The acylated/unacylated ghrelin ratio is similar in acromegaly patients during different treatment regimens

Ammar Muhammad, Patric J.D. Delhanty, Martin Huisman, Jenny A. Visser, Aart Jan van der Lelij, Sebastian J.C.M.M. Neggers

Department of Internal Medicine, Section Endocrinology, Erasmus MC, Rotterdam, the Netherlands

Published in:

The Journal of clinical endocrinology and metabolism. 2017;102:2425-2432.

ABSTRACT

Background: Data on plasma acylated ghrelin (AG) and unacylated ghrelin (UAG) levels in acromegaly are limited. High ratios of AG/UAG 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 on combination treatment of long-acting somatostatin analogues and pegvisomant. As a control, we used patients controlled with pegvisomant monotherapy, and medically naïve patients with active acromegaly.

Methods: Fasting venous blood samples (combination therapy N = 60; monotherapy N = 4; naïve N = 5) were collected and directly stabilized with AEBSF to inhibit deacylation of AG. Plasma AG and UAG levels were determined by double-antibody sandwich EIA and the AG/UAG ratio was calculated.

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

Conclusions: Although plasma AG and UAG are suppressed during combination treatment of LA-SSA and PEGV, the AG/UAG ratio remained similar. This shows that somatostatin analogues decrease both AG and UAG, which suggest that they do not alter metabolism significantly in acromegaly patients.

INTRODUCTION

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 (GOAT) and is responsible for the distinct metabolic and non-metabolic 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 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). Recently, in patients with Azlheimer's disease it was shown that rivastigmine, an acetylcholinesterase and butyrylcholinesterase inhibitor, improved appetite by increasing the AG/UAG ratio (27)

Regarding the relation of ghrelin with growth hormone (GH), it is known that ghrelin stimulates GH secretion, while ghrelin itself is reduced after GH infusion. However, the exact physiological role of ghrelin in the regulation of GH release is not entirely established.

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 controls (28-30). Ghrelin levels are elevated after surgery, while they are reduced during treatment with long-acting somatostatin analogues (LA-SSAs) (29-32). Acromegaly patients treated with the competitive GH receptor blocker pegvisomant (PEGV) have higher total ghrelin levels than patients with active disease (33).

However, to our knowledge, the effect of combination treatment with 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 between acromegaly patients under combination treatment with somatostatin analogs and pegvisomant (N = 60), and compare them to pegvisomant monotherapy (N = 4) and medically naïve acromegaly patients (N = 5).

PATIENTS AND METHODS

Study design

We prospectively recruited 69 acromegaly patients at our outpatient clinic at Erasmus University MC, Rotterdam between August 2015 and June 2016. The majority these patients were long-term biochemically controlled with combination treatment of LA-SSAs and PEGV (N = 60), 4 patients were on pegvisomant monotherapy and 5 patients were medically naïve with active acromegaly. We excluded patients with eating disorders, active malignancies, active inflammatory or infectious diseases, epilepsy and psychiatric disorders. Acromegaly patients were considered diabetic either if they were taking antidiabetic medication, or had a prior history of diabetes mellitus, or had glycated hemoglobin levels ≥ 6.5%.

In addition to measurement of plasma AG and UAG we assessed in the fasting state: glucose, insulin, HbA1c, IGF1 and GH. Also body weight and height. Serum glucose, insulin and HbA1c were determined with standard laboratory methods. The updated homeostasis model assessment (HOMA-2) was used to assess insulin resistance (HOMA-IR) and beta cell function (HOMA-IB) 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 MC, Rotterdam.

Materials

Vacutainers were obtained from Becton Dickinson (Breda, Netherlands; cat# 367899; 6 ml K2 EDTA), 4-(2-Aminoethyl) benzenesulphonyl fluoride hydrochloride (Pefabloc, SC AEBSF) was purchased from Roche Applied Science (cat# 11429876001; Almere, Netherlands). Stock solutions of 200 mg/ml AEBSF were prepared in distilled water (34, 35).

Human AG and UAG were determined by a double-antibody sandwich enzyme immunoassay (EIA) kits obtained from Bertin Pharma (Montigny-le-Bretonneux, France; A05106 and A05119, respectively) (34).

Total IGF1 concentrations were measured by chemiluminescent immunometric assay (IDS-iSYS; Immunodiagnostic Systems Limited; Boldon, United Kingdom), and were interpreted according to the sex-dependent and age-dependent ranges. GH levels were measured using the IDS-iSYS assay, this assay is free of interference from PEGV (36).

Blood collection, AEBSF treatment and storage

Overnight fasting venous blood samples for the measurement of plasma AG and plasma UAG were withdrawn and collected in EDTA tubes. One 4 ml EDTA tube per patient was collected. AEBSF was immediately added to all blood samples (dilution 1:100; final 2mg/ml) to prevent deacylation of AG to UAG (34, 37). Whole blood was mixed gently by inversion (3x) 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 each). All plasma samples were stored at -80°C until the assay was performed. AEBSF was stored for a maximum of one month after dilution.

Acylated and unacylated ghrelin ELISAs

After thawing on ice, plasma samples were centrifuged for 1 min at 1500 g 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 (34).

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 the majority of the assays. For the Bertin Pharma EIAs, the average intra-assay percent coefficient of variation (%CVs) for AG was 2.1 and for UAG 4.6. The average inter-assay %CVs for AG was 9.5 and for UAG 12.8. Their %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, Illinois) and GraphPad Prism® Version 6.04 (GraphPad Software, San Diego, USA). 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 analysed with Kruskall-Wallis test. Comparisons between patient groups were analysed by Wilcoxon signed-ranks tests and Mann-Whitney U tests. Correlation analyses were done using Spearman's rank correlation test. Data were expressed as median [interquartile range (IQR)] as they were not normally distributed. P-values of < 0.05 were considered statistically significant.

RESULTS

Clinical characteristics

Table 1 shows the patients demographics, characteristics and disease history. Plasma AG and UAG were measured in a total of 69 acromegaly patients. One third of patients (20/60) on combination treatment had previously received surgery, while 7 of 60 patients had received surgery and radiotherapy in the past. In patients on PEGV monotherapy 2 of 4 patients had received both surgery and radiotherapy. In medically naïve patients with active disease 2 of 5 patients had previously received surgery. Clinical characteristics were comparable among the groups with respect to age, sex, BMI and previous therapy. Patients on combination treatment with LA-SSAs and PEGV and patients on PEGV monotherapy had IGF1 levels within the age- and sex adjusted normal limits.

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

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
Sex - male (%)	32 (53)	2 (50)	2 (40)
Age, years, mean (range)	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			
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 x 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 (%)	14 (23)	0 (0)	1 (20)
HOMA-IR score	1.1 (0.7 - 1.5)	NA	NA
HOMA-ß score	67.2 (49.3 - 90.5)	NA	NA
PEGV dose (mg/week)	100 (60 - 160)	175 (95 - 289)	-

Data are expressed as median and interquartile range (IQR), unless specified otherwise. NA = not available

Median (IQR) AG levels were significantly lower in patients using combination treatment compared with patients using PEGV monotherapy and with medically naïve patients, 8.5 (2.9 – 21.1) pg/ml versus 60.5 (58.8 – 77.4) pg/ml (P = 0.0002 Mann-Whitney U test) versus 24.0 (12.6 – 49.7) pg/ml (P = 0.03 Mann-Whitney U test).

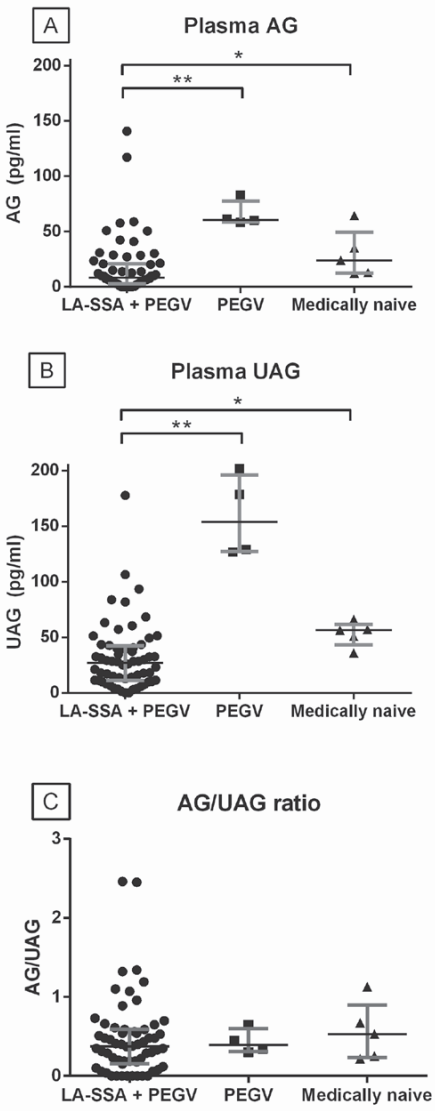

Figure 1. Plasma acylated ghrelin (AG), plasma unacylated ghrelin (UAG) and acylated ghrelin/unacylated ghrelin (AG/UAG) ratio in acromegaly patients on combination treatment of LA-SSAs and PEGV (N=60), acromegaly patients on PEGV monotherapy (N=5), and medically naïve acromegaly patients (N=4). Data are expressed as median ± interquartile range (IQR).

There was no significant difference in AG between patients using PEGV and medically naïve patients (*P* = 0.25 Wilcoxon Signed Rank test).

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, 26.9 (11.2 – 42.1) pg/ml versus 127.3 (153.7 – 196.0) pg/ml (P = 0.0002 Mann-Whitney U test), versus 56.3 (43.3 – 61.5) pg/ml (*P* = 0.03 Mann-Whitney U test). Similar to AG, UAG levels were not different between patients using PEGV and medically naïve patients (*P* = 0.13 Wilcoxon Signed Rank test).

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

The highest AG level of 140.6 pg/ml was observed in a 55-year-old male patient with long-term controlled acromegaly with lanreotide Autogel 120 mg every 4 weeks and PEGV 100 mg per week. At the time of the blood withdrawal this patient had an IGF1 level of 1.17 times the ULN. His type 2 diabetes (HbA1c 6.9%) was well regulated with metformin and he received testosterone replacement due to 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 acromegaly patient who received a transsphenoidal hypophysectomy twice in the past. At the time of the blood withdrawal she was controlled with octreotide LAR every 4 weeks and PEGV 80 mg per week.

Concentrations of plasma AG (figure 1A) and UAG (figure 1B) were very low in the LA-SSA and PEGV combination treatment group. In 17 (28%) patients on combination treatment AG levels were undetectable, while in 4 (6.7%) patients UAG levels were undetectable.

We found no statistically significant relationship in acromegaly patients between plasma AG, UAG and AG/UAG ratios versus biochemical (GH, IGF1, HbA1c levels, HOMA-IR score, HOMA-B score and clinical parameters (age, sex, BMI, diabetes, previous surgery, previous radiotherapy, PEGV dose) among the groups. Although not significant, a negative correlation (r = −0.25, *P* = 0.06) was observed between previous surgery and UAG levels in patients on combination treatment.

DISCUSSION

Our main finding was that the AG/UAG ratio was not altered in acromegaly patients 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 they lack 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 acromegaly patients is the small number of samples assessed and the use of ghrelin assays that do not distinguish between acylated and unacylated ghrelin. Commercial radioimmunoassays (RIA) and one-site competitive ELISAs measure total human serum ghrelin by using labelled unacylated ghrelin 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 are therefore highly specific and will only measure unfragmented ghrelin. Using their two-site sandwich ELISA as a comparison, Akamizu *et al* have demonstrated that about 40-60% of the total ghrelin measured by RIA is likely fragmented (38). This was further confirmed by Prudom *et al* who showed that their two-site sandwich-ELISAs for AG and UAG provided greater specificity (39). They found that dynamic changes in acyl-ghrelin were dampened and less visible in the RIAs. AG has a short half-life and in circulation it is rapidly degraded into UAG. For this reason, blocking deacylation is crucial for reliable measurements of AG and UAG (34, 37).

The observation of low AG and UAG levels during combination treatment of LA-SSA and PEGV suggests that this effect is caused by the somatostatin analogues. This finding is in line with previous studies showing that LA-SSAs suppress ghrelin levels in acromegaly patients (29, 31). Freda *et al* 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 fell significantly after treatment with LA-SSAs. They suggested that the postoperative lowering of insulin levels

and improved insulin sensitivity may have contributed to the postoperative rise of ghrelin levels (29). Another report has also suggested that acromegaly patients with greater insulin resistance have lower total ghrelin levels (28). Several studies have indicated that hyperinsulinemia inhibits AG and UAG secretion, conversely AG itself inhibits insulin secretion (14, 40-42). 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* showed that acromegaly patients using PEGV treatment had higher total ghrelin levels compared with healthy controls, patients with active acromegaly and inactive acromegaly (33). 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 (43). GH administration has been shown to suppress total ghrelin levels in GH deficient patients (44). In rodents, GH administration in cultured stomach tissue reduced total ghrelin secretion, whereas hypophysectomy increased ghrelin levels (45-47). 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 pegvisomant on ghrelin secretion, these data indirectly suggest that blockade of the GH receptor with pegvisomant leads to a positive feedback action on ghrelin secretion.

In patients using combination treatment median plasma AG levels were 8.5 pg/ml (range 0-140.6 pg/ml) and median UAG levels 26.9 pg/ml (0-177.5 pg/ml). This is considerably lower than levels that have been observed in healthy controls, measured using equivalent two-site sandwich assays and stabilized with AEBSF. Adrichem *et al* found median plasma AG levels of 57.2 pg/ml (range 10–273 pg/ml) and median plasma UAG levels of 64.9 pg/ml (range 8–331pg/ml) in 28 healthy controls, while Liu *et al* reported AG levels ranging from 43-366 pg/ml in four healthy volunteers (35, 48).

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 to 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 PWS, the AG/UAG ratio changed over time driving opposite phenotypes. While the AG/UAG ratio was low during infancy it switched to a high AG/UAG ratio at later 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 on 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 is not altered in acromegaly patients 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.

Declaration of interest

A. Muhammad received a research grant and a speaker fee from Novartis Pharma. A.J. van der Lely is a consultant for Novartis Pharma, Pfizer International, Alizé Pharma and received grants from Novartis Pharma, Ipsen Pharma International and Pfizer International. S. Neggers received research and speakers' fee grants from Ipsen Pharma International, Novartis Pharma, and Pfizer International. P.J. Delhanty, M. Huisman and J. A. Visser have nothing to declare.

Funding

This research did not receive any specific grant from any funding agency in the public, commercial or non-profit sector.

Author contributions

Conception and design: All authors
Collection and assembly of data: Ammar Muhammad
Data analysis and interpretation: All authors
Manuscript writing: All authors
Final approval of manuscript: All authors

Acknowledgements

The authors gratefully acknowledge the endocrine nurses at the Erasmus MC hospital for their excellent assistance with the collection of blood samples, and the study participants for their participation.

REFERENCES

1. **Date Y, Kojima M, Hosoda H, Sawaguchi A, Mondal MS, Suganuma T, Matsukura S, Kangawa K, Nakazato M.** Ghrelin, a novel growth hormone-releasing acylated peptide, is synthesized in a distinct endocrine cell type in the gastrointestinal tracts of rats and humans. *Endocrinology.* 2000;141:4255-4261.
2. **Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K.** Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature.* 1999;402:656-660.
3. **Tong J, Dave N, Mugundu GM, Davis HW, Gaylinn BD, Thorner MO, Tschop MH, D'Alessio D, Desai PB.** The pharmacokinetics of acyl, des-acyl, and total ghrelin in healthy human subjects. *European journal of endocrinology / European Federation of Endocrine Societies.* 2013;168:821-828.
4. **Date Y, Nakazato M, Hashiguchi S, Dezaki K, Mondal MS, Hosoda H, Kojima M, Kangawa K, Arima T, Matsuo H, Yada T, Matsukura S.** Ghrelin is present in pancreatic alpha-cells of humans and rats and stimulates insulin secretion. *Diabetes.* 2002;51:124-129.
5. **Gutierrez JA, Solenberg PJ, Perkins DR, Willency JA, Knierman MD, Jin Z, Witcher DR, Luo S, Onyia JE, Hale JE.** Ghrelin octanoylation mediated by an orphan lipid transferase. *Proceedings of the National Academy of Sciences of the United States of America.* 2008; 105:6320-6325.
6. **Lutter M, Sakata I, Osborne-Lawrence S, Rovinsky SA, Anderson JG, Jung S, Birnbaum S, Yanagisawa M, Elmquist JK, Nestler EJ, Zigman JM.** The orexigenic hormone ghrelin defends against depressive symptoms of chronic stress. *Nature neuroscience.* 2008;11: 752-753.
7. **Masuda Y, Tanaka T, Inomata N, Ohnuma N, Tanaka S, Itoh Z, Hosoda H, Kojima M, Kangawa K.** Ghrelin stimulates gastric acid secretion and motility in rats. *Biochemical and biophysical research communications.* 2000;276:905-908.
8. **Muccioli G, Broglio F, Valetto MR, Ghe C, Catapano F, Graziani A, Papotti M, Bisi G, Deghenghi R, Ghigo E.** Growth hormone-releasing peptides and the cardiovascular system. *Annales d'endocrinologie.* 2000;61:27-31.
9. **Ozcan B, Neggers SJ, Miller AR, Yang HC, Lucaites V, Abribat T, Allas S, Huisman M, Visser JA, Themmen AP, Sijbrands E, Delhanty P, Van der Lely AJ.** Does des-acyl ghrelin improve glycemic control in obese diabetic subjects by decreasing acylated ghrelin levels? *European journal of endocrinology / European Federation of Endocrine Societies.* 2013;
10. **Reed JA, Benoit SC, Pfluger PT, Tschop MH, D'Alessio DA, Seeley RJ.** Mice with chronically increased circulating ghrelin develop age-related glucose intolerance. *American journal of physiology. Endocrinology and metabolism.* 2008;294:E752-760.
11. **Yang J, Brown MS, Liang G, Grishin NV, Goldstein JL.** Identification of the acyltransferase that octanoylates ghrelin, an appetite-stimulating peptide hormone. *Cell.* 2008;132: 387-396.
12. **Delhanty PJ, Neggers SJ, van der Lely AJ.** Des-acyl ghrelin: a metabolically active peptide. *Endocrine development.* 2013;25:112-121.
13. **Barazzoni R, Zanetti M, Ferreira C, Vinci P, Pirulli A, Mucci M, Dore F, Fonda M, Ciocchi B, Cattin L, Guarnieri G.** Relationships between desacylated and acylated ghrelin and insulin sensitivity in the metabolic syndrome. *The Journal of clinical endocrinology and metabolism.* 2007;92:3935-3940.

14. **Broglio F, Koetsveld Pv P, Benso A, Gottero C, Prodam F, Papotti M, Muccioli G, Gauna C, Hofland L, Deghenghi R, Arvat E, Van Der Lely AJ, Ghigo E.** Ghrelin secretion is inhibited by either somatostatin or cortistatin in humans. *The Journal of clinical endocrinology and metabolism*. 2002;87:4829-4832.
15. **Gauna C, Meyler FM, Janssen JA, Delhanty PJ, Abribat T, van Koetsveld P, Hofland LJ, Broglio F, Ghigo E, van der Lely AJ.** Administration of acylated ghrelin reduces insulin sensitivity, whereas the combination of acylated plus unacylated ghrelin strongly improves insulin sensitivity. *The Journal of clinical endocrinology and metabolism*. 2004; 89:5035-5042.
16. **Kiewiet RM, van Aken MO, van der Weerd K, Uitterlinden P, Themmen AP, Hofland LJ, de Rijke YB, Delhanty PJ, Ghigo E, Abribat T, van der Lely AJ.** Effects of acute administration of acylated and unacylated ghrelin on glucose and insulin concentrations in morbidly obese subjects without overt diabetes. *European journal of endocrinology / European Federation of Endocrine Societies*. 2009;161:567-573.
17. **Vestergaard ET, Djurhuus CB, Gjedsted J, Nielsen S, Moller N, Holst JJ, Jorgensen JO, Schmitz O.** Acute effects of ghrelin administration on glucose and lipid metabolism. *The Journal of clinical endocrinology and metabolism*. 2008;93:438-444.
18. **Vestergaard ET, Gormsen LC, Jessen N, Lund S, Hansen TK, Moller N, Jorgensen JO.** Ghrelin infusion in humans induces acute insulin resistance and lipolysis independent of growth hormone signaling. *Diabetes*. 2008;57:3205-3210.
19. **Vestergaard ET, Hansen TK, Gormsen LC, Jakobsen P, Moller N, Christiansen JS, Jorgensen JO.** Constant intravenous ghrelin infusion in healthy young men: clinical pharmacokinetics and metabolic effects. *American journal of physiology. Endocrinology and metabolism*. 2007;292:E1829-1836.
20. **Beauloye V, Diene G, Kuppens R, Zech F, Winandy C, Molinas C, Faye S, Kieffer I, Beckers D, Nergardh R, Hauffa B, Derycke C, Delhanty P, Hokken-Koelega A, Tauber M.** High unacylated ghrelin levels support the concept of anorexia in infants with prader-willi syndrome. *Orphanet journal of rare diseases*. 2016;11:56.
21. **Cederberg H, Rajala U, Koivisto VM, Jokelainen J, Surcel HM, Keinanen-Kiukaanniemi S, Laakso M.** Unacylated ghrelin is associated with changes in body composition and body fat distribution during long-term exercise intervention. *European journal of endocrinology / European Federation of Endocrine Societies*. 2011;165:243-248.
22. **Kuppens RJ, Diene G, Bakker NE, Molinas C, Faye S, Nicolino M, Bernoux D, Delhanty PJ, van der Lely AJ, Allas S, Julien M, Delale T, Tauber M, Hokken-Koelega AC.** Elevated ratio of acylated to unacylated ghrelin in children and young adults with Prader-Willi syndrome. *Endocrine*. 2015;50:633-642.
23. **Pacifico L, Poggiogalle E, Costantino F, Anania C, Ferraro F, Chiarelli F, Chiesa C.** Acylated and nonacylated ghrelin levels and their associations with insulin resistance in obese and normal weight children with metabolic syndrome. *European journal of endocrinology / European Federation of Endocrine Societies*. 2009;161:861-870.
24. **Rodriguez A, Gomez-Ambrosi J, Catalan V, Becerril S, Sainz N, Gil MJ, Silva C, Salvador J, Barba J, Colina I, Fruhbeck G.** Association of plasma acylated ghrelin with blood pressure and left ventricular mass in patients with metabolic syndrome. *Journal of hypertension*. 2010;28:560-567.
25. **St-Pierre DH, Karelis AD, Coderre L, Malita F, Fontaine J, Mignault D, Brochu M, Bastard JP, Cianflone K, Doucet E, Imbeault P, Rabasa-Lhoret R.** Association of acylated

and nonacylated ghrelin with insulin sensitivity in overweight and obese postmenopausal women. *The Journal of clinical endocrinology and metabolism*. 2007;92:264-269.

26. **Miller JL, Lynn CH, Driscoll DC, Goldstone AP, Gold JA, Kimonis V, Dykens E, Butler MG, Shuster JJ, Driscoll DJ.** Nutritional phases in Prader-Willi syndrome. *American journal of medical genetics. Part A*. 2011;155A:1040-1049.
27. **Furiya Y, Tomiyama T, Izumi T, Ohba N, Ueno S.** Rivastigmine Improves Appetite by Increasing the Plasma Acyl/Des-Acyl Ghrelin Ratio and Cortisol in Alzheimer Disease. *Dement Geriatr Cogn Dis Extra*. 2018;8:77-84.
28. **Cappiello V, Ronchi C, Morpurgo PS, Epaminonda P, Arosio M, Beck-Peccoz P, Spada A.** Circulating ghrelin levels in basal conditions and during glucose tolerance test in acromegalic patients. *European journal of endocrinology / European Federation of Endocrine Societies*. 2002;147:189-194.
29. **Freda PU, Reyes CM, Conwell IM, Sundeen RE, Wardlaw SL.** Serum ghrelin levels in acromegaly: effects of surgical and long-acting octreotide therapy. *The Journal of clinical endocrinology and metabolism*. 2003;88:2037-2044.
30. **Kozakowski J, Rabijewski M, Zgliczynski W.** [Lowered ghrelin levels in acromegaly-normalization after treatment]. *Endokrynologia Polska*. 2006;56:862-870.
31. **Norrelund H, Hansen TK, Orskov H, Hosoda H, Kojima M, Kangawa K, Weeke J, Moller N, Christiansen JS, Jorgensen JO.** Ghrelin immunoreactivity in human plasma is suppressed by somatostatin. *Clinical endocrinology*. 2002;57:539-546.
32. **Wasko R, Jaskula M, Komarowska H, Zamyslowska H, Sowinski J, Waligorska-Stachura J.** Ghrelin concentrations in acromegalic patients in relation to the administered therapy. *Neuro endocrinology letters*. 2006;27:162-168.
33. **Roemmler J, Otto B, Arafat AM, Bidlingmaier M, Schopohl J.** Influence of pegvisomant on serum ghrelin and leptin levels in acromegalic patients. *European journal of endocrinology / European Federation of Endocrine Societies*. 2010;163:727-734.
34. **Delhanty PJ, Huisman M, Julien M, Mouchain K, Brune P, Themmen AP, Abribat T, van der Lely AJ.** The acylated (AG) to unacylated (UAG) ghrelin ratio in esterase inhibitor-treated blood is higher than previously described. *Clinical endocrinology*. 2015; 82:142-146.
35. **van Adrichem RC, van der Lely AJ, Huisman M, Kramer P, Feelders RA, Delhanty PJ, de Herder WW.** Plasma acylated and plasma unacylated ghrelin: useful new biomarkers in patients with neuroendocrine tumors? *Endocrine connections*. 2016;5:143-151.
36. **Manolopoulou J, Alami Y, Petersenn S, Schopohl J, Wu Z, Strasburger CJ, Bidlingmaier M.** Automated 22-kD growth hormone-specific assay without interference from Pegvisomant. *Clinical chemistry*. 2012;58:1446-1456.
37. **Blatnik M, Soderstrom CI.** A practical guide for the stabilization of acylghrelin in human blood collections. *Clinical endocrinology*. 2011;74:325-331.
38. **Akamizu T, Shinomiya T, Irako T, Fukunaga M, Nakai Y, Nakai Y, Kangawa K.** Separate measurement of plasma levels of acylated and desacyl ghrelin in healthy subjects using a new direct ELISA assay. *The Journal of clinical endocrinology and metabolism*. 2005;90: 6-9.
39. **Prudom C, Liu J, Patrie J, Gaylinn BD, Foster-Schubert KE, Cummings DE, Thorner MO, Geysen HM.** Comparison of competitive radioimmunoassays and two-site sandwich assays for the measurement and interpretation of plasma ghrelin levels. *The Journal of clinical endocrinology and metabolism*. 2010;95:2351-2358.

40. **Blijdorp K, van der Lely AJ, van den Heuvel-Eibrink MM, Huisman TM, Themmen AP, Delhanty PJ, Neggers SJ.** Desacyl ghrelin is influenced by changes in insulin concentration during an insulin tolerance test. *Growth hormone & IGF research : official journal of the Growth Hormone Research Society and the International IGF Research Society.* 2013; 23:193-195.
41. **Flanagan DE, Evans ML, Monsod TP, Rife F, Heptulla RA, Tamborlane WV, Sherwin RS.** The influence of insulin on circulating ghrelin. *American journal of physiology. Endocrinology and metabolism.* 2003;284:E313-316.
42. **Saad MF, Bernaba B, Hwu CM, Jinagouda S, Fahmi S, Kogosov E, Boyadjian R.** Insulin regulates plasma ghrelin concentration. *The Journal of clinical endocrinology and metabolism.* 2002;87:3997-4000.
43. **Korbonits M, Bustin SA, Kojima M, Jordan S, Adams EF, Lowe DG, Kangawa K, Grossman AB.** The expression of the growth hormone secretagogue receptor ligand ghrelin in normal and abnormal human pituitary and other neuroendocrine tumors. *The Journal of clinical endocrinology and metabolism.* 2001;86:881-887.
44. **Eden Engstrom B, Burman P, Holdstock C, Karlsson FA.** Effects of growth hormone (GH) on ghrelin, leptin, and adiponectin in GH-deficient patients. *The Journal of clinical endocrinology and metabolism.* 2003;88:5193-5198.
45. **Qi X, Reed J, Englander EW, Chandrashekar V, Bartke A, Greeley GH, Jr.** Evidence that growth hormone exerts a feedback effect on stomach ghrelin production and secretion. *Experimental biology and medicine.* 2003;228:1028-1032.
46. **Seoane LM, Al-Massadi O, Barreiro F, Dieguez C, Casanueva FF.** Growth hormone and somatostatin directly inhibit gastric ghrelin secretion. An in vitro organ culture system. *Journal of endocrinological investigation.* 2007;30:RC22-25.
47. **Tschop M, Flora DB, Mayer JP, Heiman ML.** Hypophysectomy prevents ghrelin-induced adiposity and increases gastric ghrelin secretion in rats. *Obesity research.* 2002;10: 991-999.
48. **Liu J, Prudom CE, Nass R, Pezzoli SS, Oliveri MC, Johnson ML, Veldhuis P, Gordon DA, Howard AD, Witcher DR, Geysen HM, Gaylinn BD, Thorner MO.** Novel ghrelin assays provide evidence for independent regulation of ghrelin acylation and secretion in healthy young men. *The Journal of clinical endocrinology and metabolism.* 2008;93:1980-1987.