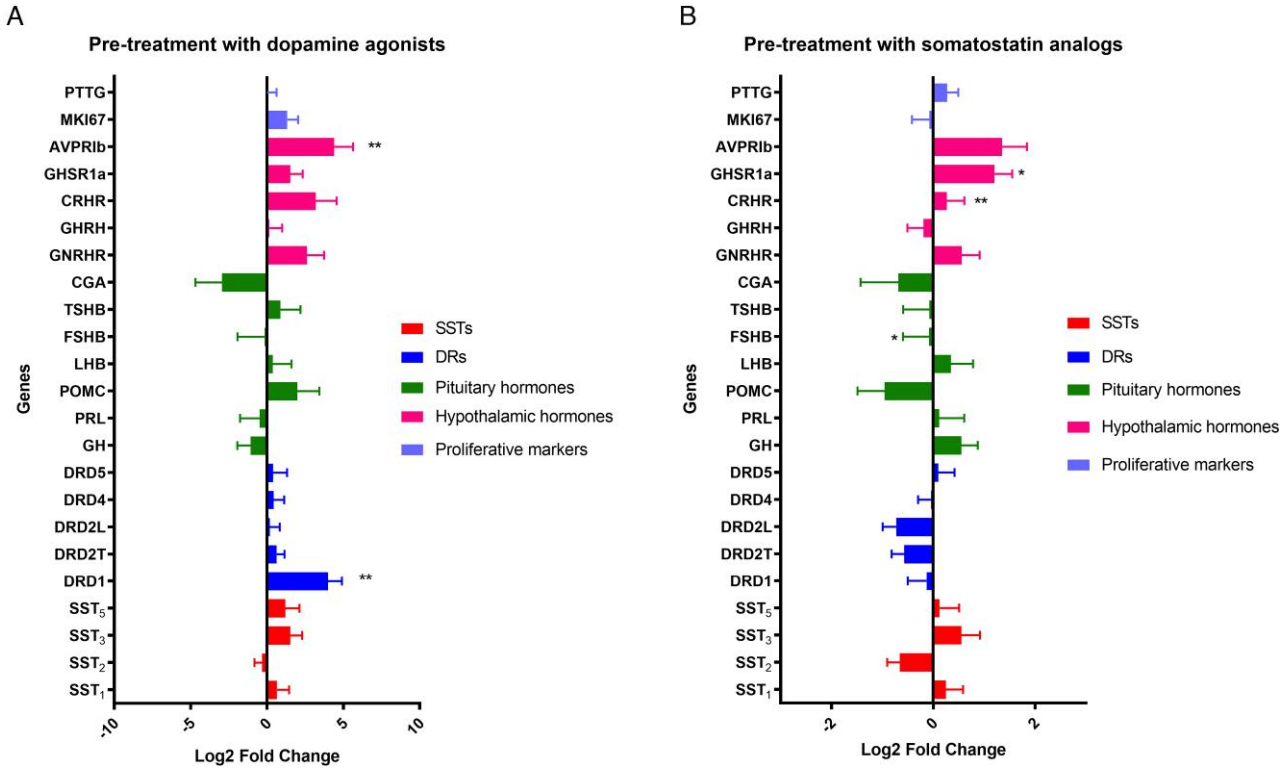


Figure 4. Effect of pre-treatment in the expression levels of *SSTR*s, *DRD*s, pituitary hormones, hypothalamic factors, and proliferative markers. (A) Pre-treatment with dopamine agonist. (B) Pre-treatment with somatostatin analogues. Legends: *SSTR*s, somatostatin receptors; *DRD*s, dopamine receptors. Results are presented in Log2 fold change ± SEM. $*P < .05$, $**P < .01$.

as the dominant *DRD* in GHomas.[35] In this sense, both isoforms of *DRD2* analyzed were included in the suprasellar, extrasellar and cavernous sinus invasion models of our study, suggesting a potential association between *DRD2* and clinical invasion. In non-functioning pituitary tumors, the expression of *DR2short*, rather than *DR2long*, has been suggested to be associated with a better *in vitro* response to DAs,[48] but data regarding their clinical relevance in acromegaly are still lacking. In addition, *DRD1* was also negatively correlated with reduction in GH levels.[46] However, no specific correlation was observed between *DRD1* with any clinical variables in our cohort.

Despite the well-known role of the pituitary tumor transforming gene (*PTTG1*) as a tumor-specific oncogene,[49] this factor was not associated with increased invasion capacity in the univariate analysis of our cohort, but it was found to be significantly associated with extrasellar, sphenoid, and cavernous sinus invasion in the multivariate analysis, suggesting that this marker may represent an important molecular target also for GHomas. Moreover, previous results have indicated that the expression of *PTTG1* has been associated with disease persistence after surgery and/or a sub-optimal response to medical therapy.[50] Thus, the expression of this marker in a tumor not yet showing an invasive behavior may prevent the clinician and probably will require a more careful follow-up, in order to detect regrowth. Other relevant molecular factors such as *AVPR1B* have been previously described in other pituitary tumors, such as ACTH-secreting pituitary tumors,[51-54] but its role in GHomas has not been described yet. Remarkably, our study revealed that *AVPR1B* expression was associated with tumor size and extrasellar and cavernous sinus invasion, suggesting a potential role in its development that requires further investigation; also, those cases showing high expression levels of this marker would require a careful follow-up after surgery.

Finally, we explored the effect of pre-treatment with SSAs and DAs on the molecular expression of hormones, receptors, and proliferation genes in these tumors.[51-53] Interestingly, we observed an up-regulation of *DRD1* in response to DAs. Previous publications have described that an increased mRNA expression levels of other *DRD*s might have a pathophysiologic importance in pituitary tumors (ie, *DRD4* has been observed in patients that respond to first-generation SSAs[35]). Therefore, our

finding of an elevated expression of *DRD1* in response to DAs should be further explored in the future. Additionally, several publications have suggested that the use of SSAs before surgery may alter the *SSTR*s tumor expression.[35,36,55] Results in this matter are contradictory,[56] as some studies report decreased *SSTR2* expression in patients with pre-operative SSAs,[57-59] while others describe similar levels of *SSTR5*,[19,36,59,60] as in our cohort, wherein mRNA expression of *SSTR*s was not altered by the pre-operative use of SSAs or DAs. Interestingly, we found in our study that that IGF-1 levels were positively correlated with GHSR1a isoform expression and that the pre-treatment with SSAs up-regulated the expression of GHSR1a suggesting that this receptor might be interesting from a pathophysiological point of view in somatotropinomas. In fact, previous studies have indicated that GHSR1a is overexpressed in patients with acromegaly and that its regulation in GH-producing cells might have some clinical implications.[61-63] Specifically, it has been reported that IGF-I could inhibit GH secretion at least in part by regulating the expression of the GHSR1a[63] and that the use of GHSR1a inverse agonists might be a potential therapeutic target in GH-producing tumors since these compounds were able to inhibit GH secretion in a dose-dependent manner and enhanced octreotide-induced GH inhibition in human somatotropinomas.[61]

The present work has some strengths and some weaknesses; among the former is having included a large number of cases, nationwide, representative of clinical practice, with a good phenotypic and molecular characterization allowing to construct mixed exploratory and predictive models. On the side of the weaknesses, it has to be mentioned 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.[64,65] In this cross-sectional study, constrained by its design and the limited prevalence of certain invasiveness variables, discussions are limited to factors' association rather than predictive outcomes. Furthermore, due to the cross-sectional design and the limited prevalence of certain variables, an external cohort for model validation was not possible. Nonetheless, our data add compelling evidence that the molecular analysis in GHomas could be a valuable, additional, tool for better understanding the pathophysiology of these tumors and for better categorizing tumor behavior; moreover, it may help in detecting tumors with a higher invasiveness capacity. Likewise, our results also revealed that the pre-operative treatment did not alter mRNA proliferation pattern of these tumors, confirming that pre-operative treatment does not negatively influence therapeutic efficacy of these drugs in those cases requiring additional treatment when only surgical debulking is feasible.

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Supplementary material

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

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Data availability

Some or all data generated or analyzed during this study are included in this published article and are available upon reasonable request.

Appendix

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