ORIGINAL RESEARCH

Hypersecretion of the α -subunit in clinically non-functioning pituitary adenomas: Diagnostic accuracy is improved by adding α -subunit/gonadotropin ratio to levels of α -subunit

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Abstract

Background: *In vitro*, the majority of clinically non-functioning pituitary adenomas (NFPAs) produce gonadotropins or their α -subunit; however, *in vivo*, measurements of α -subunit levels may not accurately detect the hypersecretion of the α -subunit.

Aim: We wanted to estimate the reference intervals and decision limits for gonadotropin α -subunit, LH and FSH levels, and α -ratio (α -subunit/LH+FSH), especially taking into consideration patient gender and menstrual status. Furthermore, we wanted to examine if the diagnostic utility of α -subunit hypersecretion was improved when the α -ratios, rather than simply the α -subunit levels, were measured in patients with NFPAs.

Material and Methods: Reference intervals for gonadotropin α -subunit serum levels and α -ratios were established in 231 healthy adults. The estimated cut-off limits were applied to 37 patients with NFPAs. Gonadotropin α -

subunit, LH and FSH levels were measured and α -ratios were calculated.

Results: In healthy adults, the cut-offs for α -subunit levels were significantly different between men and pre- and postmenopausal women: the cut-offs were 1.10, 0.48 and 3.76 IU/l, respectively. Using these estimated cut-offs, increased α -subunit levels were identified in 10 out of 37 (27%) patients with NFPAs. By adding α -ratio, in combination with α -subunit levels, 23 patients out of 37 (62%) were identified as having elevated α -subunit hypersecretion, and 22 out of these 23 patients (96%) had increased α -ratios. One premenopausal patient out of 23 had elevated α -subunit level but a normal α -ratio.

Conclusion: Our data suggest that adding the simple calculation of α -ratio improves the ability of detecting gonadotropin α -subunit hypersecretion and thereby identifying patients with NFPAs.

It is now recognized that most clinically non-functioning pituitary adenomas (NFPAs) express gonadotropin hormones or the hormone subunits, both *in vitro*, and *in vivo*.¹ *In vitro* studies and immunocytochemical analyses have demonstrated that most NFPAs synthesize glycoprotein hormones, gonadotrophins, and uncombined subunits - primarily the free α -subunit.²⁻⁴ The gonadotrophin α -subunit combines with specific β -subunits to form the three pituitary glycoprotein hormones: luteinizing hormone (LH), follicle-stimulating hormone (FSH) and thyroid-stimulating hormone (TSH) as well as human chorionic gonadotrophin (hCG) of placental origin. The α -subunit consists of 92 amino acid residues and is encoded by a single gene. The α -subunit is common to all the glycoprotein hormones, whereas the β -subunit confer functional specificity of the hormones. Importantly, only the dimers possess biologic activity.⁵ The subunits can be found in the unassociated form both within the healthy pituitary gland and in the circulation. NFPAs are rarely associated with increased levels of dimeric LH or FSH, however, they may be characterized by increased levels of uncombined subunits – especially the free α -subunit.³ The accurate detection of α -subunit in patients with NFPAs may therefore be of clinical value in diagnosis or treatment of NFPAs. In the past, there have been problems with the sensitivity and specificity of the α -subunit assay, in part because polyclonal antibodies were used.⁶

Accurate determination of hypersecretion of α -subunit requires definition of cut-off values, particularly with consideration of both gender and menstrual status. To our knowledge, these data are usually neither reported nor considered in papers on measurement of α -subunit levels (e.g., the recent study by Pineyro *et al.*⁷ The aim of this study was to estimate reference intervals and decision limits for α -subunit levels and α -ratio (α -subunit/LH+FSH), taking into consideration gender and menstrual status. Furthermore, we wanted to determine if the diagnostic utility of α -subunit hypersecretion was improved by adding

the simple calculation, α -ratio, to measurements of α -subunit in patients with NFPAs.

Materials and Methods

The study was approved by the Institutional Review Board, Region Syddanmark, and was conducted in accordance with their guidelines.

Healthy individuals

A total of 231 healthy adults were studied: 170 men (mean, 64 years; range, 24-75 years, 23 premenopausal women (mean, 30 years; range 20-42 years) and 38 postmenopausal women (mean, 53 years; range 45-73 years). None of the women had had an ovariectomy and none was taking contraceptive pills, hormone replacement therapy or other medication known to affect pituitary-ovarian function. The premenopausal females were examined during the follicular phase. Gonadotropin α -subunit, LH and FSH levels were measured and the α -ratio was calculated: α -subunit/(LH+FSH).

Patients

A total of 37 patients with newly diagnosed NFPAs were studied preoperatively: 17 women (mean, 50 years; range, 27-73 years) and 20 men (mean, 55 years; range, 26-81 years). In nine patients with NFPAs, the α -subunit levels and/or α -ratios were also determined postoperatively.

Gonadotropin α -subunit, LH and FSH levels and α -ratios in female patients above the age of 45 years were compared with corresponding levels in healthy postmenopausal women.⁸

Patients with NFPAs showed no endocrine signs or symptoms that indicated hormonal excess (such as acromegaly, gigantism, the galactorrhea-amenorrhea syndrome or Cushing's disease). Mild to moderate hyperprolactinemia, attributable to pituitary stalk compression was accepted.⁹ The diagnosis of acromegaly was made in accordance with consensus

guidelines.¹⁰ The diagnosis of Cushing was made in accordance with the recommendation of measuring cortisol in more than one 24-hour urinary collection and/or by low-dose dexamethasone suppression test.¹¹ The majority of the patients (35 of 37) had pituitary macroadenomas ≥ 10 mm as assessed by MRI or CT scans. Microadenomas were found in two patients. Immunostaining for gonadotropin α -subunit was performed in 20/37 NFPAs after pituitary surgery using methods that have previously been described.¹²

Analytical assays

Gonadotropin α -subunit levels were determined using a two-site fluoroimmunoassay based on commercial monoclonal antibodies (Medix Biochemica, Finland). The primary (capture) antibody (Clone 6601) was raised against FSH α and the secondary (detection) antibody (Clone 5501) against LH α . Labelling was performed with a DELFIA Eu-labelling kit (Wallac, Turku, Finland). The limit of detection for the α -subunit assay was 0.004 IU/l (WHO HCG-alpha 75/569, 1 μ g = 1 IU). The intra-assay coefficient of variability (CV) for α -subunit was 6.5% at 0.2 IU/l and 4.9% at 4.4 IU/l. All samples from each individual were analysed in the same analytical run. Serum LH-concentrations were determined using the AutoDEL-FIA assay (Wallac, Turku, Finland). Both monoclonal antibodies were directed against the LH β -subunit. The calibration values were traceable to international reference preparation, WHO 2nd IS 80/552, and the limit of detection for the LH-assay was 0.05 IU/l. The intra-assay CV for LH was 3.6% at 0.5 IU/l and 1.9% at 6 IU/l.

FSH concentrations were determined using the AutoDEL-FIA assay (Wallac, Turku, Finland). The two monoclonal antibodies were directed against the α -subunit and the FSH β -subunit, respectively, resulting in determination of only the intact FSH. The calibration values were traceable to international reference preparation, 2nd IRP FSH/LH 78/549, and the limit of detection for the FSH assay was 0.05 IU/l. The intra-

assay CV for FSH was 8.9% at 0.2 IU/l and 3.4% at 6.9 IU/l.

Data analysis

The reference groups and cut-off limits

We used rankit transformation of the cumulated ranked percentage as function of concentrations or ln-concentration values.^{13,14} The cut-off limits for each subunit and hormone were defined by the upper limits of the 90% CIs for the upper reference limits for the 95% reference interval in each healthy group. The CIs for each percentile were calculated, based on the formulas:

Line, describing the ln-Gaussian distribution:
 $\text{mean} + z \times s$,

where mean and s are the parameters for the ln-Gaussian distribution and z is used as a variable for describing the rankit for the cumulated percentage

$$90\% \text{ CI curves} = \text{mean} + (z \times s) \pm (1.65 \times \text{SEP}),$$

where 1.65 is the factor for 90% confidence and SEP is standard error of the percentile,

$$\text{SEP} = (s^2/n + s^2 \times z^2 / (2n + 1/2))^{1/2}$$

The values of α -subunit and α -ratio (Figure 1), and LH and FSH (data not shown) exhibited ln-Gaussian distribution, except three data sets (Table 1), which exhibited Gaussian distribution. For the ln-Gaussian distributed subgroups, the geometric mean (that is the mean on the ln-transformed scale that is back transformed by taking the anti-ln ($e^{\ln(x)}$)) and 95% reference intervals were calculated.

Test for partitioning of subgroups was performed according to Lahti *et al.*¹⁵ for the logarithmic values: the difference between the upper (or lower) reference limits of two subgroups is divided by the smallest standard deviation; if this value was above 0.75, parti-

FIGURE 1. Rankit plots illustrating the distributions of α -subunits for men and pre- and post-menopausal women. The cumulated ranked frequency distributions are shown on a rankit scale as function of natural logarithms of measured concentrations, together with the estimated ln-Gaussian distributions (straight lines) with 90 % confidence curves. The cumulated percentages are shown to the right and concentration on top. The hatched areas are illustrating the 90 % CI for men.

TABLE 1. Reference intervals and cut-off limits for gonadotropin α -subunit, LH and FSH levels, and α -ratios in healthy controls

Basal values	Men (n = 170)	Premenopausal women (n = 23)	Postmenopausal women (n = 38)
α -basal (IU/l)			
Geometric mean (ref. int.)	0.26 (0.07 to 0.93)	0.23 (0.13 to 0.39)	0.81 (0.24 to 2.69)
Cut-off *	1.10	0.48	3.76
α -ratio			
Geometric mean (ref. int.)	0.03 (0.01 to 0.09)	0.02 (0.01 to 0.05)	0.01 (0.00 to 0.03)
Cut-off *	0.11	0.07 # 0.05	0.04 # 0.05
LH (IU/l)			
Geometric mean (ref. int.)	3.9 (1.4 to 10.9)	5.2 (1.0 to 9.3) [^]	32.4 (0.1 to 64.7) [^]
Cut-off *	12.5	10.8[^]	73.6[^]
FSH (IU/l)			
Geometric mean (ref. int.)	5.5 (1.6 to 19.4)	5.8 (2.6 to 12.9)	61.9 (0 to 134.6) [^]
Cut-off *	22.9	17.1	154.8[^]

ref. int. - 95% reference interval

*: Cut-off is defined as the upper 90 % confidence limit for the upper 95 % reference limit

#: Common Cut-off for all women (see text)

All data sets were ln-Gaussian distributed except 3 data sets, marked with [^], which were Gaussian distributed, so mean values are shown instead of geometric mean values for these three components.

TABLE 2. Clinical characteristics in 23 patients with pituitary adenomas and increased gonadotropin α -subunit levels and/or α -ratios

Patients	Sex (f/m)	Age (years)	α - subunit (IU/l)	α -ratio	LH (IU/l)	FSH (IU/l)	Hormonal substitution	Immunohisto-chemistry
NFA 1	f	38	0.70	0.05	6.1	7.2	none	α -subunit, LH, FSH
NFA 2	f	35	4.01	0.56	2.6	4.5	none	/, negative
NFA 3	f	28	0.64	0.03	14.3	4.7	none	not operated
Premenopausal women cut-off			0.48	0.05	10.8	17.1		
NFA 4	f	58	2.65	0.06	5.5	42.1	none	α -subunit, LH, FSH
NFA 5	f	64	1.15	0.36	0.5	2.7	none	negative
NFA 6	f	70	2.80	0.09	2.4	30.2	H +T4	FSH
NFA 7	f	52	0.55	0.06	2.9	6.3	none	α -subunit, LH, FSH
NFA 8	f	53	1.05	0.05	2.5	20.1	none	α -subunit, LH, FSH
NFA 9	f	47	2.23	0.06	6.4	30.4	none	/, LH, FSH
NFA 10	f	61	1.08	3.72	0.2	0.1	O	/, negative
Postmenopausal women cut-off			3.76	0.05	73.6	154.8		
NFA 11	m	45	3.95	0.16	0.5	24.8	none	α -subunit, LH, FSH
NFA 12	m	81	0.39	0.23	0.4	1.3	H + T4	not operated
NFA 13	m	48	13.0	2.95	2.3	2.1	none	α -subunit, LH, FSH
NFA 14	m	75	2.05	0.13	4.6	11.7	none	not operated
NFA 15	m	51	78.5	71.4	0.1	1.0	T + GH	α -subunit, LH
NFA 16	m	50	0.35	1.94	0.1	0.1	T + H + T4	negative
NFA 17	m	57	56.7	13.8	1.4	2.7	none	α -subunit
NFA 18	m	36	76.0	21.7	0.9	2.6	none	α -subunit
NFA 19	m	72	54.5	8.93	3.3	2.8	DA	not operated
NFA 20	m	61	0.24	0.14	0.3	1.4	T + H	/, negative
NFA 21	m	26	0.25	0.11	0.4	1.9	T + H	negative
NFA 22	m	67	0.03	0.16	<0.1	0.1	H + T4	/, none
NFA 23	m	49	0.29	0.13	1.5	0.8	T	/, none
Men cut-off			1.10	0.11	12.5	22.9		

H (hydrocortisone), GH (growth hormone), T₄ (levothyroxine), T (testosterone), O (oestrogens) / a patient from Aarhus, not immunostained for α -subunit. An elevated level was defined as $\geq$ cut-off in bold.

tioning was recommended.

Results

Calculation of α -subunit levels and α -ratios in healthy individuals

The 95% reference intervals and cut-off limits of α -subunit, LH and FSH levels and α -ratio were based on the data from 231 healthy adults (Table 1). The distributions of α -subunit and α -ratio values are shown for the three groups of healthy men and pre- and post-

menopausal women in Figures 1A and 1B, respectively. All six distributions in the figures could be considered In-Gaussian as the cumulated curves were close to straight lines and generally within the 90% confidence curves. The cut-offs for α -subunit levels were significantly different between men and pre- and postmenopausal women with cut-offs of: 1.10, 0.48 and 3.76 IU/l, respectively.

The three distribution lines for α -ratios were nearly parallel and the distributions of pre- and postmenopausal women were similar. We tested if the cut-

FIGURE 2. Gonadotropin α -subunit levels and α -ratio (log) before and after surgery in nine patients with elevated levels preoperatively: α -ratio (n = 9) and α -subunit levels (n=5).

□ men
○ premenopausal women
● postmenopausal women

off points for the two subgroups of women could be combined to one single value for α -ratio. The mean $\ln\alpha$ -ratios for pre- and postmenopausal women were -3.87 and -4.53, respectively, and the standard deviations were 0.45 and 0.55, respectively. The upper reference limits were -2.99 and -3.46, with the difference 0.47, which by division with the smallest standard deviation resulted in a test value of 1.05. This value was above the 0.75 for partitioning, but not by a great degree. By combining the two cut-off points of 0.07 and 0.04 to a common of 0.05, then the percentage of individuals above this common cut-off would be 2.5 and 0.3, respectively. It was therefore decided to use the common cut-off point of 0.05 for women and 0.11 for men.

Calculation of α -subunit levels and α -ratios in NFPA patients

Increased α -subunit levels were identified in 10 out of 37 (27%) patients with NFPA. This rate was 18% (3/17) in females and 35% (7/20) in males. By calculating both α -subunit levels and α -ratio, increased gonadotropin levels were identified in 23 patients out of 37 (62%), and 22 out of these 23 patients (96%) had increased α -ratios (Table 2). Only one patient out of 23 had an elevated gonadotropin α -subunit level but a normal α -ratio - a premenopausal woman. Of the seven postmenopausal women with increased α -ratio, none had increased gonadotropin α -subunit (Table 2).

Nine patients with NFPA and significantly increased preoperative α -subunit and/or α -ratios were

examined postoperatively. All nine patients had elevated α -ratios and five of these patients also had elevated α -subunit levels. The α -subunit levels in all nine patients decreased postoperatively: the levels were reduced to mean values of 75% of preoperative values (range, 51% - 92%), and α -ratios were reduced to mean values of 63% of preoperative values (range, 22% - 95%) (Figure 2).

Calculating the fraction of the α -ratio by including TSH in the denominator (α -subunit / LH + FSH + TSH) yielded no additional information (data not shown). No patient was characterized by elevated LH and/or FSH levels and normal α -subunit/ α -ratio levels.

Immunochemistry

Immunostaining of the α -subunit was performed on surgical specimens from 20 of the 37 NFPA patients. Ten stained positively for the α -subunit and nine of these had elevated serum levels of the α -subunit. In the samples with negative immunostaining, elevated α -subunit levels in serum were found in only four patients.

Discussion

In the present study, we carefully evaluated the distribution of reference groups and found that the upper reference limits of the α -ratio for both pre- and postmenopausal women allowed for a common cut-off value, in contrast to the diverging limits for α -subunit distributions. We thoroughly assessed the serum levels of both the gonadotropin α -subunit and the intact gonadotropin in 37 patients with newly diagnosed NFPAs. Elevated α -subunit levels were found in 10 out of 37 (27%) patients with NFPAs. In addition, 22 out of 23 patients (96%) had increased α -ratios (Table 2). Only one patient out of 23, a premenopausal woman, had elevated α -subunit levels but a normal α -ratio. No postmenopausal women, of the 7 with an increased α -ratio, had increased α -subunit levels. Novel ultra-sensitive immunometric assays of α -subunit, LH and FSH were used. The reference inter-

vals describe the biology in healthy individuals and provide basic information about turn-over of the components. Most common are ln-Gaussian distributions when homogeneous subgroups are investigated¹⁴ and this is the case for all subgroups of α -subunit and α -ratio. Samejima *et al.*¹⁶ have published reference intervals for α -subunit levels; however, they did not state the age of the controls or of the patients. They established a common cut-off limit for women, pooling α -subunit levels from 42 pre- and postmenopausal women. Judging from the α -subunit levels (0.35 ± 0.62 $\mu\text{g/l}$; mean ± 2 SD) the data were not distributed in a Gaussian manner. Looking at our data (Figure 1a), it is obvious that α -subunit levels from pre- and postmenopausal women cannot be pooled, emphasizing the need for specific cut-off limits for α -subunit levels in men, pre- and postmenopausal women. In NFPAs, α -subunit levels were increased in 27% (10/37) of patients - in 18% (3/17) for women and in 35% (7/20) for men. These data are consistent with earlier studies that reported increases of 0-22% for women and 6-33% for men.¹⁷⁻²² The probable explanations for the very low number of patients with increased α -subunit levels in some of these studies may be 1) the use of polyclonal assays,⁶ 2) the lack of specific reference intervals, considering gender and menstrual status,²² and 3) the lack of α -ratio calculations. In postmenopausal women in particular, the use of α -subunit levels may not be adequate. We found that no postmenopausal patient had increased α -subunit levels, consistent with results from a previous study by Kwekkeboom *et al.*¹⁷ The necessity of using the α -ratio, especially in postmenopausal women, is underlined by the fact that in NFPAs the most frequent pituitary deficit is hypogonadism which is reported in 43.3% of patients.²³ Men and premenopausal women are both prone to hypogonadism resulting from compression of the normal gonadotroph cells. In postmenopausal women, the higher gonadotrophin levels which result from normal physiology, may be pathologically low.

In patients with TSH-secreting adenomas, the determination of α -subunit levels is an integral part of the diagnosis.^{24,25} Regarding specific reference intervals and the impact of LH and FSH levels in NFPAs, the above mentioned considerations may be applied to TSH-adenomas as well.

The preoperatively-elevated α -ratios were reduced postoperatively in all nine patients. Calculating α -ratios may increase the usefulness of the α -subunit measurements. The reduction of α -subunit levels was in agreement with another study that reported that serum α -subunit levels were reduced or normalized after surgery in patients with NFPAs.¹⁶ The α -ratio may be of even more clinical use as a tumour marker in patients with NFPAs. Remnant tumours are often found after transsphenoidal surgery on these large tumours, making adjuvant therapy necessary in many patients.²⁶ We and others have reported a positive relationship between the *in vivo* secretory potential of α -subunit gonadotropins in NFPAs and tumour reduction during medical therapy.^{27,28} The α -subunit measurements have been used to study the effects of experimental therapy; i.e., somatostatin analogues, dopamine-D2-agonists, the combination of somatostatin analogues and dopamine-D2-agonists.²⁶ Future studies will elucidate the effects of selective somatostatin analogues^{29,30} and BIM^{31,32} on α -subunit, gonadotropins, and tumour size. Temozolomide therapy for resistant pituitary adenomas including NFPAs¹² is a new experimental therapy, where monitoring of α -subunit and gonadotropins may be of value.

Immunopositivity of pituitary adenomas equates with hormone/subunit storage, but does not necessarily correlate with hormone synthesis or release³³. Accordingly, we found that positive α -subunit immunostaining did not predict elevated serum levels of the α -subunit. These findings are consistent with those of the study by Popovic & Damjanovic.³⁴ The negative α -subunit immunostaining, however, does not necessarily indicate normal α -subunit levels: this may be due to rapid secretion of the α -subunit from the cytoplasm.³⁵

Summary

1. A common cut-off limit for α -subunit levels in men, pre- and postmenopausal women does not appear to be appropriate.
2. Using the estimated cut-offs for men, pre- and postmenopausal women, 10 out of 37 (27%) NFPA patients had elevated gonadotropin α -subunit levels.
3. Calculation of the α -ratio enabled determination of a common cut-off point for both pre- and postmenopausal women.
4. 23 out of 37 patients (62%) had increased α -subunit levels and 22 of these 23 patients (96%) had elevated α -ratios.
5. The preoperatively elevated α -ratios and α -subunit levels decreased postoperatively in the nine patients who were examined.

Conclusion

These data suggest that utilizing the simple calculation of α -ratio improves the ability to detect α -subunit hypersecretion.

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