Pituitary (2019) 22:435–442
https://doi.org/10.1007/s11102-019-00973-9

Cushing's disease due to somatic *USP8* mutations: a systematic review and meta-analysis

Ingrid Quevedo Wanichi[1] · Beatriz Marinho de Paula Mariani[1] · Fernando Pereira Frassetto[2] · Sheila Aparecida Coelho Siqueira[2] · Nina Rosa de Castro Musolino[3] · Malebranche Berardo Carneiro Cunha-Neto[3] · Gilberto Ochman[3] · Valter Angelo Sperling Cescato[3] · Marcio Carlos Machado[4,5,6] · Ericka Barbosa Trarbach[6] · Marcello Delano Bronstein[4,6] · Maria Candida Barisson Villares Fragoso[1,7,8]

Published online: 4 July 2019

Abstract

Purpose Cushing's disease (CD) is a severe illness generally caused by microcorticotropinomas (MICs) and in approximately 7–20% of patients by macrocorticotropinomas (MACs). *USP8*-mutations have been identified as a major genetic cause of CD (~ 50%). Few studies have reported the distribution between MICs–MACs related to *USP8*-mutations and their genotype–phenotype correlations. Therefore, we aimed to evaluate *USP8*-mutations in a cohort of MICs–MACs from a unique center and to perform a systematic review and meta-analysis.

Methods DNA-tumor-tissues from 47 corticotropinomas (16 MICs and 31 MACs) were sequenced. Clinical-biochemical data, radiological imaging data and remission/recurrence rates were evaluated. In addition, we performed a meta-analysis of nine published series (n = 630).

Results We identified four different *USP8*-mutations previously described, in 11 out of 47 (23.4%) corticotropinomas; 8 out of 11 were MACs. The urinary cortisol levels of our patients with corticotrophin *USP8*-mutated-alleles were lower than those of patients with wild-type (WT) alleles ($p \leq 0.017$). The frequency of *USP8*-mutated-alleles among the series was approximately 30% with a higher prevalence in female-patients ($p < 0.1 \times 10^{-4}$). Among the 5 series, the remission rates were higher in patients with *USP8*-mutated-alleles than in those with the *USP8*-WT-alleles ($p < 0.1 \times 10^{-4}$).

Conclusion Our data, as well as the retrospective review of CD series associated with *USP8*-mutated alleles, show heterogeneous findings among the series. Several drawbacks included the lack of a systematic protocol to evaluate these patients before surgery and follow-up. Further prospective studies using a systematic protocol will provide more consistent information about the influence of the corticotropinomas with *USP8*-mutated alleles on the phenotype, responses to treatment and outcome of patients with CD.

Keywords Microcorticotropinomas · Macrocorticotropinomas · Ubiquitin specific peptidase 8 · Mutations

Introduction

Cushing's disease (CD) is considered to be a rare cause of hypercortisolism that is ACTH-dependent, with an incidence of 2–4 patients per million people/year and a high prevalence in female patients (aged from 25 to 40 years) [1]. The hypercortisolism caused by ACTH-secreting pituitary adenomas is associated with higher morbidity and mortality due to a severe metabolic syndrome, an increased cardiovascular risk, cerebral vascular disease and infections [2, 3].

The majority of corticotropinomas, when visible on magnetic resonance imaging (MRI) scans, are microcorticotropinomas (MICs, < 10 mm in diameter) while macrocorticotropinomas are identified in 10-20% (MACs, ≥ 10 mm in diameter) of patients. Only a few cases present invasive behavior, and exceptionally, they can reveal a malignant outcome [4].

The gold standard treatment for these patients is the neurosurgical resection of the pituitary tumor; the remission of

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s11102-019-00973-9) contains supplementary material, which is available to authorized users.

✉ Maria Candida Barisson Villares Fragoso
maria.villares@hc.fm.usp.br

Extended author information available on the last page of the article

hypercortisolism is approximately 76% of MICs and 43% of MACs, with the recurrence rates ranging from 23 to 33%, dependent on the referred centers [5]. Patients without remission or with recurrence of hypercortisolism need the beneficial combination of other treatments such as medical therapy and/or irradiation/radio therapy and surgery of the sellar region. The most important drugs in clinical practice to control hypercortisolism act as inhibitors of ACTH release or cortisol secretion or block its receptor (dopamine agonists and somatostatin analogs; ketoconazole, metyrapone, mitotane and mifepristone, respectively) [6, 7].

CD can be associated with genetic syndromes, which are mainly present as pituitary adenomas, such as multiple endocrine neoplasia type 1 (*MEN1*), familial isolated pituitary adenoma (*AIP*), multiple endocrine neoplasia type 4 (*CDKN1B*), McCune-Albright syndrome (*GNAS*) and Carney complex (*PRKAR1A*), among others [8].

A recent robust study by Reincke M et al. reported the new major finding of mutations in the ubiquitinase ubiquitin-specific peptidase 8 gene (*USP8*) in 35% of corticotropinomas (10 MICs and 7 MACs) [9]. The *USP8*-mutations produce USP8 protein hyperactivity leading to an upregulated pathway of the epidermal growth factor receptor (EGFR) that causes cell proliferation and ACTH secretion (Fig. 1) [9].

Other groups detected somatic *USP8* mutations in corticotrophin adenomas, but the frequency of *USP8*-mutated alleles in pituitary adenomas and the clinical-hormonal-radiological imaging correlations and the rate of remission/recurrence showed a great variability among the published series [9, 11–18].

To the best of our knowledge, no systematic review and meta-analysis of all series has been compiled until now. We thought to analyze all published series and to add the patients with Cushing´s disease from a unique tertiary center of Hospital das Clínicas of University of Sao Paulo, Brazil for genetic analysis of *USP8*.

Materials and methods

This study was approved by the Ethical Committee of the Hospital das Clínicas of the University of Sao Paulo, Brazil (#56235216.0.0000.0068), and the procedures were

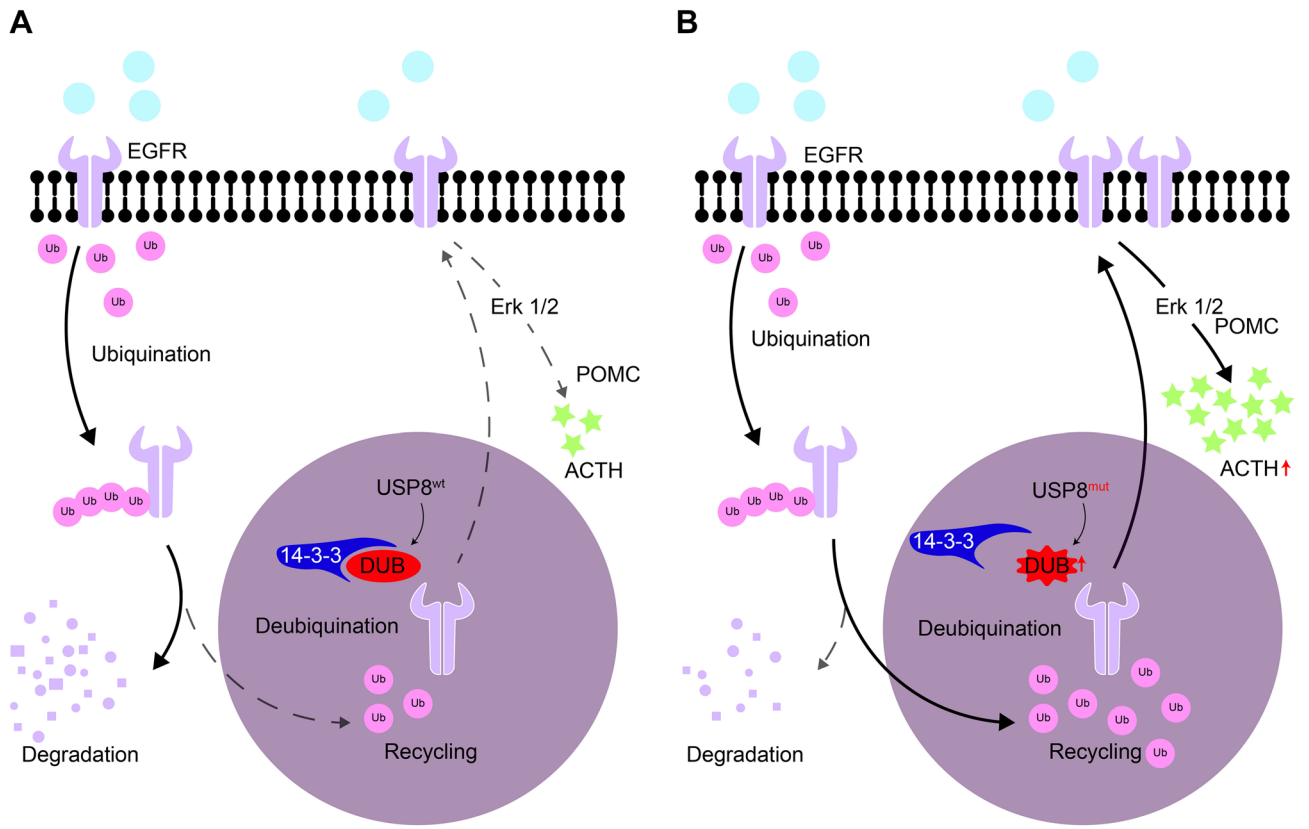

Fig. 1 Schematic representation of *USP8* -WT and *USP8* -Mutated lead to increase ACTH and tumorigenesis. **a** *USP8*-WT—Normal activity of EGFR internalization and degradation via ubiquitination activity in cytoplasm and its nuclear recycling. **b** *USP8*-mutated—Ectopic nuclear deubiquitination activity causing increasing the recycling of EGFR. EGFR trigger ERK1/2 pathway and increase cytoplasmatic ACTH secretion and leading to corticotropinomas

performed in accordance with the 1964 Helsinki Declaration and its later amendments. All patients or their legal guardians responsible signed the written informed consent.

We focused our analysis on the frequency of somatic *USP8* mutations of corticotropinomas and aimed to correlate the clinical features, the hormone levels, the MRI pituitary imaging data and patients outcomes (remission or recurrence of hypercortisolism). In addition, we systematically searched the medical electronic databases (PubMed—MEDLINE—Web of Science—Cochrane database) for review and meta-analysis.

We retrospectively evaluated 47 corticotropinomas (16 MICs and 31 MACs) followed up for at least 12 months and up to 252 months at Hospital das Clinicas of University of Sao Paulo. The histological analysis confirmed the diagnosis of ACTH-secreting pituitary adenomas on the basis of the presence of fragmented reticulin and positive ACTH immune-staining above 50% along with the clinical characteristics of these patients.

We collected the clinical, biochemical, hormonal, MRI scan data before and after the surgery from all patients, and we constructed a database platform.

The MRI scans of the pituitary were performed in all patients using a 1.5 -T MRI system (Signa, GE Medical Systems, Milwaukee, WI, USA) with gadopentetate dimeglumine contrast-enhanced injection in a bolus before image acquisition.

The tumor lesion was considered to be a MIC when the maximum diameter size was less than 10 mm, and a tumor with a diameter equal to or greater than 10 mm was classified as a MAC.

Database

Hormonal testing before and after surgery included, basal plasmatic ACTH, early-morning serum cortisol, 24 h urinary cortisol, midnight salivary cortisol, and cortisol after a dexamethasone suppression test (DST) with low doses of dexamethasone; the samples were collected from almost all 47 patients. The remission criterion of the hypercortisolism was defined as a serum cortisol level below 2.0 µg/dL (routinely performed 4–5 days after surgery) [19]; in addition, all patients required glucocorticoid replacement due to hypocortisolism. Hydrocortisone was introduced until full adrenal axis recovery. Remission status was considered to occur if no recurrence was detected for at least 6 months after surgery. The recurrence of CD was identified when the clinical symptoms of hypercortisolism were evident and/or the 24 h urinary/midnight cortisol levels increased and/or there was a nonsuppression of cortisol after a DST (F > 1.8 µg/dL or above 50 nmol/L—after a 1 mg overnight orally administration) were detected. The clinical, hormonal, molecular and imaging data from 47 patients before surgery are described in the Supplementary data.

USP8 sequencing

Of the 47 patients, DNA was obtained from 34 fresh-frozen corticotropinomas or from 13 formalin-fixed conserved tissues using the TRIzol protocol and the fixed formalin paraffin embedded (FFPE) tissue DNA mini kit (QIAGEN), respectively. Primers for exon 14 for *USP8* amplification were designed according to fresh and formalin-fixed tissues (Supplementary data). The amplified fragments of the *hotspot* region of *USP8* were sequenced by standard protocols (Sanger) and the chromatograms were analyzed using Sequencher DNA Sequence Analysis software v4.10.1 (Gene Codes Corporation). Both DNAs extracted from FFPE and frozen tissues presented high quality for *USP8* amplification and sequencing.

Immunohistochemistry

To confirm the presence of corticotropic tumor cells from fresh and fixed formalin tissues hematoxylin and eosin staining and fragmented reticulin slides were generated for each sample with positive immunostaining for ACTH on the slides. Immunohistochemistry was performed on 20 pituitary ACTH-secreting adenomas (five with somatic *USP8* mutations), there were, formalin-fixed and sectioned into 3 µm sections that were fixed onto silanized slides. The USP8 protein stain was visualized using rabbit anti-USP8 (Abcam) at a dilution of 1:100. Two expert pathologists (#SACS and #FPF) performed a blind analysis, and both presented concordance of the data analyzed (data not shown).

Statistical analysis

In our study, three effect measures were considered in the analyses as follows: hazard ratio (HR), proportion and standardized mean difference (SMD). The SMD was calculated as the mean difference between the WT and *USP8*-mutated allele groups divided by the standard deviation of the outcomes among the patients. The pooled measurements and their 95% confidence intervals (CIs) were calculated by the inverse variance method. To combine the proportion measure (*USP8*-mutated), we performed a Freeman-Tukey double arcsine transformation to stabilize the variances. The random or fixed effects models were fitted to estimate the pooled HR, proportion and SMD. The heterogeneity was examined using the Q-statistic (Cochran's Q: the Chi square statistic for the test of heterogeneity and the measure of inconsistency given by the I^2 statistic). We considered a fixed-effect model when the measure of inconsistency was lower than 40%. Publication bias was verified by a funnel plot. Sensitivity analysis

was performed to test the influence of individual studies on the summary estimate by plotting the summary estimate in the absence of each study. All statistical analyses and forest plots were created using the R 3.5 (R Foundation for Statistical Computing) meta package and RevMan 5.0. All statistical tests were two-sided, and we adopted a significance level of 5% [20].

Results

The clinical, hormonal and radiological imaging data from the 47 patients with CD and the systematic review database of all series are summarized in the Supplementary data.

Genetic analysis of USP8 of the Brazilian Cohort

Forty-seven corticotrophin tissues were evaluated in this study, and we identified four previously described pathogenic allelic variants of *USP8* (p.Ser718Pro, p.Ser718Cys, p.Pro720Arg and p.Pro720Gln) in 11 pituitary adenomas (3 MICs, 8 MACs) and (11/47–23.4%). Of note,seven out of 17 were previously described as *USP8*-WT alleles [10]. The mutations described were: p.Ser718Pro, p.Ser718Cys, p.Pro720Arg, p.Pro720Gln, the affected amino acids (718 and 720) are highly conserved among species and all mutations are located close to the binding site of the UBPY enzyme with the 14-3-3 protein [10]. All but one of the *USP8*-mutated tumors (10/11, 90.9%) were from female patients. We observed a tendency toward more corticotropinomas with the somatic *USP8* mutations in MACs (n = 8) than in MICs (n = 3).

Systematic analysis of the ten series

The frequency of the somatic *USP8*- mutated alleles from 677 patients with CD from nine published series (630 patients) plus our cohort (47 patients) was 33% ($I^2 = 83\%$, 95% CI: 0.33 [0.24; 0.42]). Interestingly, one series of 120 Chinese patients identified the somatic *USP8*- mutated alleles in 62.5% of them by whole-exome sequencing. In our statistical analysis without this specific group, we observed in the heterogeneity from $I^2 = 83\%$ to $I^2 = 22\%$, ($I^2 = 22\%$, 95% CI: 0.29 [0.25; 0.34]) [12] (Supplementary data).

Characteristics of the Brazilian cohort

Our cohort was predominantly composed of adult Caucasian patients (41/47—87.2%, 6/47 Afro-descendants) and the female/male ratio was 6.8:1. The mean age was 31.3 years (ranging from 8 to 69 years—standard deviation [SD]: 13.25) (Table 1). On the basis of MRI scans of 47 pituitary tumors, we classified 16 MICs and 31 MACs. In the MAC group, we observed a significant difference between the ages of the genders in which males were younger than females (mean age, years, 35 ± 13.16 SD vs. mean age,

Table 1 Clinical and radiological features from 47 Brazilian patients with Cushing`s disease associated with USP8- WT alleles or with USP8-mutated alleles

Characteristics	All patients (n = 47)	*USP8*-WT (n = 36)	*USP8*-mutated (n = 11)	*p* value*
Female n (%)	41 (87.2%)	31 (75.6%)	10 (24.4%)	1
Male n (%)	6 (12.7%)	5 (83.4%)	1 (16.6%)	
Mean age ± SD, years old	31.3 ± 13.25	31.17 ± 12.28	29.20 ± 16.7	0.84
Median tumor size diameter ± SD, mm	11 ± 10.8	10.0 ± 12.45	13 ± 3.73	0.18
MACs n (%)	31 (65.9%)	23 (74.2%)	8 (25.8%)	0.50
MICs n (%)	16 (34%)	13(81.3%)	3 (18.7%)	
Remission after surgery, n (%)	21 (44.6%)	14 (66.6%)	7 (33.3%)	0.49
Mean ± SD ACTH (pg/mL)	74 ± 116.39	76 ± 42.24	69 ± 10.20	0.87
Median ± SD salivary F 00 h (µg/dL)	0.54 ± 79.06	0.58 ± 96.13	0.52 ± 0.48	0.46
Median ± SD serum F (µg/dL) after DST (1 mg)	9.6 ± 8.05	12.1 ± 8.47	7.7 ± 7.70	0.63
Mean ± SD 24 h UC	683.42 ± 842.92	721.5 ± 932.59	388 ± 433.33	**0.017**
Median ± SD serum F (MACs)	24.7 ± 12.57 (n = 31)	27 ± 13.28 (n = 23)	21.15 ± 7.48 (n = 8)	**0.048**
Median ± SD 24 h UC (MACs)	700.85 ± 622.20 (n = 31)	775 ± 677.47 (n = 23)	383.75 ± 370.43 (n = 8)	**0.016**

References: ACTH < 46 pg/mL; midnight cortisol salivary < 0.13 ug/dL; serum cortisol 8 am 5–25 ug/dL; serum cortisol after 1 mg of dexamethasone test < 1.8 ug/dL; 24 h urinary cortisol (30–300 ug/dL 24 h)

Statistical analysis: Test *U* of Mann–Whitney

n Number, *WT* Wild-type, *SD* Standard deviation, *MACs* Macrocorticotropinomas, *MICs* Microcorticotropinomas, *F* Cortisol, *UC* Urinary cortisol

Bold values are statistically significant ($p < 0.05$)

*Comparison of patients of somatic *USP8*-mutated alleles versus *USP8*-WT alleles ($p < 0.05$)

years, 15 ± 2.36 SD, respectively, $p = 0.007$—Mann–Whitney U test, Table 2).

Regarding the hormonal data, we identified a significant difference between those in the MICs and MACs only when we analyzed the 24 h urinary cortisol (UC) levels. The MICs had a fivefold increase, and the MACs had a 2.3-fold increase compared to the upper limit of the normal range of UC ($p = 0.023$), independent of *USP8*-mutated alleles (Table 2).

We also identified a significant difference between the recurrence rates in younger patients (median age: 23 years ± 5.9 SD) and those in older patients (median age 35.5 years old ± 14.17 SD), $p = 0.047$ (Table 2). In addition, the recurrence rate in patients with MICs (80%) was higher than that in patients with MACs (20%) but was independent of *USP8 status* ($p = 0.047$) (Table 2). Regarding the hormonal data of patients with the presence or absence of a somatic *USP8*-mutated allele, the plasmatic ACTH and the midnight salivary cortisol levels were similar (mean: 64.1 ± 10.2 SD vs. mean: 85.45 ± 42.2 SD, Mann–Whitney U test; median 0.58 ± 96.13 SD vs. 0.52 ± 0.42 SD, Mann–Whitney U test, respectively). Nevertheless, the 24 h UC levels were lower in the patients with the somatic *USP8*-mutated allele (mean 388 ± 433.33 SD vs. median 721.5 ± 932.59 SD, Mann–Whitney U test, $p \leq 0.017$) than in patients with the WT allele (Table 1). In the MAC group, we observed a significant difference between the median serum basal cortisol of patients with the presence or absence of somatic *USP8*-mutated alleles (median 21.15 ± 7.48 SD vs. median 27 ± 13.28 SD, respectively, [$p = 0.048$]) and among the 24 h UC levels of patients with the presence or absence of somatic *USP8*-mutated alleles (median 383.75 ± 370.43 SD vs. median 775 ± 677.47 SD, respectively, $p = 0.016$) (Table 1). The remission status after surgery was observed in 21 out of 47 patients (44%), and 7 of these 21 patients were identified to have somatic *USP8*-mutated alleles (33.3%) (Table 1). In the recurrent group, no significant differences were observed among the patients with the presence or absence of somatic *USP8*-mutated alleles.

Overall clinical data of all patients with or without somatic USP8-mutated alleles from ten series

Of the 677 patients analyzed, 514 were females (76%) and 216 of them had *USP8*-mutated alleles (32%) ($I^2 = 0\%$, 95% CI: 2.63 [1.83, 3.79]) (Supplementary statistical meta-analysis data). Of the male patients (n = 163), only 24 of them had the *USP8*-mutated allele (24%). In only two cohorts, we find a statistically significant difference between the genders [11, 12].

The age among the patients presented great variability once there were four series with pediatric (until 12 years of age) and adolescents (12–15 years of age) individuals. Therefore, the mean age ranged in patients with somatic *USP8*-mutated alleles was from 15.1 to 46 years of age, and the mean age ranged of patients with somatic *USP8*-WT alleles was from 13.1 to 53 years of age ($I^2 = 26\%$, 95% CI: 0.10 [−0.16; 0.23%], $p = 0.23$) (Supplementary statistical meta-analysis). Only one study [16] presented a statistically significant difference related to the age between the somatic *USP8*-mutated and *USP8*-WT alleles, although in this cohort no children were evaluated, and the younger adult patients showed a higher prevalence of somatic *USP8*-mutated alleles.

The MRI analysis identified 290 MICs and 238 MACs, of which, 121 (41.7%) and 73 (30.6%) were identified to have *USP8*-mutated alleles ($I^2 = 63\%$, 95% CI: 1.22 [0.77; 1.93]) (Supplementary statistical meta- analysis 5). Two series showed a significant difference, and both of them indicated

Table 2 Clinical, hormonal, radiological data and outcomes from all the 47 Brazilian patients

Characteristics	Females (n)/median age ± SD	Males (n)/median age ± SD	*p* value*
MACs (n = 31)	(27)/35 years ± 13.16	(4)/15 years ± 2.36	0.007
Characteristics	N	Number of fold of increase –24 h UC ± SD	*p* value*
MICs	16	5 ± 3.80	0.023
MACs	31	2.3 ± 8.47	
Characteristics	N	Mean age ± SD	*p* value*
Remission	16 out of 21	35.5 years ± 14.17	0.047
Recurrence	5 out of 21	23 years ± 5.9	
Characteristics	MICs	MACs	*p* value*
Recurrence rate	4/5 (80%)	1/5 (20%)	0.047

N Number of cases, *SD* Standard deviation, *MACs* Macrocorticotropinomas, *MICs* Microcorticotropinomas, *UC* Urinary cortisol

Statistical analysis: Test *U* of Mann–Whitney p* < 0.05

that the somatic *USP8*-mutated alleles were more frequent in MICs [12, 13]. The median size of tumors containing the somatic *USP8*-mutated allele was 9 mm (range: 3–29 mm), and the median size of tumors containing the somatic *USP8*-WT allele was 10 mm (range: 2–64 mm).

The hormonal patterns among the series were quite similar. The basal plasmatic ACTH was analyzed in four series, and only one of them identified a significant difference between the somatic *USP8*-WT and somatic *USP8*-mutated alleles, in which the lower levels of ACTH were associated with a somatic mutation for *USP8* ($I^2 = 35\%$, 95% CI: 0.80 [0.37; 1.22%]) (Supplementary statistical meta- analysis 6) [15]. Only two series could be systematically evaluated for 24 h UC levels according to the data. One series and our cohort presented a similarity without significant difference between the groups (mutated and WT) ($I^2 = 0\%$, 95% CI: 0.20 [−0.30; 0.70%]) (Supplementary statistical meta-analysis 7) [14].

Remission and recurrence

The number of patients who exhibited the remission criteria was 272 of the 369 patients (73.7%), and those who had the presence of the somatic *USP8*-mutated allele were 128 out of 272 (47%) patients; and 144 had the somatic *USP8*-WT allele (53%) [12, 13, 15, 16]. Among the five-series analyzed, two showed significant differences, and the somatic *USP8*-mutated allele was shown to confer a greater chance for remission ($I^2 = 40\%$, 95% CI: 1.77 [1.30; 2.40]) and ($I^2 = 40\%$, 95% CI: 1.30 [1.12; 1.50]), respectively [13, 15]. Using a systematic analysis that included four series and our cohort, the probability of remission was found to be higher in patients with the somatic *USP8*-mutated allele than in patients with the *USP8*-WT allele ($I^2 = 40\%$, 95% CI: 1.29 [1.16; 1.42] (Supplementary statistical meta- analysis 8). The risks of recurrence among the series were quite similar ($I^2 = 40\%$, 95% CI: 1.45 [0.89; 2.36]) (Supplementary statistical meta- analysis 9).

Discussion

This study aimed to improve our comprehension of patients with CD who are either carriers or were not carriers of the somatic *USP8* mutation through a systematic review of a total of 677 patients from ten series. The main concerns were as follow: the frequency of *USP8*-mutated alleles among patients with CD; the phenotype-genotype correlation; the frequency of MICs and MACs; the hormonal pattern; the rates of remission and the recurrence of hypercortisolism after transsphenoidal surgery.

The frequency of the somatic *USP8*-mutated allele in our cohort was 23.4%, which is slightly below of the median-proportion observed in all series analyzed (33%). Otherwise, one cohort calls our attention, which includes a group of 120 Chinese patients with CD, and the authors identified that 62.5% of these patients had the somatic *USP8*-mutated allele by whole-exome sequencing. Statistical analysis without this specific group (120 Chinese patients) decreased the heterogeneity and the frequency of the somatic *USP8*-mutated alleles was approximately equal among the others series. If this discrepancy is linked with to ethnicity, it needs to be better evaluated in further collaborative multicenter studies. Regarding demographic characteristics, we endorse a disproportionate frequency of somatic *USP8*-mutated alleles in females versus those carrying the *USP8*-mutated alleles in males in all series analyzed. However, only two series show the same significant difference between the female/male ratio related to the carrier and noncarrier status of the somatic *USP8*-mutated allele [11, 12]. Because Cushing's disease is more common frequent in female patients, insights about the role of estrogens in the stimulation of the tumor growth and possibly providing the environment for tumor growth to occur in patients with somatic mutations have been speculated. This hypothesis has been supported by the presence of estrogen receptors in human pituitary tumor cells and in vitro studies about the stimulatory effect of estrogens on murine corticotrophin cells [11, 21, 22]. Four series are in accordance with to the young age of clinical presentation associated with the somatic *USP8*-mutated alleles; this median difference in patient age showed a short variability between *USP8*- mutated alleles and *USP8*- WT alleles, almost 1–2 years in almost all four series. However, only Albani A et al. with a cohort of 48 adult patients observed a significant difference (median age: *USP8*-WT 53 years; *USP8*-mutated, 46 years.) [16]. When we analyzed all series regarding tumor size and aggressive behavior, we found an overlap among the MIC-*USP8*-WT × MIC-*USP8*-mutated and MAC-*USP8*-WT x MAC-*USP8*-mutated corticotropinomas [10–18]. The superimposed findings may be caused by the way the authors described the MRI data, such as the median and range of maximum size, the absolute value of size and Knosp classification and the distribution of MICs or MACs among patients with Cushing's disease [23]. Hayashi K et al. comprehensively and meticulously described all the details of approximately 60 corticotropinomas and they found that there was a smaller maximum tumor diameter size; the MICs and the tumors (Knosp 0) were frequently detected with somatic *USP8*-mutated alleles [13].

In our cohort, we observed a tendency toward more somatic *USP8* mutations in MACs than in MICs, and this finding is in accordance with the cohort series by Perez-Rivas et al, in which 52% of carriers were identified in MACs [10]. Other series have reported that, *USP8*-mutated alleles were identified 2–3 times more often in MICs than they were in MACs [12–14]; one series reported the same proportion of *USP8*-mutated alleles between MICs and MACs and the two other authors did not evaluate this issue [15].

The hormone pattern (independent of somatic *USP8*-mutated allele) of the urinary 24 h UC levels in our cohort presented a significant difference between MICs and MACs. In the MICs, the median upper limit of the assay was five times, and in the MACs, it was 2.3 times. Recently, our group observed in a cohort of 243 MICs versus 74 MACs that 38% of MICs presented more than three times the upper limit of 24 h UC levels, while the MACs presented 23% [4]. Including our cohort, only five series evaluated the UC levels, and three out of five noted significantly higher levels of UC in patients carrying the somatic *USP8*-mutated alleles. However, only two series could be systematically analyzed, and this correlation did not reach significance, this is possibly due to the small number of cases available from the other series. Using basal plasma levels of ACTH, we noted in our cohort that there patients with *USP8*-mutated alleles tended to have higher ACTH levels. Nevertheless, the controversial levels of ACTH among four out of the ten series systematically analyzed (due to the lack of standard deviation for six of the series) were described; this finding is perhaps due to bias selection among the MICs-MACs *USP8*-WT alleles and the carriers as ACTH levels are generally higher in MACs than those in MICs [4, 24–27]. The systematic analysis of the remission and recurrence rates among five out of ten series showed a significant association between the presence of the *USP8*-mutated alleles and remission compared with that of the *USP8*-WT alleles among the five series and singly in two of the series analyzed ($I^2 = 40\%$, 95% CI: 1.29 [1.16; 1.42]) [13, 15]. Interestingly, only Faucz F et al. presented a significant recurrence rate in a cohort of pediatric patients with *USP8*-mutated alleles; these data need to be analyzed in a larger cohort using the same concept of remission and recurrence [14]. Our cohort contributed with 11 patients who had the somatic *USP8* mutations (3 MICs and 8 MACs) analyzed from a unique tertiary center. In addition, we performed a retrospective systematic review of 677 published patients with Cushing's disease (528/677 could be classified as having 290 MICs and 238 MACs) and analyzed them for the somatic *USP8* mutations.

Recently, Chen J et al. reported recurrent mutations in *USP48* and in *BRAF* in WT *USP8* and both mutant genes increased the activity and transcription of the gene encoding POMC, providing a new mechanism for ACTH production in corticotropinomas. Interestingly, mutated corticotroph tumor cells were sensitive to the BRAF inhibitor, vemurafenib [28].

Our data, as well as the systematic retrospective review of Cushing's disease series associated with *USP8*-mutated alleles showed us a heterogeneous finding among the series. The correlations of the somatic genotype–phenotype were variable among the series analyzed with several drawbacks due to the lack of a systematic protocol to evaluate these patients. In conclusion, further multicenter prospective studies with a taskforce will yield more consistent information about the influence of the mainly somatic genetic cause of Cushing's disease, regarding the phenotype, response to treatment and outcome of these patients.

Acknowledgements We thank Prof. Margaret de Castro for providing us with the *USP8-mutated* control DNA, and we appreciate all the staff of Laboratorio de Investigacao Medica LIM 42; LIM 25 and Histocell Laboratório de Anatomia Patológica. We also thank Mariana Funari and Vinicius Calsavara for the genetic analysis and meta-analysis, respectively.

Funding This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) – Finance Code 001 to IQW.

Compliance with ethical standards

Conflict of interest The authors declare that have no conflicts of interest.

Ethical approval This study was approved by the Ethical Committee of the Hospital das Clinicas of the University of Sao Paulo, Brazil (#56235216.0.0000.0068) and the procedures were performed in accordance with the 1964 Helsinki Declaration and its later amendments. The signing of informed consent was required.

References

1. Lindholm J, Juul S, Jørgensen JO, Astrup J, Bjerre P, Feldt-Rasmussen U, Hagen C et al (2001) Incidence and late prognosis of cushing's syndrome: a population-based study. J Clin Endocrinol Metab 86:117–123
2. Javanmard P, Duan D, Geer EB (2018) Mortality in patients with endogenous cushing's syndrome. Endocrinol Metab Clin North Am 47:313–333
3. Dekkers OM, Biermasz NR, Pereira AM, Roelfsema F, van Aken MO, Voormolen JH, Romijn JA (2007) Mortality in patients treated for Cushing's disease is increased, compared with patients treated for nonfunctioning pituitary macroadenoma. J Clin Endocrinol Metab 92:976–981
4. Machado MC, Alcantara AE, Pereira AC, Cescato VA, Castro Musolino NR, de Mendonça BB, Bronstein MD et al (2016) Negative correlation between tumour size and cortisol/ACTH ratios in patients with cushing's disease harbouring microadenomas or macroadenomas. J Endocrinol Invest 39:1401–1409
5. Nieman LK, Biller BM, Findling JW, Murad MH, Newell-Price J, Savage MO, Tabarin A et al (2015) Treatment of cushing's syndrome: an endocrine society clinical practice guideline. J Clin Endocrinol Metab 100:2807–2831
6. Bertagna X, Guignat L (2013) Approach to the cushing's disease patient with persistent/recurrent hypercortisolism after pituitary surgery. J Clin Endocrinol Metab 98:1307–1318
7. Feelders RA, Hofland LJ (2013) Medical treatment of cushing's disease. J Clin Endocrinol Metab 98:425–438
8. Sbiera S, Deutschbein T, Weigand I, Reincke M, Fassnacht M, Allolio B (2015) The new molecular landscape of cushing's disease. Trends Endocrinol Metab 26:573–583
9. Reincke M, Sbiera S, Hayakawa A, Theodoropoulou M, Osswald A, Beuschlein F, Meitinger T et al (2015) Mutations in the

deubiquitinase gene USP8 cause cushing's disease. Nat Genet 47:31–38
10. de Araújo LJ, Lerario AM, de Castro M, Martins CS, Bronstein MD, Machado MC, Trarbach EB et al (2017) Transcriptome analysis showed a differential signature between invasive and non-invasive corticotrophinomas. Front Endocrinol 8:55
11. Perez-Rivas LG, Theodoropoulou M, Ferraù F, Nusser C, Kawaguchi K, Stratakis CA, Faucz FR et al (2015) The Gene of the ubiquitin-specific protease 8 is frequently mutated in adenomas causing cushing's disease. J Clin Endocrinol Metab 100:E997–1004
12. Ma ZY, Song ZJ, Chen JH, Wang YF, Li SQ, Zhou LF, Mao Y et al (2015) Recurrent gain-of-function USP8 mutations in cushing's disease. Cell Res 25:306–317
13. Hayashi K, Inoshita N, Kawaguchi K, Ibrahim Ardisasmita A, Suzuki H, Fukuhara N, Okada M et al (2016) The USP8 mutational status may predict drug susceptibility in corticotroph adenomas of cushing's disease. Eur J Endocrinol 174:213–226
14. Faucz FR, Tirosh A, Tatsi C, Berthon A, Hernández-Ramírez LC, Settas N, Angelousi A et al (2017) Somatic USP8 gene mutations are a common cause of pediatric cushing disease. J Clin Endocrinol Metab 102:2836–2843
15. Losa M, Mortini P, Pagnano A, Detomas M, Cassarino MF, Pecori Giraldi F (2019) Clinical characteristics and surgical outcome in USP8-mutated human adrenocorticotropic hormone-secreting pituitary adenomas. Endocrine 63:240–246
16. Albani A, Pérez-Rivas LG, Dimopoulou C, Zopp S, Colón-Bolea P, Roeber S, Honegger J et al (2018) The USP8 mutational status may predict long-term remission in patients with cushing's disease. Clin Endocrinol 89(4):454–458
17. Weigand I, Knobloch L, Flitsch J, Saeger W, Monoranu CM, Höfner K, Herterich S et al (2019) Impact of USP8 gene mutations on protein deregulation in cushing's disease. J Clin Endocrinol Metab 104(7):2535–2546
18. Ballmann C, Thiel A, Korah HE, Reis AC, Saeger W, Stepanow S, Köhrer K et al (2018) Mutations in pituitary cushing adenomas-targeted analysis by next-generation sequencing. J Endocr Soc 2:266–278
19. Machado MC, Fragoso MCBV, Moreira AC, Boguszewski CL, Vieira Neto L, Naves LA, Vilar L et al (2018) A review of cushing's disease treatment by the Department of Neuroendocrinology of the Brazilian Society of Endocrinology and Metabolism. Arch Endocrinol Metab 62:87–105
20. Higgins JP, Thompson SG, Deeks JJ, Altman DG (2003) Measuring inconsistency in meta-analyses. BMJ 327:557–560
21. Chaidarun SS, Swearingen B, Alexander JM (1998) Differential expression of estrogen receptor-beta (ER beta) in human pituitary tumors: functional interactions with ER alpha and a tumor-specific splice variant. J Clin Endocrinol Metab 83:3308–3315
22. Oomizu S, Honda J, Takeuchi S, Kakeya T, Masui T, Takahashi S (2000) Transforming growth factor-alpha stimulates proliferation of mammotrophs and corticotrophs in the mouse pituitary. J Endocrinol 165:493–501
23. Knosp E, Steiner E, Kitz K, Matula C (1993) Pituitary adenomas with invasion of the cavernous sinus space: a magnetic resonance imaging classification compared with surgical findings. Neurosurgery 33(4):610–617
24. Katznelson L, Bogan JS, Trob JR, Schoenfeld DA, Hedley-Whyte ET, Hsu DW, Zervas NT et al (1998) Biochemical assessment of cushing's disease in patients with corticotroph macroadenomas. J Clin Endocrinol Metab 83:1619–1623
25. Woo YS, Isidori AM, Wat WZ, Kaltsas GA, Afshar F, Sabin I, Jenkins PJ et al (2005) Clinical and biochemical characteristics of adrenocorticotropin-secreting macroadenomas. J Clin Endocrinol Metab 90:4963–4969
26. Selvais P, Donckier J, Buysschaert M, Maiter D (1998) Cushing's disease: a comparison of pituitary corticotroph microadenomas and macroadenomas. Eur J Endocrinol 138:153–159
27. Losa M, Barzaghi RL, Mortini P, Franzin A, Mangili F, Terreni MR, Giovanelli M (2000) Determination of the proliferation and apoptotic index in adrenocorticotropin-secreting pituitary tumors: comparison between micro- and macroadenomas. Am J Pathol 156:245–251
28. Chen J, Jian X, Deng S, Ma Z, Shou X, Shen Y, Zhang Q et al (2018) Identification of recurrent USP48 and BRAF mutations in Cushing's disease. Nat Commun 9:3171

Affiliations

Ingrid Quevedo Wanichi[1] · Beatriz Marinho de Paula Mariani[1] · Fernando Pereira Frassetto[2] · Sheila Aparecida Coelho Siqueira[2] · Nina Rosa de Castro Musolino[3] · Malebranche Berardo Carneiro Cunha-Neto[3] · Gilberto Ochman[3] · Valter Angelo Sperling Cescato[3] · Marcio Carlos Machado[4,5,6] · Ericka Barbosa Trarbach[6] · Marcello Delano Bronstein[4,6] · Maria Candida Barisson Villares Fragoso[1,7,8]

1 Laboratório de Hormônios e Genética Molecular (LIM/42) do Hospital das Clinicas da Disciplina de Endocrinologia e Metabologia da Faculdade de Medicina da, Universidade de São Paulo, Avenida Dr.Enéas de Carvalho Aguiar, 155 - 2 andar bloco 6, São Paulo CEP 05403900, Brazil

2 Departamento de Patologia do Hospital das Clinicas da Faculdade de Medicina da, Universidade de São Paulo, São Paulo, Brazil

3 Unidade de Neuroendocrinologia da Divisão de Neurocirurgia Funcional, Instituto de Psiquiatria do Hospital das Clinicas da Faculdade de Medicina da Universidade de São Paulo, São Paulo, Brazil

4 Unidade de Neuroendocrinologia da Disciplina de Endocrinologia e Metabologia da Faculdade de Medicina da, Universidade de São Paulo, São Paulo, Brazil

5 Endocrinology Service, AC Camargo Cancer Center, São Paulo, Brazil

6 Laboratorio de Endocrinologia Celular e Molecular (LIM/25) do Hospital das Clinicas da Disciplina de Endocrinologia e Metabologia da Faculdade de Medicina da, Universidade de São Paulo, São Paulo, Brazil

7 Unidade de Suprarrenal da Disciplina de Endocrinologia e Metabologia da Faculdade de Medicina da, Universidade de São Paulo, São Paulo, Brazil

8 Clinica de Bases do Instituto do Câncer do Estado de São Paulo, ICESP, São Paulo, Brazil