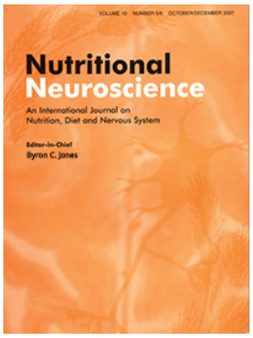

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Association between des-acyl ghrelin at fasting and predictive index of muscle derangement, metabolic markers and eating disorders: a cross-sectional study in overweight and obese adults

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ABSTRACT

Background: This study aimed to analyse the impact of des-acyl and acyl ghrelin (AG) on a wide range of muscular and metabolic markers and in order to discover the possible relationships and interactions of des-acylated ghrelin (DAG) on eating disorders.

Materials & Methods: A total of 88 subjects (64 women and 24 men, with a mean age of 43 years and a mean body mass index (BMI) of 30.20 ± 3.27 kg/m^2) were enrolled in the cross-sectional study.

Results: The findings showed that for each unit of increase of free fat mass index (FFMI), levels of DAG decreased by −41.11 pg/mL ($p < 0.05$). Moreover, similar associations with DAG were found for insulin ($\beta = -30.67$; $p < 0.001$), leptin ($\beta = -0.64$; $p < 0.05$), body weight ($\beta = -14.36$; $p < 0.001$), and free fat mass (FFM) ($\beta = -30.67$; $p < 0.001$). In addition, associations were found between DAG and resting energy expenditure (REE) ($\beta = -0.84$; $p = 0.05$) and the binge eating scale (BES) in which a unit increase of the BES score Q3 (depression) correlated with a decrease of DAG levels ($\beta = -9.98$; $p = 0.08$). Further, a unit increase of AG/DAG ratio correspond with an increase in body weight ($\beta = 12.20$; $p < 0.05$), BMI ($\beta = 4.70$; $p < 0.05$) and fat mass ($\beta = 7.30$; $p < 0.05$). However, the AG/DAG ratio was not associated with FFMI ($\beta = 2.61$; $p = 0.165$) and FFML/BMI ($\beta = -0{,}064$; $p = 0.625$).

Conclusion: This study suggests that higher levels of DAG at fasting are indices of poor muscle mass, insulin resistance and depression.

KEYWORDS
Ghrelin; des-acyl ghrelin; muscle; leptin; muscle atrophy; body composition; satiety; energy expenditure

Introduction

The history of des-acyl ghrelin (DAG) started in 1999, when Kojima et al. [1] described this peptide in the same report that presented ghrelin to the scientific community. A little over twenty years has elapsed since ghrelin was identified as a natural ligand of the growth hormone (GH) secretagogue receptor type 1a (GHSR1a). In addition to ghrelin, the preproghrelin gene-derived peptides include acyl ghrelin (AG), DAG, and obestatin [2]. AG is produced primarily in the stomach and exerts its central and peripheral effects through GHSR1a [1]. The acylation of the ghrelin peptide is catalyzed by the enzyme ghrelin O-acyl transferase (GOAT), which is mostly expressed in the stomach and intestine [2].

DAG represents by far the most abundant form (~90%) of circulating ghrelin, whereas AG constitutes only 10% of the circulating hormone. Nevertheless, the acylated and not the des-acyl form is able to interact with the GHS-R1a receptors and activate the inositol triphosphate pathway [3]. The reason being the inability of DAG to bind and activate the GHSR1ain the submicromolar, physiological range [1]. In spite of this fact, an increasing number of reports support the notion that DAG may have intrinsic activities in a variety of physiological and pathophysiological situations [4]. For instance, DAG can either antagonize or support the activities of AG, while in other cases, DAG works completely independently of AG [5].

The two major molecular forms of ghrelin peptide, AG and DAG, exist in the stomach and hypothalamus. Acylation is indispensable for the binding of ghrelin to the GHS-R1a [6]. Ghrelin enhances feeding via the neuronal pathways of neuropeptide Y and orexin, which act as orexigenic peptides in the hypothalamus [7].

The ghrelin plays an important role on nutritional state and on body composition, because of upregulation

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of hunger, particularly in overweight and obese conditions [8].

More specifically, in vivo experiments show that DAG directly targeting ARC neurons, and could be potentially considered an anorexigenic hormone [9,10]. At the same time, more than one study in animals suggests a positive association between ghrelin administration (in particular DAG) and muscle trophism [11].

The possible associations between muscle metabolism and ghrelin has also been explored only in few studies in human, because may stimulate lipolysis directly in skeletal muscle in healthy subjects but other studies report controversial results, which suggest that ghrelin has different and more complicated mechanisms of action on human muscle tissue [12,13]

By now, what is still controversial in humans is if the ghrelin is a muscle trophic factor only by increasing appetite and food intake or if the positive effects are independent from the calorie intake, as reported in vitro animals and preliminary studies on humans (Figure 1).

The inefficacy of endogenous ghrelin and leptin in properly regulating hunger (and weight) in overweight obese and anorectic subjects also suggests that a condition called 'ghrelin resistance' may be accounted together with the leptin resistance. In humans, ghrelin resistance hasn't been defined yet, even if in literature it is explained to be a physiological adaptation to a chronic energy disbalance [13].

In animals, the ghrelin resistance consists in an inefficacy of ghrelin in improving hunger and nutritional state despite the administration of ghrelin [14]. An experimental study by Briggs et al. also reports that ghrelin resistance in vivo is a product of neuroendocrine and neuronal mechanisms that decrease ghrelin production and suppresses general responsiveness to ghrelin, and this represents an attempt to defend neutral energy balance [15].

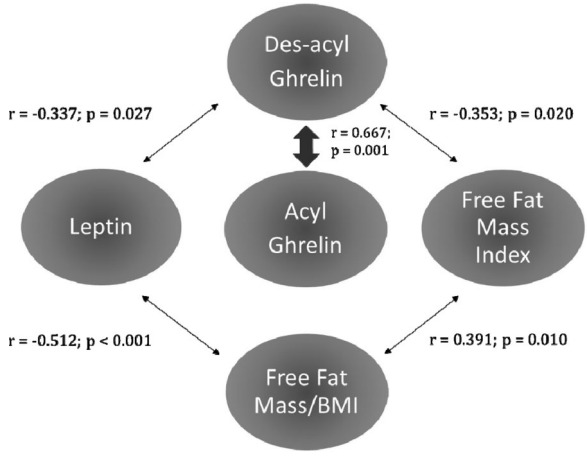

Figure 1. Conceptual model explaining the role of leptin as a mediator where there is a negative association between des-acyl ghrelin and leptin and des-acyl ghrelin with free fat mass index.

In this context, the primary aim of this study is to investigate, in a cohort of metabolically healthy overweight and obese subjects, the possible associations between hunger/satiety indicators at fasting (AG, DAG, total ghrelin, AG/DAG, leptin) and a wide range of parameters involving nutritional state, body composition, lipid profile, glucose metabolism and binge eating disorders.

Materials and methods

Participants

The study was approved by the institutional review board at the University of Pavia and the Ethics Committee of the Department of Internal Medicine and Medical Therapy at the University of Pavia, Italy and carried out in accordance with the principles of the Helsinki Declaration. Written informed consent for the study was obtained for all participants before they entered the study.

Healthy males and females between 25 and 45 years, with a body mass index (BMI) greater than 25 and less than 35 kg/m^2 were eligible for the study. All subjects had to provide complete medical histories, and all underwent a physical examination, anthropometric assessment and routine laboratory testing. Patients were excluded if they suffered from any hepatic or renal disease, diabetes, unstable cardiovascular disease, uncontrolled hypertension, an eating disorder (diagnosed bulimia), had active cancer, had undergone surgery for weight loss, had taken any weight loss medication or had met the Diagnostic and Statistical Manual-IV (DSM-IV) criteria for depressive disorder as determined by the Structured Clinical Interview for DSM-IV Axis 1 Disorders (SCID-1) [16].

Female patients were also excluded if they were pregnant or lactating, or had entered menopause. Alcohol intake, smoking habits and physical activity were recorded. Sedentary and non-smoking subjects, who did not drink more than 6 glasses of wine a week and did not consume hard liquor, were admitted to the study.

Procedures and study design

The cross-sectional study was performed at the Endocrinology and Clinical Nutrition Unit of Azienda di Servizi alla Persona di Pavia at the University of Pavia (Italy) and at the Dietetic and Metabolic Unit. After 12 h of fasting and abstinence from water since midnight, the

subjects arrived at around 8:00 am and blood samples were taken for routine analysis and for the measurements of leptin, ghrelin, insulin, glycerol and free fatty acid (FFA) levels. The assessment of resting energy expenditure (REE) and respiratory quotient (RQ) by indirect calorimetry, as well as the assessment of body composition by dual-energy X-ray absorptiometry (DXA) and anthropometry measurements was carried out in the fasting state at baseline. Twenty four-hour urine sample were brought by the subjects on the same morning for urinary nitrogen assessment of protein.

Body composition measurement

Bone mineral density and body composition including fat mass (FM), free fat mass (FFM), and lean mass was measured at baseline by using a Lunar Prodigy DXA scanner (GE Medical Systems, Waukesha, WI, USA) as previously described [17].

Assessment of resting energy expenditure

Respiratory exchange measurements using indirect calorimetry (Deltatrac Monitor II MBM-200, Datex Engstrom Division, Instruments Corp. Helsinki, Finland) were used to estimate REE, adhering to the recommended measurement conditions [18]. REE was calculated from O_2 and CO_2 volumes, as well as, urinary nitrogen using the Weir formula [19], and is expressed as kcal/day.

Anthropometry

Body weight and height were measured using conventional techniques and the body mass index (BMI) was calculated as weight/height squared (kg/m^2). Anthropometric variables were measured by a single investigator.

Biochemical analyses

Fasting venous blood samples were drawn between 08.00 and 10.00 am with the subjects in a sitting position. Clinical Chemistry parameters were detected on the Roche Cobas Integra 400 plus analyzer (Roche Diagnostics, Basel, Switzerland), using specially designed commercial kits provided by the manufacturer. In particular, total serum cholesterol, triglycerides, high density lipoprotein (HDL) cholesterol, total proteins, total bilirubin, iron, glucose, uric acid, creatinine, transaminase alanine aminotransferase, aspartate aminotransferase and gamma glutamyl transferase were measured using enzymatic-colorimetric methods. Low density lipoprotein (LDL) cholesterol was calculated with the Friedewald formula [20] for those specimens with triglyceride levels less than 400 mg/dL (<4.5 mmol/L). The C-reactive protein (CRP) was determined by Nephelometric High-Sensitivity CRP assay (Dade Behring, Marburg, Germany). Hemochrome was measured using a Coulter automated cell counter MAX-M (Beckman Coulter, Inc., Fullerton, USA). Serum insulin levels were measured on a Roche Elecsys 2010 analyzer (Roche Diagnostics, Basel, Switzerland) using dedicated commercial electrochemiluminescent immunoassays. Insulin resistance was evaluated using the homeostasis model assessment (HOMA) method [21] and the quantitative insulin-sensitivity check index (QUICKI) [22].

Serum concentrations of FFA and glycerol were determined by a quantitative colorimetric assay (BioAssay Systems, Hayward, CA, USA). Plasma AG and DAG levels were measured using an enzyme immunometric assay based on a double-antibody sandwich technique (BioVendor, Brno, Czech Republic). Serum leptin levels were measured using an enzyme linked immunosorbent assay (ELISA) (R&D Systems Inc., Minneapolis, MN, USA). Serum vascular endothelial growth factor (VEGF) levels were measured using ELISA kit (R&D Systems Inc., Minneapolis, MN, USA).

Assessment of depressive symptoms and health-related quality of life

Depressive symptoms, assessed by the Beck depression inventory (BDI) [23] and health-related quality of life, assessed by the Short-Form 36-Item Health Survey (SF-36) [24], were evaluated at baseline and after 2 months in both groups.

Statistical analysis

All analyses were performed using Statistical Package for the Social Sciences, version 22.0 (SPSS Inc., Chicago, IL, USA). Descriptive statistics representing raw data for each of the three categories and the full sample were provided, including means, standard deviations and frequencies, where appropriate.

After the verification of the normal distribution of the continuous variables, data were analyzed and statistically compared between groups using one-way analysis of variance (ANOVA). Variances were considered to be statistically significant for p value < 0.05. Linear regression was used to predict the effects of ghrelin on different metabolic outcomes. Selection of independent variables was performed manually to identify the strongest model.

Results

Baseline characteristics of the sample are shown in Table 1. A total of 88 subjects (64 women and 24 men) with a mean age of 43 years were enrolled following the inclusion/exclusion criteria. Moreover, the study result revealed that the mean of BMI was 30.20 ± 3.27 kg/m^2, and subjects were metabolically healthy with normal glucose and cholesterol levels.

Table 2 shows the associations between a path of different biomarkers with DAG and AG. The data showed that for each unit of increase of the FFMI resulted in the decrease of DAG(−41.11; $p < 0.005$). Associations with DAG were also found for insulin ($\beta = -30.67$; $p < 0.001$), leptin ($\beta = -0.64$; $p < 0.05$), body weight ($\beta = -14.36$; $p < 0.001$), and for FFM ($\beta = -30.67$; $p < 0.001$).

In Table 3, are shows the relationship between des-acyl ghrelin with others variables (considering the mediator effect of leptin). For each unit increase of the ratio of AG/DAG there was a significant decrease of weight ($\beta = 12.20$; $p < 0.05$), BMI ($\beta = 4.70$; $p < 0.05$), and FM ($\beta = -7.30$; $p < 0.05$).

Table 1. Baseline characteristics of subjects.

Variables	Mean; SD
Age (year)	43.26 ± 9.29
Des-acyl ghrelin (pg/mL)	408.37 ± 274.60
Acyl ghrelin (pg/mL)	94.37 ± 66.46
Total Cholesterol (mg/dL)	205.60 ± 33.75
HDL Cholesterol (mg/dL)	53.72 ± 13.36
LDL Cholesterol (mg/dL)	109.09 ± 31.85
Cholesterol/HDL (n)	4.02 ± 1.11
Triglycerides (mg/dL)	113.93 ± 74.38
CRP (mg/dL)	0.40 ± 0.54
Glycemia (mg/dL)	90.10 ± 9.58
Insulin (mcIU/mL)	10.14 ± 4.58
HOMA-IR (score)	2.29 ± 1.16
QUICKI (score)	0.34 ± 0.04
ApoA-I (mg/dL)	120.71 ± 27.36
ApoB (mg/dL)	80.62 ± 26.00
Leptin (ng/dL)	223.45 ± 157.21
VEGF (pg/ml)	309.71 ± 226.29
GLP-1 (pmol/L)	1.28 ± 0.50
Height (m)	1.65 ± 0.07
Weight (kg)	82.82 ± 12.06
BMI (kg/m^2)	30.20 ± 3.27
Fat Mass (kg)	33.94 ± 12.06
Lean Mass (kg)	45.71 ± 9.87
Android Fat (%)	49.71 ± 5.82
Gynoid Fat (%)	46.86 ± 9.37
FFMI (score)	27.49 ± 4.94
FFM/BMI (*n*)	1.51 ± 0.29
Estimated Basal Metabolism (Kcal/day)	1467.36 ± 319.88
RQ (*n*)	0.80 ± 0.13
Protein (%)	20.22 ± 15.66
Carbohydrate (%)	49.00 ± 7.07
Fat (%)	53.83 ± 25.63
Beck Depression Inventory (score)	7.51 ± 6.38
Binge Eating Scale (score)	9.16 ± 8.05
BMR (kcal/day)	1462.34 ± 301.89
Level of Education	
Primary	0.5
Intermediate	3.3
Secondary	55.5
Diploma/University	40.7
Total	100.0
Socioeconomical status	
Employee	68.3
Nonemployee	31.7
Total	100

Abbreviations: ApoA-I, apolipoprotein A-I; ApoB, apolipoprotein B; BMI, body mass index; BMR, basal metabolic rate; CRP, C-reactive protein; FFMI, free fat mass index; GLP-1, glucagon-like peptide-1; HDL, high-density lipoprotein; HOMA-IR, homeostatic model assessmentof insulin resistance; LDL, low-density lipoprotein;QUICKI, quantitative insulin-sensitivity check index;RQ, respiratory quotient; VEGF, vascular endothelial growth factor.

Discussion

The primary aim of this study has been an attempt to explain the relationship between DAG and muscle metabolism markers and satiety in overweight and obese adults.

The first finding of this study is that there is a linear relationship between low levels of DAG and many positive outcomes linked to muscle mass and metabolic health. In addition, we have kept in mind the strong negative association between levels of DAG and FFM index. Specifically, high plasma levels of DAG influenced FFM and insulin. These findings are supported by the study of Zhang et al. [25] who demonstrated that overexpression of ghrelin from the fatty acid-binding protein-4 (FABP4) promoter impairs the development of white adipose tissues, and alters glucose tolerance and insulin sensitivity in mice.

Furthermore, we show that DAG affects binge eating disorders, which is in line with previous research that has also suggested that ghrelin is involved with eating disorders [26]. As shown in the review of Méquinion et al. [27], typically elevated plasma levels of ghrelin and DAG found in anorexia nervosa patients was not sufficient to provide physiological signal of hunger leading to nutrient intake. Moreover, the authors suggested that the paradoxical increase in plasma ghrelin levels might be the result of resistance to ghrelin in anorexia nervosa. This is an important endeavor because this data has been confirmed using the association with a standardized variable such as the AG/DAG ratio.

Similar negative associations of DAG have also been found on glucagon-like peptide-1(GLP-1) hormone level and on body weight and BMI. This finding has been confirmed by Djurhuus et al. [28], who reported an inverse relationship between GLP-1 and ghrelin and raised the question of a common hypothalamic control mechanism of the two appetite-regulating peptides.

It has to be noted that in this study, it was assumed that obese individuals often have increased appetite despite normal plasma levels of the main orexigenic hormone ghrelin. This has been supported by literature that shows that DAG decreases food intake and disrupts the

Table 2. Effect of Des-acyl and acyl ghrelin (adjusted for sex, age, and BMI) on a path of bio-body markers.

	Des-acyl Ghrelin (pg/mL)			Acyl Ghrelin (pg/mL)		
Variables	*B*	CI 95%	*P* value	*B*	CI 95%	Sig.
FFMI (score)	−41.11	−75.48; −6.75	**0.05**	−4.73	−13.98; 4.51	0.30
TC (mg/dL)	0.93	−1.62; 3.49	0.46	−0.10	−0.71; 0.50	0.73
CRP (mg/dL)	−23.22	−147.45; 100.99	0.70	−1.11	−30.51; 28.29	0.93
Glycemia (mg/dL)	−7.99	−16.80; 0.80	**0.07**	−1.43	−3.55; 0.68	0.18
Insulin (mcIU/mL)	−30.67	−48.45; −12.88	**0.001**	−4.78	−9.33; −0.23	**0.05**
ApoA-I (mg/dL)	4.09	0,02; 8,17	***P* < 0.05**	0.07	−0.93; 1.08	0.88
ApoB (mg/dL)	−0.54	−4,53; 3,44	0.78	−0.10	−1.04; 0.83	0.82
Leptin (ng/dL)	−0.64	−1.26; −0.03	**0.05**	0.002	−0.15; 0.15	0.98
VEGF (pg/ml)	−0.21	−0.68; 0.24	0.34	−0.05	−0.16; 0.06	0.35
GLP-1 (pmol/L)	−71.28	−254.42; 111.85	0.42	−19.60	−78.98; 39.77	0.49
Weight (kg)	−14.36	−23.76; −4.97	**0.01**	0.10	2.36; 2.57	0.93
BMR (kcal/day)	−0.84	−1.69; −0.14	**0.05**	−0.06	−0.27; 0.14	0.52
Fat Mass (kg)	−14.37	−27.75; −1.00	**0.05**	1.14	−2.17; 4.47	0.48
Free Fat Mass (kg)	−30,88	−51.95; −9.82	**.001**	−2.71	−8.14; 2.71	0.31
Android Fat (%)	−15.27	−33.21; 2.67	0.09	0.31	−4.08; 4.70	0.88
Gynoid Fat (%)	9.48	−10.29; 29.26	0.33	4.72	0.24; 9.20	**0.05**
BDI (score)	−6.60	−22.35; 9.15	0.40	−4.72	−8.16; −1.27	**0.001**
BES (score)	−9.98	−21.18; 1.20	0.08	−2.64	−5.26; −0.01	**0.05**

Abbreviations: ApoA-I, apolipoprotein A-I; ApoB, Apolipoprotein B;BDI, Beck Depression Inventory; BES, binge eating scale; BMR, basal metabolic rate; CRP, C-reactive protein; FFMI, free fat mass index; GLP-1, glucagon-like peptide-1;TC, total cholesterol; VEGF, vascular endothelial growth factor.

Table 3. Relationship between des-acyl ghrelin with others variables (considering the mediator effect of leptin).

Factor* (independent)	Predictor* (dependent)	*β*	*P*-value	CI 95% Lower	CI 95% Higher
AG/DAG >	HDL Cholesterol	−29.021	0.281	−82.657	24.616
AG/DAG >	LDL Cholesterol	4.819	0.841	−43.555	53.193
AG/DAG >	Triglycerides	−99.299	0.080	−211.153	12.555
AG/DAG >	CRP	−0.249	0.619	−1.253	0.756
AG/DAG >	Glycemia	−1.443	0.856	−17.406	14.520
AG/DAG >	Insulin	−0.296	0.923	−6.437	5.846
AG/DAG >	HOMA Index	−0.204	0.797	−1.798	1.389
AG/DAG >	ApoA-I	−25.900	0.109	−57.802	6.003
AG/DAG >	ApoB	7.974	0.644	−26.590	42.537
AG/DAG >	Noradrenalin	23.573	0.296	−21.374	68.520
AG/DAG >	Adrenaline	−0.724	0.786	−6.092	4.644
AG/DAG >	Adiponectin	−45.760	0.331	−139.821	48.301
AG/DAG >	VEGF	−53.748	0.718	−352.922	245.426
AG/DAG >	GLP-1	−0.229	0.712	−1.530	1.071
AG/DAG >	Weight	12.201	***P* < 0.05**	0.250	24.152
AG/DAG >	BMI	4.761	***P* < 0.05**	0.930	8.592
AG/DAG >	Waist Circumference	8.098	0.195	−4.322	20.518
AG/DAG >	Hip Circumference	3.838	0.359	−4.522	12.199
AG/DAG >	Waist-Hip Ratio	0.044	0.435	−0.069	0.158
AG/DAG >	Total Body Water	2.180	0.434	−3.391	7.751
AG/DAG >	Extracellular Water	−0.075	0.951	−2.527	2.376
AG/DAG >	BMR	88.930	0.275	−73.325	251.186
AG/DAG >	Fat Mass	7.375	***P* < 0.05**	1.001	13.750
AG/DAG >	Free Fat Mass	4.092	0.299	−3.765	11.949
AG/DAG >	Android Fat Mass	0.581	0.848	−5.506	6.668
AG/DAG >	Gynoid Fat Mass	1.847	0.555	−4.422	8.117
AG/DAG >	Android/Gynoid Ratio	−0.033	0.693	−0.201	0.135
AG/DAG >	BQ	246.123	0.205	−139.562	631.808
AG/DAG >	FFMI	2.609	***P* < 0.05**	−1.120	6.339
AG/DAG >	FFM/BMI	−0.064	0.625	−0.329	0.200

Abbreviations: ApoA-I, apolipoprotein A-I; ApoB, Apolipoprotein B;BMI, body mass index; BMR, basal metabolic rate; BQ, Beck Questionnaire, CRP, C-reactive protein;FFMI, free fat mass index; GLP-1, glucagon-like peptide-1; HDL, high-density lipoprotein;HOMA, homeostatic model assessment; LDL, low-density lipoprotein; VEGF, vascular endothelial growth factor.

*The linear regression model has been adjusted for regressors age and gender.

fasted motor activity of the antrum in freely moving conscious rats [29]. Of potential clinical importance is that research suggests that DAG might be a functional inhibitor of ghrelin and that DAG can suppress ghrelin levels in humans [5].

Our findings are in accordance with Delhanty et al. [5], which highlight the controversial aspects of the AG/DAG ratio. Although available data are still contradictory, several researches have indicated that obese mice and humans might have lower DAG levels than normal-

weight subjects whereas AG levels are similar, indicating that obesity could reflect a relative DAG deficiency.

Despite the interesting association found between DAG and BES, this resulted not strong enough to reach the statistical significance; instead a negative statistically significant association was found between AG and BES and between AG and BDI. This suggests that low total ghrelin may still be a stronger indicator of binge eating disorders.

Additionally, blood samples were collected in the morning, but we know that ghrelin levels have a sinusoidal evolution during the 24 h and that they are highly dependent on patient meal habits; due to this fact, our study may show multiple bias linked to the nature of this hormone, which has a pulsatory and not constant secretion [30]. Finally, due to the instability of ghrelin molecule in blood and plasma samples, a portion of AG desacylates during the storage of samples [31].

On the other hand, the purpose of this study was to give preliminary evidence of the associations between ghrelin and multiple biological markers, thus assuming that hormone secretion was similar for all patients.

This study suggests that an inverse association exists between DAG and muscle index. Furthermore, the findings of the present study suggest that higher levels of DAG at fasting are indices of poor muscle mass and insulin resistance. The AG/DAG ratio is also associated with FFM and may be verified as an index of sarcopenia.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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