The Acylated/Unacylated Ghrelin Ratio Is Similar in Patients With Acromegaly During Different Treatment Regimens

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Background: Data on plasma acylated ghrelin (AG) and unacylated ghrelin (UAG) levels in acromegaly are limited. High AG/UAG ratios are linked with type 2 diabetes, obesity, and hyperphagia (*e.g.*, in Prader-Willi syndrome).

Objective: To assess fasting plasma AG and UAG levels, and the AG/UAG ratio in acromegaly patients receiving combination treatment of long-acting somatostatin analogs (LA-SSAs) and pegvisomant (PEGV; n = 60). We used as controls acromegaly patients whose disease was controlled with PEGV monotherapy and medically naïve patients with active acromegaly.

Methods: Fasting venous blood samples were collected and directly stabilized to inhibit deacylation of AG. Plasma AG and UAG levels were determined by double-antibody sandwich enzyme immunoassay, and the AG/UAG ratio was calculated.

Results: Plasma AG and UAG levels were significantly lower in patients with acromegaly receiving combination treatment [median, interquartile range (IQR): AG: 8.5 pg/mL, 2.9 to 21.1 pg/mL; UAG: 26.9 pg/mL, 11.2 to 42.1 pg/mL] compared with patients using PEGV alone [AG: 60.5 pg/mL (IQR, 58.8 to 77.4 pg/mL); UAG: 153.7 pg/mL (IQR, 127.3 to 196.0 pg/mL)] and medically naïve patients with acromegaly [AG: 24.0 pg/mL (IQR, 12.6 to 49.7 pg/mL); UAG: 56.3 pg/mL (IQR, 43.4 to 61.5 pg/mL)]. However, AG/UAG ratios were similar in all groups.

Conclusions: Although plasma AG and UAG are suppressed during combination treatment with LA-SSAs and PEGV, the AG/UAG ratio remained similar. This shows that SSAs decrease both AG and UAG levels, which suggests that they do not alter metabolism significantly in acromegaly patients. **(*J Clin Endocrinol Metab* 102: 2425–2432, 2017)**

Ghrelin is a small peptide hormone secreted mainly by neuroendocrine X/A cells in the stomach (1, 2). In the circulation, it consists of two isoforms: acylated ghrelin (AG) and unacylated ghrelin (UAG). Both isoforms are detectable in equal amounts in the circulation (3). AG differs from UAG in being acylated by attachment of a medium-chain fatty acid at its serine-3 residue. AG is acylated by the intracellular enzyme ghrelin *O*-acyl transferase and is responsible for the distinct metabolic and nonmetabolic effects of ghrelin *in vivo* (4–11). AG acts on the hypothalamus through the growth hormone secretagogue receptor (GHSR1a) and is known to be diabetogenic, orexigenic, and obesogenic. UAG does not bind to the GHSR1a receptor at physiological concentrations and, therefore, was considered to be inactive. However, recent studies have shown

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Abbreviations: %CV, percent coefficient of variation; AEBSF, 4-(2-aminoethyl) benzenesulphonyl fluoride hydrochloride; AG, acylated ghrelin; ELISA, enzyme-linked immunosorbent assay; GH, growth hormone; GHSR1a, growth hormone secretagogue receptor; HbA1c, glycated hemoglobin; HOMA-IR, homeostasis model assessment-insulin resistance; IGF1, insulinlike growth factor 1; IQR, interquartile range; LA-SSA, long-acting somatostatin analog; PEGV, pegvisomant; RIA, radioimmunoassay; UAG, unacylated ghrelin.

that UAG is able to counteract the metabolic effects of AG (9, 12).

Because AG and UAG have distinct biological effects and can affect each other, the AG/UAG ratio may be a more important parameter than individual levels of AG and UAG (13–19). For example, elevated AG/UAG ratios have been associated with diabetes, obesity, and hyperphagia (13, 20–25). Hyperphagia is a hallmark of Prader-Willi syndrome, a rare cause of genetic, early-onset obesity, which is characterized by elevated total ghrelin levels but changing AG/UAG ratios throughout life (20, 22, 26).

It is known that ghrelin stimulates growth hormone (GH) secretion, whereas ghrelin itself is reduced after GH infusion (11, 27–29). However, the exact physiological role of ghrelin in the regulation of GH release is not entirely established (30–33).

In acromegaly, a prototype disease characterized by excessive GH levels, the emerging picture from previous studies is that, in medically naïve patients during active disease, total ghrelin levels are lowered compared with levels in control subjects (27, 34, 35). Ghrelin levels are elevated after surgery, whereas they are reduced during treatment with long-acting somatostatin analogs (LA-SSAs) (34–37). Patients with acromegaly treated with the competitive GH receptor blocker pegvisomant (PEGV) have higher total ghrelin levels than patients with active disease (38).

However, to our knowledge, the effect of combination treatment with long-acting somatostatin analogs (LA-SSAs) and PEGV on plasma AG and UAG levels in acromegaly remains unknown. Similarly, AG and UAG levels have not been assessed together in patients with acromegaly.

Therefore, the aim of this study was to assess fasting plasma AG and UAG levels and to determine the AG/UAG ratio in patients with acromegaly who were receiving combination treatment with somatostatin analogs and PEGV (n = 60), and compare them with the ratios in patients receiving PEGV monotherapy (n = 4) and in medically naïve patients with acromegaly (n = 5).

Patients and Methods

Study design

We prospectively recruited 69 patients with acromegaly at our outpatient clinic at the Erasmus University Medical Center, Rotterdam, The Netherlands, between August 2015 and June 2016. For most of these patients, their acromegaly was biochemically controlled in the long term with combination treatment of LA-SSAs and PEGV (n = 60), four patients were treated with PEGV monotherapy, and five patients were medically naïve with active acromegaly. We excluded patients with eating disorders, active malignancies, active inflammatory or infectious diseases, epilepsy, and psychiatric disorders. Patients with acromegaly were considered diabetic either if they were taking antidiabetic medication or had a history of diabetes mellitus, or had glycated hemoglobin (HbA1c) levels ≥6.5%.

In addition to measurement of plasma AG and UAG, we assessed in the fasting state: glucose, insulin, HbA1c, insulinlike growth factor 1 (IGF1), and GH levels. The patients' body weight and height also were measured. Serum glucose, insulin, and HbA1c values were determined with standard laboratory methods. The updated homeostasis model assessment was used to assess insulin resistance (HOMA-IR) and β-cell function from pairs of fasting glucose and insulin levels.

All patients gave their written informed consent, and the study was approved by the Medical Ethics Committee of Erasmus University MC, Rotterdam.

Materials

Vacutainers were obtained from Becton Dickinson (6 mL of di-potassium EDTA; catalog no. 367899; Breda, The Netherlands); 4-(2-aminoethyl) benzenesulphonyl fluoride hydrochloride [(AEBSF) Pefabloc SC; Sigma-Aldrich, St. Louis, MO] was purchased from Roche Applied Science (catalog no. 11429876001; Almere, The Netherlands). Stock solutions of 200 mg/mL AEBSF in distilled water were prepared (39, 40).

Human AG and UAG levels were determined by double-antibody sandwich enzyme immunoassay kits obtained from Bertin Pharma (catalog nos. A05106 and A05119, respectively; Montigny-le-Bretonneux, France) (39).

Total IGF1 concentrations were measured by chemiluminescent immunometric assay (IDS-iSYS; Immunodiagnostic Systems, Boldon, United Kingdom) and were interpreted according to the sex-dependent and age-dependent ranges. GH levels were measured using the IDS-iSYS assay, which is free of interference from PEGV (41).

Blood collection, AEBSF treatment, and storage

Overnight-fasting venous blood samples for the measurement of plasma AG and plasma UAG were drawn and collected in EDTA tubes. One 4-mL EDTA tube per patient was collected. AEBSF was immediately added to all blood samples (1:100 dilution; final concentration, 2 mg/mL) to prevent deacylation of AG to UAG (39, 42). Whole blood was mixed gently by inversion (three times) and stored on water ice (4°C) until centrifugation at 2500*g* at 4°C for 5 minutes. Plasma of these venous blood samples was then rapidly aliquoted in four 1.5-mL Eppendorf tubes (300 μL in each). All plasma samples were stored at −80°C until the assay was performed. AEBSF was stored for a maximum of 1 month after dilution.

Acylated and unacylated ghrelin enzyme-linked immunosorbent assays

After thawing on ice, plasma samples were centrifuged for 1 minute at 1500*g* and 4°C, and kept on ice before

transferring to the assay plates. All samples were measured in duplicate (50 μL/well), according to the manufacturer's protocol (39).

A sigmoidal third-order cubic polynomial fitting was used to determine concentrations from the calibration curves. This resulted in r^2 values >0.99 in most of the assays. For the Bertin Pharma EIAs, the average intra-assay percent coefficient of variation (%CV) was 2.1 for AG and 4.6 for UAG. The average interassay %CV was 9.5 for AG and 12.8 for UAG. The %CVs were assessed over six assays. The lower limit of detection was 4 pg/mL.

Statistical analysis

Analyses were performed using SPSS software (version 24 for Windows; SPSS Inc., Chicago, IL) and GraphPad Prism® version 6.04 (GraphPad Software, San Diego, CA). The Kolmogorov-Smirnov test was used to test normality of variables (data were considered to be normally distributed when $P > 0.05$). Comparisons across all groups were analyzed with the Kruskall-Wallis test. Comparisons between patient groups were analyzed by Wilcoxon signed-ranks tests and Mann-Whitney *U* tests. Correlation analyses were done using the Spearman rank-correlation test. Data were reported as median [interquartile range (IQR)] because they were not normally distributed. $P < 0.05$ was considered statistically significant.

Results

Clinical characteristics

Table 1 shows the patients' demographics, characteristics, and disease history. Plasma AG and UAG levels were measured in a total of 69 acromegaly patients. One-third of patients (20 of 60) who received combination treatment had previously undergone surgery, whereas 7 of 60 patients had undergone surgery and radiotherapy in the past. In patients receiving PEGV monotherapy, two of four patients had undergone both surgery and radiotherapy. In medically naïve patients with active disease, two of five patients had previously undergone surgery. Clinical characteristics were comparable among the groups with respect to age, sex, body mass index, and previous therapy. Patients receiving combination treatment with LA-SSAs and PEGV and patients receiving PEGV monotherapy had IGF1 levels within the age- and sex-adjusted normal limits.

Figure 1 shows the median fasting levels of AG and UAG, and the AG/UAG ratio. Levels of AG and UAG were significantly different between the groups (Kruskal-Wallis test: AG, $P = 0.004$; UAG, $P = 0.005$).

Median (IQR) AG levels were significantly lower in patients using combination treatment compared with patients using PEGV monotherapy and with medically naïve patients [respective Mann-Whitney *U* test results: 8.5 pg/mL (2.9 to 21.1 pg/mL) vs 60.5 pg/mL (58.8 to 77.4 pg/mL), $P = 0.0002$; vs 24.0 pg/mL (12.6 to 49.7 pg/mL), $P = 0.03$]. There was no significant difference in AG between patients using PEGV and the medically naïve patients (Wilcoxon signed-rank test: $P = 0.25$).

Although UAG levels were higher than AG levels in all groups, they showed a similar pattern. Median (IQR) UAG levels were significantly lower during combination treatment compared with the other groups (respective Mann-Whitney *U* test results: [26.9 pg/mL (11.2 to 42.1 pg/mL)

Table 1. Characteristics of All Patients in the Three Study Groups

Parameters	LA-SSA + PEGV	PEGV	Medically Naïve Acromegaly
No. of patients	60	4	5
Male sex, no. (%)	32 (53)	2 (50)	2 (40)
Age, mean (range), y	54 (27–81)	62 (44–82)	44 (29–62)
BMI, kg/m^2	28.8 (26.0–31.7)	30.9 (24.1–34.9)	30.3 (26.1–35.4)
Previous therapy, no. (%)			
Surgery	20 (33)	0	2 (40)
Radiotherapy	0	0	0
Surgery and radiotherapy	7 (11.7)	2 (50)	0
IGF1, nmol/L	25.7 (22.2–32.3)	27.0 (22.4–36.0)	96.1 (64.0–166.0)
IGF1 × ULN	0.99 (0.85–1.12)	1.09 (0.90–1.28)	3.63 (2.20–4.50)
GH, μg/L	4.6 (1.7–8.6)	3.1 (0.7–17.6)	15.4 (11.7–112.0)
Fasting glucose, mmol/L	6.1 (5.6–6.8)	4.6 (4.3–4.9)	5.5 (5.1–6.2)
HbA1c, %	5.9 (5.7–6.2)	5.6 (5.5–5.9)	5.6 (5.3–6.4)
Diabetes mellitus, no. (%)	14 (23)	0 (0)	1 (20)
HOMA-IR score	1.1 (0.7–1.5)	NA	NA
HOMA β-cell score	67.2 (49.3–90.5)	NA	NA
PEGV dose, mg/wk	100 (60–160)	175 (95–289)	—

Data are expressed as median and IQR, unless otherwise specified.

Abbreviations: —, not applicable; BMI, body mass index; NA, not available; ULN, upper limit of normal.

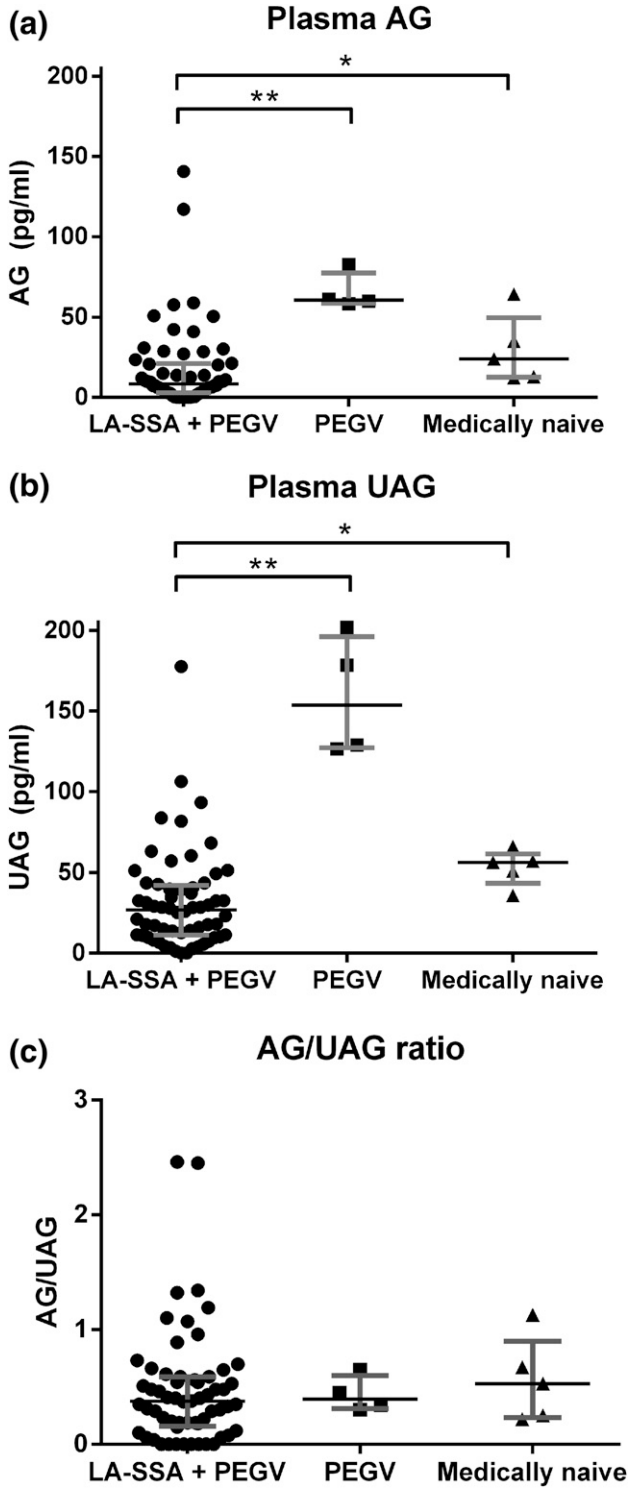

Figure 1. In patients with acromegaly who were treated with combination therapy consisting of LA-SSAs and PEGV (n = 60), patients with acromegaly receiving PEGV monotherapy (n = 5), and medically naïve patients with acromegaly (n = 4), (a) plasma AG, (b) plasma UAG, and (c) AG/UAG ratios are depicted. Data are expressed as median ± IQR. (a, b) $*P < 0.05$; $**P < 0.001$.

vs 127.3 pg/mL (153.7 to 196.0 pg/mL), $P = 0.0002$; vs 56.3 pg/mL (43.3 to 61.5 pg/mL), $P = 0.03$])]. Similar to AG, UAG levels were not different between patients using PEGV and medically naïve patients (Wilcoxon signed-rank test: $P = 0.13$).

AG/UAG ratios were not significantly different between the groups ($P = 0.65$). The median (IQR) AG/UAG ratio was 0.38 (0.16 to 0.59) in the combination group vs 0.40 (0.31 to 0.60) in the PEGV monotherapy group vs 0.53 (0.24 to 0.90) in medically naïve patients.

The highest AG level of 140.6 pg/mL was observed in a 55-year-old man with long-term controlled acromegaly treated with lanreotide Autogel (Ipsen, Paris, France) 120 mg every 4 weeks and PEGV 100 mg/wk. At the time of the blood sampling, this patient had an IGF1 level of 1.17 times the upper limit of normal. His type 2 diabetes (HbA1c, 6.9%) was well regulated with metformin and he received testosterone replacement because of hypogonadism.

In another patient, we observed the second highest AG level of 117.1 pg/mL and the highest observed UAG level of 177.5 pg/mL. This was a 27-year-old patient with acromegaly who had undergone a transsphenoidal hypophysectomy twice. At the time of the blood sampling, her condition was controlled with octreotide long-acting release every 4 weeks and PEGV 80 mg/wk.

Concentrations of plasma AG [Fig. 1(a)] and UAG [Fig. 1(b)] were very low in the LA-SSA and PEGV combination treatment group. In 17 patients (28%) receiving combination treatment, AG levels were undetectable, whereas in four patients (6.7%), UAG levels were undetectable.

We found no statistically significant relationship between plasma AG, UAG, and AG/UAG ratios vs biochemical parameters [*i.e.*, GH, IGF1, HbA1c levels, HOMA-IR score, HOMA β-cell score, and clinical parameters (age, sex, body mass index, diabetes, previous surgery, previous radiotherapy, PEGV dose)] among the groups of patients with acromegaly. Although not statistically significant, a negative correlation ($r = -0.25$; $P = 0.06$) was observed between previous surgery and UAG levels in patients receiving combination treatment.

Discussion

Our main finding was that the AG/UAG ratio was not altered in patients with acromegaly during different treatment regimens. AG and UAG levels were suppressed during combination treatment with LA-SSAs and PEGV, compared with patients using PEGV alone and with medically naïve active acromegaly. This is in line with literature on total ghrelin levels, although previous studies lacked information on the ratios between AG/UAG. It is questionable whether higher single AG and UAG levels have a physiological role during PEGV

treatment and active disease if the ratios remain the same. Therefore, assessment of total ghrelin assessment is probably not clinically useful.

The AG/UAG ratio is probably more clinically relevant than measurement of total ghrelin. The main problem with previous studies examining ghrelin levels in patients with acromegaly is the small number of samples assessed and the use of ghrelin assays that do not distinguish between acylated and unacylated ghrelin. Commercial radioimmunoassays (RIAs) and one-site competitive enzyme-linked immunosorbent assays (ELISAs) measure total human serum ghrelin by using labeled UAG as a tracer and a polyclonal antibody against the C-terminal end of human ghrelin. These assays overestimate ghrelin levels because they measure peptide fragments as well as full-length peptide. These fragments exist naturally in the circulation and lack the *N*-terminal region, but can also be artificially produced during the assay procedure. Two-site ELISAs use antibodies directed against both ends of the peptide and, therefore, are highly specific and will only measure unfragmented ghrelin. Using their two-site sandwich ELISA as a comparison, Akamizu *et al.* (43) have demonstrated that about 40% to 60% of the total ghrelin measured by RIA is likely fragmented. This was further confirmed by Prudom *et al.* (44) who showed that their two-site sandwich ELISAs for AG and UAG provided greater specificity. They found that dynamic changes in AG were dampened and less visible in the RIAs. AG has a short half-life and, in circulation, it is rapidly degraded to UAG. For this reason, blocking deacylation is crucial for reliable measurements of AG and UAG (39, 42).

The observation of low AG and UAG levels during combination treatment of LA-SSA and PEGV suggests that this effect is caused by the SSAs. This finding is in line with previous studies showing that LA-SSAs suppress ghrelin levels in patients with acromegaly (34, 36). Freda *et al.* (34) evaluated fasting and serum ghrelin levels after an oral glucose tolerance test in patients with active acromegaly at baseline and after either surgery or administration of LA-SSAs. They observed that fasting total ghrelin levels were higher in patients after surgery, but these levels fell significantly after treatment with LA-SSAs. The researchers suggested that the postoperative lowering of insulin levels and improved insulin sensitivity may have contributed to the postoperative rise of ghrelin levels (34). Another report also suggested that patients with acromegaly with greater insulin resistance have lower total ghrelin levels (27). Several studies have indicated that hyperinsulinemia inhibits AG and UAG secretion; conversely, AG itself inhibits insulin secretion (14, 45–47). However, we could only assess insulin levels in the combination group and, therefore, cannot draw any conclusions on the difference in insulin sensitivity between the groups.

The observation of higher AG and UAG levels in patients using PEGV suggests that PEGV itself can stimulate AG and UAG. Roemmler *et al.* (38) showed that acromegaly patients using PEGV had higher total ghrelin levels compared with healthy control subjects, patients with active acromegaly, and those with inactive acromegaly. This finding suggests that treatment with PEGV might disrupt the feedback loop of ghrelin and GH, leading to elevated ghrelin levels. The ghrelin receptor GHSR1a is expressed in normal pituitary and somatotroph adenomas (48). GH administration has been shown to suppress total ghrelin levels in GH-deficient patients (49). In rodents, GH administration in cultured stomach tissue reduced total ghrelin secretion, whereas hypophysectomy increased ghrelin levels (50–52). These results support the notion that GH exerts a negative feedback action on ghrelin secretion. Although there are no studies evaluating the direct effect of PEGV on ghrelin secretion, these data indirectly suggest that blockade of the GH receptor with PEGV leads to a positive feedback action on ghrelin secretion.

In patients using combination treatment, median plasma AG levels were 8.5 pg/mL (range, 0 to 140.6 pg/mL) and median UAG levels were 26.9 pg/mL (range, 0 to 177.5 pg/mL). This is considerably lower than levels that have been observed in healthy control subjects, measured using equivalent two-site sandwich assays and stabilized with AEBSF. Adrichem *et al.* (40) found median plasma AG levels of 57.2 pg/mL (range, 10 to 273 pg/mL) and median plasma UAG levels of 64.9 pg/mL (range, 8 to 331 pg/mL) in 28 healthy control subjects, whereas Liu *et al.* (53) reported AG levels ranging from 43 to 366 pg/mL in four healthy volunteers.

AG and UAG exert distinct effects on glucose homeostasis and insulin sensitivity. AG has diabetogenic actions; it induces insulin resistance and reduces insulin secretion. However, UAG displays antidiabetogenic actions (13–19). UAG alone or in combination with AG improves insulin sensitivity through the suppression of AG levels in obese subjects with type 2 diabetes (9). Studies have shown that insulin-resistant obese subjects have an elevated AG/UAG ratio compared with insulin-sensitive obese subjects (13, 24, 25), which can be explained by a relative UAG deficiency in obese subjects (23). Conversely, low AG/UAG ratios are associated with an improved metabolic state (21).

Prader-Willi syndrome is characterized by distinct nutritional phenotypes, from anorexia in infancy to hyperphagia and obesity in childhood (26). Recently, it was revealed that although total hyperghrelinemia was observed at all ages throughout life in Prader-Willi

syndrome, the AG/UAG ratio changed over time, driving opposite phenotypes. Although the AG/UAG ratio was low during infancy, it switched to a high AG/UAG ratio later in life (20, 22).

These data illustrate that AG and UAG have opposing actions, and that the AG/UAG ratio yields more physiological importance than measurement of absolute levels of AG and UAG. Although our patients receiving combination therapy had lower AG and UAG levels, the AG/UAG ratio was similar between all groups. This suggests that treatment with LA-SSAs and PEGV does not alter the relation of AG with respect to UAG.

In summary, the plasma AG/UAG ratio was not altered in patients with acromegaly during medical treatment. Absolute levels of individual assessments of AG and UAG, however, were lower than observed during the assessment of total ghrelin levels. Assessment of the AG/UAG ratio is more clinically relevant because it is a better reflection of the physiological bioactive state of ghrelin than measurement of total ghrelin. Therefore, we recommend assessment of AG and UAG separately and calculation of the AG/UAG ratio.

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