

Evaluation of Hypothalamic–Pituitary–Adrenal Axis by the GHRP2 Test: Comparison With the Insulin Tolerance Test

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Context: GH-releasing peptide 2 (GHRP2) stimulates the hypothalamic–pituitary–adrenal axis (HPA) through the GH secretagogue receptor (GHSR) in the hypothalamus, in which ghrelin is a natural ligand. Therefore, the GHRP2 test (GHRP2T) could be used instead of the insulin tolerance test (ITT).

Objective: Can the GHRP2T replace the ITT for evaluation of HPA?

Design: The present retrospective study analyzed the clinical features and laboratory data from 254 patients admitted for evaluation of hypopituitarism who underwent both GHRP2T and ITT. We analyzed the association between the maximum cortisol level (Fmax) during both tests. Adrenocortical insufficiency was diagnosed by ITT. The suitability of GHRP2T was examined using the receiver operating characteristic curve.

Results: A strong correlation was found between Fmax measured using both tests ($r = 0.777$, $P < 0.0001$). However, the sensitivity (64%) and specificity (79%) showed that the GHRP2T was not suitable for clinical use. Various factors influenced the correlation, probably through their effects on ghrelin and/or GHSR, including functional adenoma ($P < 0.05$) and sex ($P < 0.05$). No substantial correlation was found between Fmax measured using both tests in patients with prolactinoma ($n = 30$). The exclusion of patients with functional adenoma revealed no factors that affected the association in male patients; however, age and menstruation significantly influenced it in female patients ($P < 0.05$). Analysis of the data from male subjects without functional adenoma ($n = 104$) showed high sensitivity (95%) and specificity (85%) for the GHRP2T.

Conclusion: ITT can be substituted with GHRP2T for assessment of HPA in male patients free of functional adenoma.

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Freeform/Key Words: gender, ghrelin, growth hormone-releasing peptide 2 test, hypothalamic, pituitary-adrenal axis, insulin tolerance test

The hypothalamic–pituitary–adrenal (HPA) axis is the most critical system for life, and secondary adrenocortical insufficiency is associated with a poor prognosis and low quality of life [1, 2]. The insulin tolerance test (ITT) is the reference standard for evaluation of this axis

Abbreviations: BMI, body mass index; CRH, corticotropin-releasing hormone; CV, coefficient of variation; Fmax, maximum cortisol level; FT4, free T4; GHD, GH deficiency; GHRP2, GH-releasing peptide 2; GHRP2T, GH-releasing peptide 2 test; GHS, GH secretagogue; GHSR, GH secretagogue receptor; HPA, hypothalamic–pituitary–adrenal axis; PVN, paraventricular nucleus; ROC, receiver operating characteristic.

[3, 4], which can be conducted by an experienced endocrinologist. Because the ITT involves the induction of severe hypoglycemia (blood glucose level of <40 mg/dL), patients can become uncomfortable and will require close observation during the test. Moreover, ITT is contraindicated in patients with ischemic heart disease and those with epilepsy. In clinical practice, the corticotropin-releasing hormone (CRH) test or synacthen test is used as an alternative test for ITT. However, discrepancies between these two tests and the ITT have been reported in some cases, mainly because the two tests are not designed to evaluate hypothalamic function [5, 6]. Therefore, a need exists for another low-risk and accurate test for evaluation of the HPA axis.

The GH-releasing peptide 2 (GHRP2), one of the GH secretagogues (GHSs) for the evaluation of the capacity of GH secretion in Japan, is also known to stimulate HPA axis via the GHS receptor (GHSR) in the hypothalamus [7, 8]. Therefore, it is possible that the GHRP2 test (GHRP2T) could be used as a substitute for the ITT. Kano *et al.* [9] compared the GHRP2T and ITT in 15 patients suspected of hypopituitarism and concluded that the GHRP2T was a suitable alternative to the ITT for the assessment of HPA axis function. In contrast, the study by Arimura *et al.* [10] in 47 patients suspected of having a hypothalamic–pituitary disorder concluded that the GHRP2T did not have the same predictive value as the ITT. These results suggest that more studies of larger population samples are needed to confirm the usefulness of the GHRP2T as a substitute for the ITT and to explain the differences between the two tests.

The present retrospective study was designed to further compare the GHRP2T and ITT in the evaluation of HPA. Thus, we compared the clinical features and laboratory data from 254 patients who had undergone both the ITT and the GHRP2T. We analyzed the correlation between the maximum cortisol (Fmax) values recorded during the two tests and the factors that influenced this correlation using regression models.

1. Subjects and Methods

A. Subjects

The subjects were recruited from among all 258 patients who had been admitted previously to the Osaka University Hospital from January 2009 to April 2015 and had undergone the ITT and the GHRP2T for evaluation of pituitary function. We excluded three patients with Cushing disease because of an apparent cortisol excess and one patient with prolactinoma because of a lack of GH data during the ITT. Thus, the study included 254 patients. Table 1 lists the characteristics and laboratory data of the subjects, including age, sex, body mass index (BMI), underlying disease, sexual status (testosterone in the morning for males and menstruation status for females), and free T4 (FT4) level, which were collected from the medical records. The underlying diseases included nonfunctional adenoma in 118 patients (46.2%) and functional adenoma in 38 patients (30 with prolactinoma, 5 with acromegaly, 3 with TSH-producing tumor). Thirty-three patients were admitted for an assessment of miscellaneous conditions, including idiopathic (n = 17), suspicion of secondary adrenocortical insufficiency by medication (n = 5), amenorrhea (n = 3), short stature (n = 2), menstrual irregularity (n = 1), meningioma (n = 1), suspicion of pituitary apoplexy (n = 1), body weight loss (n = 1), primary hypoparathyroidism (n = 1), and hemochromatosis (n = 1). The human ethics committee of Osaka University approved the study protocol (approval no. 17080) and was performed according to the Declaration of Helsinki.

B. Procedures for ITT and GHRP2T

ITT was performed as follows. At 8 AM, after an overnight fast, a cannula was inserted into the forearm vein. After 30 minutes of bed rest, 0.1 U/kg of regular insulin (Eli Lilly Japan KK, Kobe, Japan) was injected IV. Venous blood samples were withdrawn before and at 15, 30, 45, 60, 90, and 120 minutes after IV administration of insulin for measurement of glucose,

Table 1. Clinical Characteristics and Biochemical Data of Enrolled Patients

Characteristic	Value
Sex	
Male	112
Female	142
Age, y	47.1 ± 16.9
BMI, kg/m ²	23.1 ± 3.8
Fmax using ITT, µg/dL	15.9 ± 6.5
Fmax using GHRP2T, µg/dL	14.6 ± 6.2
Secondary adrenocortical insufficiency, n	154 (60.6)
GHD, n	137 (53.9)
FT4, ng/dL	0.9 (0.8–1.1)
Sexual status	
Dysfunction	128
Normal	126
Underlying disease	
Nonfunctional adenoma	118
Functional adenoma	38
Rathke cleft cyst	25
Craniopharyngioma	17
Empty sella	12
Hypophysitis	5
Germinoma	3
Isolated ACTH deficiency	3
Other	33

Data presented as mean ± SD, n (%), or median (IQR, first and third quartiles).
Abbreviation: GHD, GH deficiency.

cortisol, and GH. Another dose of insulin was administered to induce hypoglycemia, which was defined as a blood glucose level of <40 mg/dL and/or one-half of the baseline blood glucose level.

The GHRP2T was conducted as follows. At 8 AM, after an overnight fast, a cannula was inserted into the forearm vein. After 30 minutes of bed rest, 100 µg of GHRP2 (Kaken Seiyaku, Tokyo, Japan) was injected IV. Venous blood samples were withdrawn before and at 15, 30, 45, and 60 minutes after the administration of GHRP2.

The two tests were performed within 4 days in no particular order. Secondary adrenocortical insufficiency and GH deficiency (GHD) were diagnosed by ITT (secondary adrenocortical insufficiency, peak serum cortisol level <18 µg/dL; GH deficiency, peak serum GH level <3 ng/mL).

C. Laboratory Tests

Blood samples were collected after overnight fast. The serum cortisol level was measured using a chemiluminescent enzyme immunoassay (Access cortisol kit, Beckman Coulter, Tokyo, Japan). This kit has a sensitivity of 0.4 µg/dL, with an intra-assay coefficient of variation (CV) of <4.3% and interassay CV of <5.9%, with a normal range of 4.5 to 24.5 µg/dL. The serum GH level was measured using a chemiluminescent enzyme immunoassay kit (Access hGH kit, Beckman Coulter), with a sensitivity of 0.002 ng/mL and CV for the inter- and intra-assays of <20% and <15%, respectively, with a normal range of <2.7 ng/mL. The serum total testosterone level was measured using a chemiluminescent enzyme immunoassay (Access Testosterone kit, Beckman Coulter), with a sensitivity of 0.1 ng/mL, intra- and interassay CV of <10%, and normal range of 2.70 to 10.3 ng/mL. The serum FT4 level was measured using an enzyme immunoassay (TOSOH-II ST AIA-PACK FT4, Tosoh Bioscience Inc., Tokyo, Japan) with a CV for the intra- and interassays of <3.9% and a normal range of 0.8 to 1.6 ng/dL.

D. Statistical Analysis

Data from the clinical characteristics and laboratory tests are presented as the mean \pm SD. However, data for FT4 showed a skewed distribution and are, therefore, expressed as the median and interquartile range (IQR; first and third quartiles). The Pearson correlation coefficient was used to assess the relationship between the Fmax and other variables. Receiver operating characteristic (ROC) analysis was used to assess the predictive cutoff point of Fmax in the GHRP2T and sensitivity and specificity for normal adrenal function diagnosed using the ITT. FT4 was log transformed for normal distribution. Normal sexual status was defined as male by a normal testosterone level and female by menstruation or hormone replacement therapy. None of the male patients had a testosterone level greater than the normal range. Sex, underlying disease, GHD, and sexual status were categorical variables and all others were continuous variables. Fmax measured in the GHRP2T was the dependent variable in the regression analysis, and Fmax measured in the ITT, age, sex, BMI, underlying disease (functional adenoma or not), GHD, LogFT4, and sexual status were the independent variables. In all subjects, we tested for potential interactions by adding interaction terms (each variable \times maximum cortisol in ITT) to the regression model. Regarding female subjects free of functional adenoma, age and sexual status were tested separately because these factors are related to each other. All *P* values were two-sided, and *P* < 0.05 denoted the presence of a statistically significant difference. JMP Pro software, version 10.0.2, for Windows (SAS Institute, Cary, NC) and R, version 3.1.0 (R Development Core Team, Vienna, Austria) were used for statistical analysis.

2. Results

A. Characteristic of Subjects

The study included 254 patients (112 males and 142 females) with a mean age of 47.1 ± 16.9 years and BMI of 23.1 ± 3.8 kg/m² (Table 1). The mean Fmax was 15.9 ± 6.5 and 14.6 ± 6.2 μ g/dL in the ITT and GHRP2T, respectively. The number of patients with secondary adrenocortical insufficiency or GH deficiency diagnosed using the ITT was 154 (60.6%) and 137 (53.9%), respectively (secondary adrenocortical insufficiency, peak serum cortisol level <18 μ g/dL; GH deficiency, peak serum GH level <3 ng/mL). The median FT4 was 0.9 ng/dL (IQR, 0.8 to 1.1). Approximately one-half of the patients (*n* = 126; 49.6%) were considered to have normal sexual status. No adverse reactions were recorded during the two tests.

B. Correlation Between Fmax Using GHRP2T and Fmax Using ITT

A strong and statistically significant relationship between the Fmax measured in GHRP2T and that recorded in ITT was found (Fig. 1A; *r* = 0.777, 95% CI, 0.723 to 0.821; *P* < 0.001). Figure 1B shows the diagnostic power of Fmax measured in the GHRP2T as determined by ROC analysis. The sensitivity and specificity of GHRP2T do not reach the useful level of clinical practice (64% and 79%, respectively; area under the curve, 0.802; 95% CI, 0.748 to 0.855). The effect of the interaction of various factors on this relationship is summarized in Table 2. Functional adenoma and sex were statistically significant interaction factors (*P* < 0.05). The correlation coefficient (*r*) in males (*r* = 0.858; 95% CI, 0.799 to 0.900) was apparently better than that in females (*r* = 0.659; 95% CI, 0.555 to 0.743; Supplemental Fig. 1). Although the BMI tended to be an interaction factor (*P* = 0.079), no apparent difference was found on the correlation coefficient dividing two or four groups according to the median or quantile of BMI (data not shown).

C. Effect of Underlying Disease on Fmax–GHRP2T to Fmax–ITT Correlation

In patients with functional adenoma, the correlation between Fmax–GHRP2T and Fmax–ITT was still statistically significant, although weaker (*r* = 0.375; 95% CI, 0.063 to 0.620; *P* < 0.05).

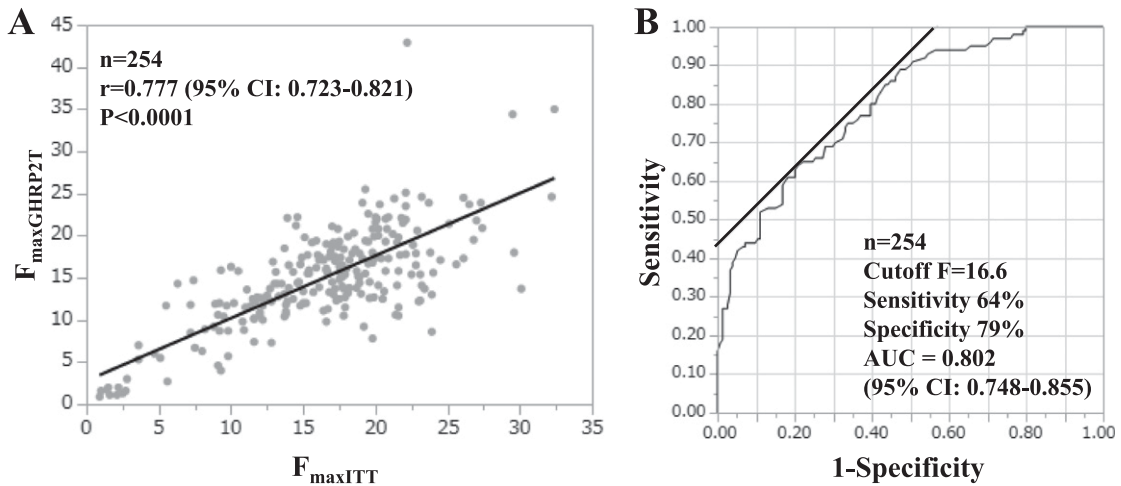


Figure 1. (A) Correlation between Fmax measured by GHRP2T ($F_{\max\text{GHRP2T}}$) and ITT ($F_{\max\text{ITT}}$). Pearson correlation coefficient was used to assess the relationship between $F_{\max\text{GHRP2T}}$ and $F_{\max\text{ITT}}$. (B) ROC analysis assessed the predictive cutoff value of $F_{\max\text{GHRP2T}}$, sensitivity, and specificity for normal adrenal function diagnosed using the ITT. AUC, area under the curve.

Analysis of the data from the 30 patients with prolactinoma showed no statistically significant relationship ($r = 0.020$; 95% CI, -0.343 to 0.377 ; $P = 0.918$; Fig. 2A). A similar finding was observed in the 5 patients with acromegaly (Fig. 2B). Data from the three patients with a TSH-producing tumor were not analyzed owing to the small sample number.

D. Fmax–GHRP2T to Fmax–ITT Correlation in Male and Female Patients Without Functional Adenoma

We also analyzed the correlation in male and female patients who had been confirmed to have no functional adenoma. A statistically significant and strong relationship was found between Fmax–GHRP2T and Fmax–ITT in males ($n = 104$; $r = 0.879$; 95% CI, 0.826 to 0.916 ; $P < 0.001$; Fig. 3A). ROC analysis showed clinically acceptable diagnostic power for GHRP2T in males without functional adenoma (sensitivity, 95%; specificity, 85%; area under the curve, 0.947 ; 95% CI, 0.910 to 0.985 ; Fig. 3B). The predictive cutoff value determined by ROC analysis was $15.8 \mu\text{g/dL}$ for males without functional adenoma. Simultaneously, we did not identify any statistically significant interaction factors in males (Table 3). Secondary adrenocortical insufficiency could be completely ruled out in male patients with an Fmax–GHRP2T of $>19.8 \mu\text{g/dL}$.

Table 2. Interaction Terms Between Maximum Cortisol Measured Using ITT and GHRP2T in the Regression Model

Interaction Term	Regression Coefficient β	P Value
Functional adenoma \times $F_{\max\text{ITT}}$	-0.121	< 0.05
Sex \times $F_{\max\text{ITT}}$	-0.087	< 0.05
BMI \times $F_{\max\text{ITT}}$	-0.078	0.079
Age \times $F_{\max\text{ITT}}$	-0.038	0.422
GHD \times $F_{\max\text{ITT}}$	-0.068	0.161
LogFT4 \times $F_{\max\text{ITT}}$	-0.019	0.642
Sexual status \times $F_{\max\text{ITT}}$	0.062	0.206

Fmax in GHRP2T was the dependent variable; maximum cortisol in ITT ($F_{\max\text{ITT}}$), age, sex, BMI, underlying disease (functional adenoma or not), GHD, LogFT4, and sexual status were independent variables. We tested the potential interactions by adding the interaction terms (each variable \times $F_{\max\text{ITT}}$) to the regression model. Abbreviation: $F_{\max\text{ITT}}$, Fmax value using the ITT.

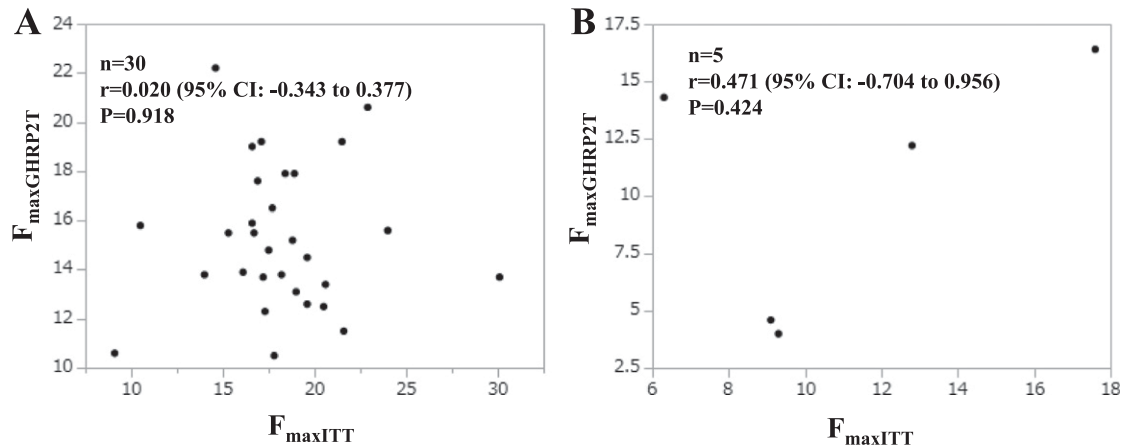


Figure 2. Correlation between Fmax measured using the GHRP2T ($F_{\max\text{GHRP2T}}$) and ITT ($F_{\max\text{ITT}}$) in patients with functional adenoma. Pearson correlation coefficient was used to assess the relationship between $F_{\max\text{GHRP2T}}$ and $F_{\max\text{ITT}}$ of patients with (A) prolactinoma ($n = 30$) and (B) acromegaly ($n = 5$).

(sensitivity, 46%; specificity, 100%) and was confirmed in male patients with an Fmax of $<12.8 \mu\text{g/dL}$ (sensitivity, 100%; specificity, 66%). However, in females, the correlation between Fmax–GHRP2T and Fmax–ITT was statistically significant but not strong ($n = 112$; $r = 0.692$; 95% CI, 0.581 to 0.778; $P < 0.001$). Age and sexual status were statistically significant interactive factors (Table 3).

3. Discussion

The main finding of the present study was that the GHRP2T can be used instead of the ITT in male patients without functional adenoma for the assessment of the HPA. Based on our data, secondary adrenocortical insufficiency can be confirmed or ruled out in male patients with an Fmax of <12.8 to $>19.8 \mu\text{g/dL}$ using the GHRP2T, respectively. The results of two previous similar studies were controversial with regard to the correlation between the two tests [9, 10].

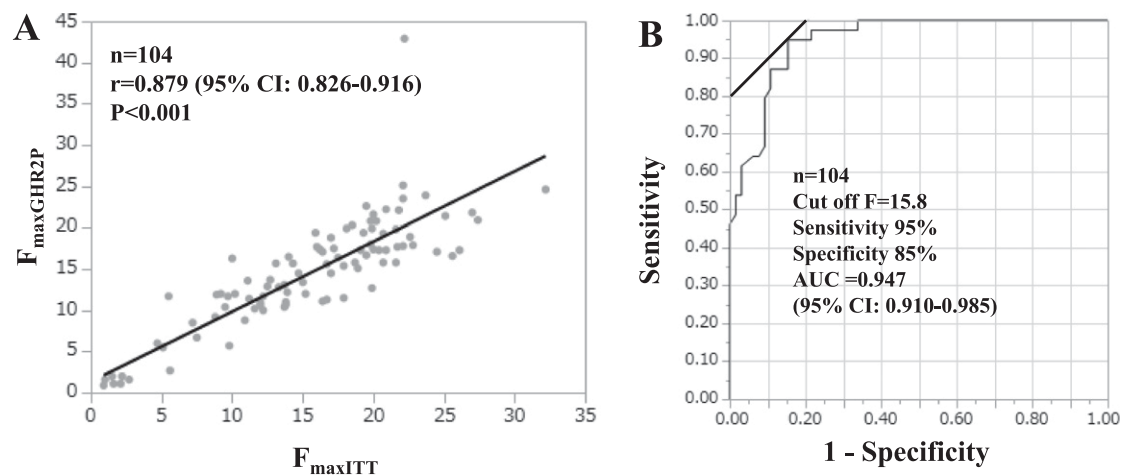


Figure 3. (A) Correlation between maximum cortisol level measured by GHRP2T ($F_{\max\text{GHRP2T}}$) and ITT ($F_{\max\text{ITT}}$) for male patients free of functional adenoma. Pearson correlation coefficient was used to assess the relationship between $F_{\max\text{GHRP2T}}$ and $F_{\max\text{ITT}}$ in male patients without functional adenoma ($n = 104$). (B) ROC analysis assessed the predictive cutoff value of $F_{\max\text{GHRP2T}}$, sensitivity, and specificity for normal adrenal function diagnosed using the ITT in this group. AUC, area under the curve.

Table 3. Sex-Specific Interaction Factors in Patients Free of Functional Adenoma

Interaction Term	Regression Coefficient β	P Value
Male patients (n = 104)		
BMI \times F _{maxITT}	-0.051	0.332
Age \times F _{maxITT}	0.010	0.864
GHD \times F _{maxITT}	-0.006	0.930
LogFT4 \times F _{maxITT}	-0.006	0.914
Sexual status \times F _{maxITT}	0.004	0.946
Female patients (n = 112)		
Age		
BMI \times F _{maxITT}	-0.116	0.161
Age \times F _{maxITT}	-0.139	<0.05
GHD \times F _{maxITT}	-0.033	0.659
logFT4 \times F _{maxITT}	-0.054	0.359
Sexual status		
BMI \times F _{maxITT}	-0.112	0.196
Sexual status \times F _{maxITT}	0.154	< 0.05
GHD \times F _{maxITT}	-0.040	0.600
LogFT4 \times F _{maxITT}	-0.077	0.214

In male patients, the Fmax in GHRP2T was the dependent variable, and F_{maxITT}, age, BMI, GHD, LogFT4, and sexual status were the independent variables. In female patients, the Fmax in GHRP2T was the dependent variable, and F_{maxITT}, BMI, GHD, LogFT4, age, and sexual status were the independent variables.

Abbreviation: F_{maxITT}, Fmax value using the ITT.

However, the female subjects formed ~50% (7 of 15) or >50% (29 of 47) of the subjects in those two studies, and they did not include sufficient numbers of patients to allow for a proper analysis of the interaction factors. In contrast, we analyzed the data of 254 patients and were able to identify two interaction factors (*i.e.*, functional adenoma and sex) in the two tests.

Different mechanisms operate in the initial part of stimulation of the HPA in the two tests. GHSs, including GHRP2, are thought to stimulate directly the parvocellular paraventricular nucleus (PVN) in the hypothalamus through the GHSR [11, 12]. In the PVN, CRH and arginine vasopressin neurons are activated, and such activation enhances ACTH secretion from the pituitary [13, 14]. With regard to the ITT, the mechanism that stimulates the HPA is still not completely understood. Hypoglycemia is sensed in the ventromedial nucleus of the hypothalamus [15]. Signals from this nucleus are thought to activate the PVN and promote ACTH secretion from the pituitary [16]. The mechanism involved in HPA activation during the ITT is independent of GHSs or GHSR, because ghrelin does not change during the ITT [17, 18]. Such differences in the mechanism could be the reason for our recommendation that GHRP2T can be used as a substitute test (instead of ITT) for limited types of patients.

In female mice, estradiol has been reported to attenuate the orexigenic action of ghrelin [19]. However, GHSR gene expression is significantly greater in the lateral hypothalamic area of female rats compared with male rats [20]. Other studies have reported that estradiol benzoate upregulated the mRNA expression levels of GHSR in the arcuate nucleus in fasting and fed female mice [21]. These effects of ghrelin and the different expression levels of GHSR mRNA might explain why sex was a statistically significant interaction factor in our study. A statistically significant difference was found in the background parameters between the male and female patients, such as age (50.4 ± 16.8 vs 44.5 ± 16.5 years; $P < 0.01$), BMI (23.9 ± 3.1 vs 22.4 ± 4.2 kg/m²; $P < 0.001$), functional adenoma (7.1% vs 21%; $P < 0.01$), GHD (67% vs 44%; $P < 0.001$), and normal sexual status (60% vs 42%; $P < 0.01$). The potential interactions between these parameters were simultaneously tested by the regression model, and sex was a statistically significant independent interaction factor.

Our results showed no interactive effect for age and low testosterone levels on the correlation between Fmax–GHRP2T and Fmax–ITT in male patients free of functional adenoma. In this regard, Iranmanesh *et al.* [22] reported that 5 α -dihydrotestosterone reduced GHRP2-induced cortisol secretion in healthy men. This discrepancy can be explained by the

different concentrations of this hormone. In their study [22], 5 α -dihydrotestosterone was 2.6-fold that of the control, an indication of hormone excess [23]. In our study, some patients showed a hormonal deficiency but none had hormone excess. In the study by Iranmanesh *et al.* [22], age was also reported to correlate positively with Fmax after GHRP2 stimulation. This relationship was also reported after CRH and CRH/ arginine vasopressin stimulation [24, 25]. The reason for such correlation was explained by the hypersensitivity of the HPA at the hypothalamus (PVN) and pituitary level in elderly subjects [26, 27]. The route beyond the PVN is common for both the ITT and the GHRP2T. In our study, the effect of aging on the sensitivity was canceled out because we compared the results of the two tests in all subjects.

Another interactive factor we detected in the present study was functional adenoma, with excess levels of prolactin, GH, and TSH. In particular, no substantial Fmax–GHRP2T/Fmax–ITT correlation in patients with prolactinoma (n = 30). However, only little information was available for ghrelin in those patients with prolactinoma. A recent study reported the presence of significantly greater levels of ghrelin in patients with prolactinoma than in the controls [28]. However, in these patients, no statistically significant correlation was found between the ghrelin levels and the percentage of body fat, although a negative correlation was found in healthy subjects. These results suggest the disturbance of ghrelin regulation by prolactin. Hyperprolactinemia is known to increase dopamine secretion from the hypothalamus, which acts as a negative feedback mechanism for prolactin [29]. Furthermore, a recent study demonstrated colocalization of the dopamine receptor and GHSR in the hypothalamus and reported that a dopamine agonist attenuated the orexigenic effect of ghrelin [30]. In our study, the presence of dopamine excess in the hypothalamus could have interfered with the results of the GHRP2T in the patients with prolactinoma.

The present study had a limitation. We did not include healthy control subjects. Ethically, the ITT should be avoided as much as possible because of the associated risk of hypoglycemia. The underlying diseases and other hormonal deficiencies in our patients could have influenced the results of each test. However, we compared the results of the two tests in each patient, which allowed us to cancel out the possible effects of these factors.

In conclusion, the present study has demonstrated that functional adenoma and sex of the patient influenced the correlation between the GHRP2T and ITT results when evaluating the HPA. The GHRP2T can be used instead of the ITT for evaluation of the HPA with clinical accuracy in male patients without functional adenoma.

Acknowledgments

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