CLINICAL STUDY

Acylated and nonacylated ghrelin levels and their associations with insulin resistance in obese and normal weight children with metabolic syndrome

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Abstract

Background: Ghrelin, a peptide mainly derived from the stomach, plays a pivotal role in the regulation of food intake, energy metabolism, and storage, as well as in insulin sensitivity. Ghrelin circulates in acylated (A-Ghr) and nonacylated (NA-Ghr) forms, and their potential differential associations with insulin resistance (IR) in childhood obesity remain undefined.

Objective: We investigated the associations of ghrelin forms with IR in normal weight and obese children and the impact of metabolic syndrome (MS) on their plasma values.

Design: A total of 210 children in four subgroups of normal weight/obese children with and without components of MS were studied. Fasting blood glucose, insulin, lipid profile, and acylated and total ghrelin were examined. IR was determined by a homeostasis model assessment (HOMA) of IR.

Results: In the entire population, plasma insulin and HOMA-IR were associated negatively with T-Ghr and NA-Ghr, but positively with the ratio of A/NA-Ghr after adjustment for age, gender, and Tanner stage. Obese metabolically abnormal children had lower T-Ghr and NA-Ghr, but comparable A-Ghr and a higher A/NA-Ghr ratio than obese metabolically normal subjects. Compared with lean healthy children, lean metabolically abnormal subjects had higher A-Ghr and the A/NA-Ghr ratio, but comparable T-Ghr and NA-Ghr. A multiple regression analysis showed that A-Ghr and the A/NA-Ghr ratios were positively associated with HOMA-IR, independent of age, gender, Tanner stage, and body mass index (or waist circumference) and other components of MS.

Conclusions: A-Ghr excess may negatively modulate insulin action in obese and nonobese children, and may contribute to the association of IR and MS.

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Introduction

Ghrelin, a 28-amino acid peptide predominantly produced by the stomach, is expressed in many other central and peripheral tissues including hypothalamus, liver, kidney, pituitary gland, endocrine pancreas, and adipose tissue (1, 2). In addition to potent GH-releasing activity, ghrelin is involved in the control of food intake and energy metabolism at central levels as well as at peripheral levels influencing the endocrine pancreatic function and glucose and lipid metabolism (3, 4). In humans, circulating levels of ghrelin are increased in anorexia and cachexia, but are reduced in obesity, insulin resistance (IR) and type 2 diabetes. (1, 4–6). Several *in vitro* and *in vivo* studies have shown that ghrelin may regulate insulin and glucose metabolism (7–11). Ghrelin was shown to inhibit insulin secretion from pancreatic islets in rodents (9). Intravenous administration of ghrelin to healthy young men

impaired insulin sensitivity (10). In obese women with polycystic ovary syndrome, ghrelin induced a significant decrease in insulin concentrations; an increase (though not significant) in glucose levels was also observed (11). Earlier studies on ghrelin and energy balance and metabolism were performed by total ghrelin assays. However, two forms of ghrelin have been described recently: acylated ghrelin (A-Ghr) and nonacylated ghrelin (NA-Ghr). NA-Ghr is the major circulating form and constitutes 80–90% of circulating ghrelin. Although it was originally thought that NA-Ghr lacked endocrine and biological actions, more recent findings suggest that both A-Ghr and NA-Ghr may mediate peripheral biological actions; indeed, there is a suggestion that both may act antagonistically (1, 3, 12–16). Some studies have provided evidence that A-Ghr and NA-Ghr have different effects on insulin metabolism. Gauna *et al.* reported that glucose output by primary hepatocytes is stimulated by A-Ghr and is inhibited by

NA-Ghr (13). Broglio *et al.* demonstrated that NA-Ghr counteracted the stimulatory effect of A-Ghr on glucose release, and that it was able to antagonize the influence of A-Ghr on insulin secretion and glucose metabolism (14). Under pharmacological concentrations, A-Ghr induced IR, whereas a combination of NA-Ghr and A-Ghr improved insulin sensitivity (15, 16). These results suggest that A-Ghr may act as a diabetogenic factor and may therefore potentially modulate a deterioration of insulin sensitivity.

The relation of A-Ghr to NA-Ghr may also have a clinical significance in metabolic disorders as it has been demonstrated recently in adults. Obesity altered circulating ghrelin profile, and relative A-Ghr excess was suggested to contribute to obesity-associated IR in metabolic syndrome (MS) (17). Yet, both increased A-Ghr physiological concentrations, and elevated A/NA-Ghr ratios were associated with IR in obese and overweight postmenopausal women (18). Taken together, these findings provide some evidence of differential associations of ghrelin forms with insulin action in obese adults. However, relatively little information exists in obese children (19, 20). There is ample evidence suggesting that the complications of obesity seen with adults begin in early childhood (21, 22). IR, hypertension, glucose intolerance, and type 2 diabetes mellitus have all been associated with childhood obesity (23, 24). The concomitant occurrence of these abnormalities, referred to as MS, accrues a significant risk for the development of cardiovascular disease and diabetes during early adulthood, if not during childhood. Obesity in children is in rapid expansion across the world, and studies in this age group are important.

Therefore, the aims of this study were to explore in children i) the associations of ghrelin secretion profiles, including T-Ghr, A-Ghr, NA-Ghr, and the A/NA-Ghr ratio, with IR independently of obesity, as well as of other components of MS; and ii) the impact of MS and its single components on ghrelin forms.

Methods

Subjects

Over a 15-month period, children and adolescents with primary obesity (body mass index (BMI) equal to or higher than the age- and sex-specific 97th percentile (25)) were consecutively enrolled at the Department of Pediatrics, Sapienza University of Rome, if they met the following criteria: no renal disease; no type 1 or 2 diabetes; no condition known to influence body composition, insulin action, or insulin secretion (e.g. glucocorticoid therapy, hypothyroidism, and Cushing's disease); and no history of alcohol consumption and smoking (where appropriate). At enrollment, their visit included physical examination (i.e. the measurements

of weight, standing height, waist circumference (WC), and BMI, and the determination of the stage of puberty according to the criteria of Tanner (26, 27)), measurements of body composition (including the determination of lean body mass and total fat mass), and laboratory tests. WC was obtained at the midpoint between the lowest rib and the iliac crest. The measurement was made at the end of a normal expiration while the subjects were in a standing position. The degree of obesity was quantified using Cole's least mean square method, which normalizes the skewed distribution of BMI and expresses BMI as a SDS. This measure gives age- and sex-specific estimates of the distribution median, the variation coefficient, and the degree of skew by a maximum-likelihood fitting technique (28). Systolic blood pressure (BP) and diastolic BP were measured twice at the right arm after a 10-min rest in the supine position using an automated oscillatory system (Dinamap Vital Signs Monitor, Model 1846 SX; Criticon Incorporated, Tampa, FL, USA). Body composition was determined using a total body scanner (Hologic QDR-4500W, Waltham, MA, USA, which uses fan-beam scanning) in array mode. This equipment uses a switched pulse stable dual energy X-ray operating at 100 and 140 kV. The data were analyzed using the software version 11.2.

Over the same study period, normal weight children, defined as those with BMI appropriate for gender and age (BMI <85th), were recruited to the study if they had no history of chronic disease; no history of use of medications that would affect insulin action or insulin secretion; no history of alcohol consumption and smoking (where appropriate); and their parents consented to participate in the study. At enrollment, their visit included physical examination (i.e. the measurements of weight, standing height, WC, and BMI, and the determination of the stage of puberty), measurements of body composition, and laboratory tests.

Both obese and normal weight children denied any significant change in weight during the last 3 months prior to the enrollment in the study.

The study was approved by the hospital ethics committee, and informed consent was obtained from the subjects' parents prior to the assessment.

Laboratory data

Blood samples were taken from each subject after an overnight fast for the measurement of circulating concentrations of glucose, insulin, triglycerides, total cholesterol and high-density lipoprotein (HDL) cholesterol, T-Ghr, and A-Ghr. In order to prevent the degradation and loss of the octanoyl group on the serine 3 position of ghrelin after centrifugation, A-Ghr samples were processed with 50 µl of 1 M HCl and with the addition of 10 µl phenylmethylsulfonyl fluoride per 1 ml plasma. Plasma immunoreactive T-Ghr and A-Ghr

values were measured in duplicate with commercial RIA using ^{125}I -labeled bioactive human A-Ghr as a tracer and a rabbit polyclonal antibody raised against full-length T-Ghr and against the serine 3 position of the octanoylated portion of A-Ghr respectively (Linco Research, Inc., St Charles, MO, USA). According to the supplier's specifications, inter- and intra-assay coefficient of variations (CV) were 14.7–17.8 and 3.3–10.0% respectively for T-Ghr, and were 9.6–16.2 and 6.5–9.5% respectively for A-Ghr. NA-Ghr values were calculated as T-Ghr – A-Ghr (17, 18). Insulin concentrations were determined using RIA with polyclonal antibodies (CIS Bio International, Schering SA, Gif-Sur-Yvette Cedex, France; detection limit, 2.0 mU/l; inter- and intra-assay CV, 6.4–8.8 and 4.2–8.2% respectively). We measured the remaining analytes on a COBAS INTEGRA 800 analyzer (Roche Diagnostics). Total cholesterol, HDL cholesterol, and triglyceride concentrations were assessed with the cassettes COBAS INTEGRA total cholesterol version 2; HDL cholesterol was assessed with version 3; triglyceride was assessed according to International Federation of Clinical Chemistry (IFCC) respectively by enzymatic colorimetric methods; and glucose concentration was assessed with the cassette version 3 by a hexokinase method (Roche Diagnostics).

Definitions

Because there is still no universally accepted definition of MS in children and adolescents, the criteria used in pediatric studies have been variably adapted from adult standards with the use of gender- and age-dependent normal values (29–34). For the American Heart Association (AHA) definition, MS is diagnosed in the presence of any three of the following five constituent risks: obesity as determined by WC; hypertension; low HDL values; elevated triglyceride values; glucose impairment (34). Our definition was based on the AHA definition, but it used pediatric reference standards for BP, WC, triglycerides, and HDL cholesterol. The glucose cutoff was identical to that used in the AHA definition. In our study, obesity was defined as a BMI ≥ 97 th percentile adjusted for age and sex (25) and/or a WC ≥ 90 th percentile for age and sex (35); hypertriglyceridemia as triglycerides ≥ 95 th percentile for age and sex (36); low HDL cholesterol as concentrations ≤ 5 th percentile for age and sex (36); elevated BP as systolic or diastolic BP ≥ 95 th percentile for age and sex (37); and impaired fasting glucose as glucose ≥ 5.6 mmol/l (26). IR was determined by a homeostasis model assessment (HOMA) of IR. Scores were calculated as the product of the fasting serum insulin concentration (mU/l) and the fasting serum glucose concentration (mmol/l) divided by 22.5. HOMA-IR changes during childhood depended on the age, gender, and pubertal stage (38). We have considered HOMA-IR values ≥ 95 th percentile as an indicator of

IR. We therefore defined MS in the presence of any three of the following five constituent risks: obesity; hypertension; low HDL cholesterol; elevated triglycerides; impaired fasting glucose and/or IR.

Statistical analysis

Statistical analyses were performed by SPSS (version 13.0; SPSS Inc, Chicago, IL, USA). Data are expressed either as frequencies or as means with 95% confidence intervals (CIs). Distributions of continuous variables were examined for skewness and kurtosis, and were logarithmically transformed when appropriate. Geometric means are reported for ghrelin forms, insulin, and HOMA-IR values. Differences between groups were tested for significance using ANOVA for quantitative variables with the Bonferroni correction for multiple comparisons, and using χ^2 test for qualitative variables. Pearson's correlation and linear regression coefficients were used to examine the relationship between variables. First, multiple linear regression analyses were performed to assess the associations of HOMA-IR values with ghrelin forms (considered one at a time) after adjustment for age, gender, Tanner stage, and BMI (or WC) (model 1); and second, the model was adjusted for age, gender, Tanner stage, BMI (or WC), and other components of MS including triglycerides, HDL cholesterol, and systolic BP. Log HOMA-IR, log T-Ghr, log A-Ghr, log NA-Ghr, and the log A/NA-Ghr ratios were used for correlations, and log HOMA-IR was used as the dependent variable in multiple regression analyses. *P* values < 0.05 were considered statistically significant.

Results

Clinical and biochemical characteristics of the study population

A total of 210 children and adolescents were included in the study. Participants were 34 obese children with normal blood glucose, insulin concentrations, lipid profile, and BP considered as the phenotypically obese metabolically normal (POMN) group; 79 with one component or more components of MS, the phenotypically obese metabolically abnormal (POMA) group; 55 normal weight children with normal blood glucose, insulin values, lipid profile, and BP, the normal weight metabolically normal (NWMN) group; and 42 children with normal weight and with one component or more components of MS, the normal weight metabolically abnormal (NWMA) group.

Baseline anthropometric and metabolic characteristics of the four groups of participants are presented in Table 1. Children in the POMA group were older than those in the POMN and NWMA groups, and had higher BMI, total fat, and lean mass than the other groups. BMI-SDS as well as systolic BP and diastolic BP were higher in obese children than in normal weight

Table 1 Anthropometric and metabolic characteristics of obese and normal weight children.

	POMA (n=79)	POMN (n=34)	NWMA (n=42)	NWMN (n=55)
Age (year)	10.0 (9.4–10.7)*, a	8.7 (7.7–9.6)	8.4 (7.2–9.6)	9.3 (8.3–10.2)
Male, n (%)	44 (56)	22 (65)	23 (55)	24 (44)
Tanner stage, n (%)				
I	27 (34)	19 (56)	30 (72)	27 (49)
II–III	25 (32)	13 (38)	9 (21)	21 (38)
IV–V	27 (34)	2 (6)	3 (7)	7 (13)
BMI (kg/m ²)	26.0 (25.3–26.8) [‡] , [¶] , b	23.7 (22.6–24.8) [‡] , [¶]	16.0 (15.5–16.6)	16.6 (16.0–17.1)
BMI–SDS	2.16 (2.0 to 2.3) [‡] , [¶]	2.11 (2.0–2.2) [‡] , [¶]	–0.09 (–0.32–0.13)	–0.12 (–0.33–0.08)
Waist circumference (cm)	81.1 (78.2–83.7) [‡] , [¶]	80.9 (78.2–83.7)	67.1 (63.9–70.4) [§]	61.8 (58.0–65.6)
Systolic BP (mmHg)	115 (113–116) [‡] , [¶]	115 (113–117) [‡] , [¶]	107 (105–108)	105 (104–107)
Diastolic BP (mmHg)	75 (72–76) [‡] , [¶]	75 (72–77) [‡] , [¶]	66 (65–68)	65 (64–67)
Total fat mass (kg)	20.6 (18.8–22.4) [‡] , [¶] , b	15.4 (13.2–17.6) [‡] , [¶]	4.6 (3.8–5.8)	5.7 (4.7–6.7)
Total lean mass (kg)	30.3 (28.0–32.6) [‡] , [¶] , b	23.5 (20.6–26.4) [‡] , [§]	16.2 (13.9–18.5)	19.0 (16.8–21.3)
Total cholesterol (mg/dl)	177 (165–190)*, [¶] , a	149 (138–160) [‡]	194 (178–210) [¶]	151 (145–157)
HDL cholesterol (mg/dl)	41 (39–43)* [¶]	45 (43–48) [§]	46 (42–50) [§]	53 (49–57)
Triglycerides (mg/dl)	135 (110–161)*, [¶] , c	72 (62–82)	95 (77–114) [§]	60 (53–65)
Fasting glucose (mmol/l)	4.82 (4.72–4.91) ^a	4.59 (4.45–4.73)	4.70 (4.53–4.84)	4.72 (4.62–4.82)
Fasting insulin (mU/l)	19.0 (17.0–21.3) [‡] , [¶] , c	10.4 (9.0–12.2) [¶]	9.3 (7.4–11.7) [¶]	6.2 (5.4–7.1)
HOMA-IR values	4.0 (3.6–4.6) [‡] , [¶] , c	2.1 (1.8–2.4) [¶]	1.9 (1.5–2.4) [¶]	1.3 (1.1–1.5)
Total ghrelin (ng/l)	208 (183–241) [‡] , [¶] , a	302 (243–376)* [§]	403 (330–487)	428 (354–513)
Acylated ghrelin (ng/l)	78 (70–89) [‡]	81 (69–93) [‡]	110 (92–136) [¶]	84 (74–96)
Nonacylated ghrelin (ng/l)	97 (76–125) [‡] , [¶] , a	171 (109–269) [§]	238 (166–340)	318 (251–403)
Acylated to nonacylated ghrelin ratio (%)	81 (60–110)*, [¶] , a	47 (29–76) [§]	47 (31–71) [§]	26 (21–34)

ANOVA with the Bonferroni correction for multiple comparisons: * $P < 0.05$, [‡] $P < 0.001$, [‡] $P < 0.0001$ versus NWMA; [§] $P < 0.05$, [¶] $P < 0.001$, [¶] $P < 0.0001$ versus NWMN; ^a $P < 0.05$, ^b $P < 0.001$, ^c $P < 0.0001$ versus POMN.

Results are expressed as n (%), mean (95% CI), or geometric mean (95% CI) for log-transformed variables. POMA, phenotypically obese metabolic abnormal; POMN, phenotypically obese metabolic normal; NWMA, normal weight metabolically abnormal; NWMN, normal weight metabolically normal.

subjects, but were similar within the same weight category. WC was higher in obese children than in normal weight subjects, but was similar in the POMA and POMN groups. In contrast, WC was higher in the NWMA group than in the NWMN group. Mean values of triglycerides, insulin, and HOMA-IR were significantly higher in the POMA group than in the other three groups. Yet, within the normal weight category, the NWMA children had higher triglycerides, insulin, and HOMA-IR values than the NWMN group. The NWMA group had higher total cholesterol followed respectively by the POMA, POMN, and NWMN groups, while the POMA patients had lower HDL cholesterol

followed respectively by the POMN, NWMA, and NWMN children. T-Ghr and NA-Ghr were significantly lower in the POMA children than in the other groups, while the A/NA-Ghr ratio was significantly higher. In contrast, A-Ghr levels were significantly higher in the NWMA children than in the other groups.

Ghrelin profiles in relation to clinical, anthropometric, and biochemical parameters

Within the entire study population, after adjustment for age, gender, and Tanner stage, insulin levels and HOMA-IR values were negatively related with T-Ghr

Table 2 Partial correlation coefficients between total, acylated, nonacylated, and acylated to nonacylated ghrelin ratio profiles and anthropometric and metabolic variables within the entire study population adjusted for age, gender, and Tanner stage.

	Total ghrelin	Acylated ghrelin	Nonacylated ghrelin	Acylated to nonacylated ghrelin ratio
BMI (kg/m ²)	–0.374 [‡]	–0.126	–0.349 [‡]	0.262 [‡]
Waist circumference (cm)	–0.234 [‡]	0.013	–0.254 [‡]	0.233 [‡]
Total fat mass (kg)	–0.341 [‡]	–0.121	–0.300 [‡]	0.221 [‡]
Total lean mass (kg)	–0.374 [‡]	–0.217 [‡]	–0.329 [‡]	0.206 [‡]
Systolic BP (mmHg)	–0.273 [‡]	–0.022	–0.298 [‡]	0.258 [‡]
Diastolic BP (mmHg)	–0.250 [‡]	–0.014	–0.275 [‡]	0.246 [‡]
Triglycerides (mg/dl)	–0.256 [‡]	–0.095	–0.202 [‡]	0.142 [*]
Total cholesterol (mg/dl)	–0.153 [*]	0.083	–0.142 [*]	0.161 [*]
HDL cholesterol (mg/dl)	0.229 [‡]	0.044	0.204 [‡]	–0.166 [*]
Fasting glucose (mmol/l)	–0.099	–0.016	–0.113	0.095
Fasting insulin (mU/l)	–0.292 [‡]	0.016	–0.296 [‡]	0.273 [‡]
HOMA-IR values	–0.303 [‡]	0.013	–0.309 [‡]	0.284 [‡]

* $P < 0.05$; [‡] $P < 0.001$; [‡] $P < 0.0001$.

and NA-Ghr, whereas they were positively associated with the A/NA-Ghr ratio (Table 2). BMI, WC, lean and fat mass, and systolic BP and diastolic BP, as well as total cholesterol and triglycerides were correlated negatively with T-Ghr and NA-Ghr, but positively with the A/NA-Ghr ratio also after adjusting for age, gender, and Tanner stage (Table 2). In contrast, HDL cholesterol was correlated positively with T-Ghr and NA-Ghr but negatively with the A/NA-Ghr ratio. A-Ghr was not significantly correlated with metabolic parameters, including insulin and HOMA-IR. Of the anthropometric characteristics, lean mass was significantly and negatively associated with A-Ghr.

When the associations were restricted to the normal weight groups, insulin values and HOMA-IR remained significantly negatively associated with T-Ghr ($r = -0.256$, $P < 0.05$, and $r = -0.259$, $P < 0.05$ respectively) as well as with NA-Ghr ($r = -0.344$, $P < 0.01$, and $r = -0.338$, $P < 0.01$ respectively), and positively associated with the A/NA-Ghr ratio ($r = 0.347$, $P < 0.01$, and $r = 0.334$, $P < 0.05$ respectively) after adjustment for age, gender, and Tanner stage. Moreover, in these children, A-Ghr was also significantly associated with insulin and HOMA-IR ($r = 0.239$, $P < 0.05$, and $r = 0.219$, $P < 0.05$ respectively) after adjustment for age, gender, and Tanner stage.

Ghrelin profiles and MS

Out of the 210 children included in the study, 39 (18.5%) were identified as having MS. Compared with the children without MS, those with MS had a higher mean age (10.6 (95% CI, 9.8–11.4) vs 9.1 (8.6–9.6) years; $P < 0.05$), as well as a BMI-SDS (2.2 (2.1–2.3) vs 0.95 (0.75–1.15); $P < 0.0001$), WC (78.5 (76.1–80.3) vs 70.0 (68.7–71.8) cm; $P < 0.0001$), systolic BP (115 (112–117) vs 110 (108–111) mmHg; $P < 0.0001$),

Figure 1 Geometric mean concentrations (95% CI) of total ghrelin in the entire population (white bars), normal weight children (gray bars), and obese patients (black bars) according to the presence of metabolic syndrome. * $P < 0.0001$ versus nonmetabolic counterpart; ** $P < 0.05$ versus normal weight children with metabolic syndrome.

Figure 2 Geometric mean concentrations (95% CI) of total ghrelin in the entire population (white bars), normal weight children (gray bars), and obese patients (black bars) according to the number of metabolic syndrome components. * $P < 0.0001$ and ** $P < 0.001$ for trend.

diastolic BP (75 (72–77) vs 69 (68–70) mmHg; $P < 0.0001$), triglycerides (183 (128–238) vs 82 (74–90) mg/dl; $P < 0.0001$), insulin (29 (23–35) vs 12 (11–13) mU/l; $P < 0.0001$), and HOMA-IR values (6.1 (4.9–7.4) vs 2.4 (2.2–2.7); $P < 0.0001$), but had lower HDL cholesterol values (39 (36–43) vs 47 (45–49) mg/dl; $P < 0.0001$). Glucose and total cholesterol levels did not differ between the two groups. The mean concentrations of T-Ghr were significantly lower in patients with MS than in those without MS (194 (95% CI, 160–235) vs 330 (296–365) ng/l; $P < 0.0001$; Fig. 1), and they decreased with the number of the components of MS (420 (347–508) vs 376 (317–445) vs 236 (201–279) vs 193 (160–235) ng/l) in subjects with 0, 1, 2, and ≥ 3 components of MS, $P < 0.0001$ for trend (Fig. 2). Similarly, NA-Ghr concentrations were significantly lower in patients with MS than in those without MS (83 (57–120) vs 196 (166–235) ng/l; $P < 0.0001$), and they decreased with the number of components of MS (311 (245–395) vs 230 (172–311) vs 114 (84–148) vs 83 (55–119) ng/l) in subjects with 0, 1, 2, and ≥ 3 components of MS, $P < 0.0001$ for trend. No significant differences were found for A-Ghr within the entire population (Figs 3 and 4). In contrast, the A/NA-Ghr ratio was significantly higher in all the patients with MS than in those without MS (100 (65–155) vs 44 (36–53)%; $P < 0.001$); Fig. 5), and they increased with the number of the components of MS (26 (21–33) vs 41 (30–56) vs 73 (51–104) vs 100 (65–157)%) in subjects with 0, 1, 2, and ≥ 3 components of MS, $P < 0.0001$ for trend (Fig. 6).

Out of the 39 patients with MS, 30 (76.9%) belonged to the group of obese children, and 9 (23.0%) to the normal weight group. Compared with normal weight subjects with MS, obese children with MS had a similar mean age as well as mean values for systolic BP and diastolic BP and HDL cholesterol, but had higher values

Figure 3 Geometric mean concentrations (95% CI) of acylated ghrelin in the entire population (white bars), normal weight children (gray bars), and obese patients (black bars) according to the presence of metabolic syndrome. * $P < 0.01$ versus nonmetabolic counterpart; ** $P < 0.01$ versus obese children with metabolic syndrome.

for triglycerides (189 (125–236) vs 130 (55–210) mg/dl; $P < 0.05$), glucose (4.85 (4.69–5.01) vs 4.28 (3.97–4.58) mmol/l; $P < 0.01$), and HOMA-IR (5.7 (4.6–7.0) vs 4.4 (3.0–6.9); $P < 0.05$) and had lower values for total cholesterol (171 (154–188) vs 204 (146–262) mg/dl; $P < 0.05$). T-Ghr (Fig. 1) and NA-Ghr were lower in obese children with MS than in normal weight patients with MS, while the A/NA-Ghr ratio was higher (Fig. 5). In contrast, A-Ghr was higher in normal weight children with MS than in obese patients with MS (Fig. 3).

We performed a multiple linear regression analysis to investigate the independent association of the single components of MS with ghrelin forms. WC, systolic BP, triglycerides, HDL cholesterol, glucose, and insulin values, along with age, gender, and Tanner stage, were included in the model. To avoid colinearity, diastolic BP and BMI were not included. WC (unstandardized coefficient (95% CI), -0.104 (-0.167 to

Figure 4 Geometric mean concentrations (95% CI) of acylated ghrelin in the entire population (white bars), normal weight children (gray bars), and obese patients (black bars) according to the number of metabolic syndrome components. * $P < 0.01$ for trend.

Figure 5 Geometric mean concentrations (95% CI) of acylated/nonacylated ghrelin ratio in the entire population (white bars), normal weight children (gray bars) and obese patients (black bars) according to the presence of metabolic syndrome. * $P < 0.001$ versus nonmetabolic counterpart; ** $P < 0.05$ versus normal weight children with metabolic syndrome.

-0.042); $P < 0.05$), systolic BP (-0.013 (-0.026 to -0.0001); $P < 0.05$), triglycerides (-0.002 (-0.003 to -0.001); $P < 0.01$), and insulin (-0.126 (-0.267 to -0.011); $P < 0.05$) were independently and negatively associated with total ghrelin concentrations. In contrast, the A/NA-Ghr ratio was positively associated with systolic BP (0.032 (0.008–0.057); $P < 0.01$), triglycerides (0.002 (0.0001–0.004); $P < 0.05$), and insulin (0.473 (0.227–0.718); $P < 0.0001$). If insulin was removed from the model, WC was significantly associated with the A/NA-Ghr ratio (0.04 (0.002–0.077); $P < 0.05$).

Multivariate regression analysis of the association between HOMA-IR values and plasma ghrelin forms

Multiple linear regression analysis was used to evaluate the roles of ghrelin profiles as independent predictors of IR. As shown in Table 3, A-Ghr and the A/NA-Ghr ratios were significantly positively associated with HOMA-IR after adjustment for age, gender, Tanner stage, and BMI (or WC) as well as after adjustment for age, gender, Tanner stage, BMI (or WC), and other components of MS including systolic BP, triglycerides, and HDL cholesterol. In contrast, T-Ghr and NA-Ghr were negatively associated with HOMA-IR after correction for anthropometric and metabolic variables (Table 3).

Discussion

Recent research has focused on the role of ghrelin in the regulation of both insulin action and glucose homeostasis. Most available data indicate a negative association between total ghrelin and insulin concentrations (5, 39, 40). However, with respect to IR, the relevance of NA versus A-Ghr has been assessed in very few studies

Figure 6 Geometric mean concentrations (95% CI) of acylated/nonacylated ghrelin ratio in the entire population (white bars), normal weight children (gray bars), and obese patients (black bars) according to the number of metabolic syndrome components. * $P < 0.0001$, ** $P < 0.001$, *** $P < 0.01$ for trend.

performed in adult patients (17, 18, 41). In a group of 60 overweight or obese postmenopausal women (31 insulin sensitive and 29 insulin resistant), St-Pierre *et al.* (18) demonstrated that both increased acylated ghrelin physiological concentrations and elevated A/NA-Ghr ratios were associated with IR. In a group of 45 adult MS patients, of whom 33 were obese and 12 were nonobese, Barazzoni *et al.* (17) demonstrated that plasma insulin and HOMA-IR were associated negatively with T-Ghr and NA-Ghr, but positively with A-Ghr and the A/NA-Ghr ratio. Compared to nonobese patients, obese MS patients had lower T-Ghr and NA-Ghr but comparable A-Ghr, and had a higher A/NA-Ghr ratio. BMI and WC were positively related to HOMA-IR. However, opposite associations between the A/NA-Ghr ratio and HOMA-IR remained significant after adjustment for sex and BMI (or WC). Yet, in the same study, Barazzoni *et al.* showed that ten obese individuals without MS (age-, sex-, BMI-, and WC-matched to obese MS patients) had lower T-Ghr but higher A-Ghr than 15 age- and sex-matched healthy nonobese controls. T-Ghr and A-Ghr were comparable in obese individuals with or without MS. In a very recent study (41) involving 80 adult subjects

(19 obese with type 2 diabetes; 20 obese with impaired glucose tolerance; 20 obese with normoglycemia; and 21 lean with normoglycemia), Rodriguez *et al.* demonstrated that obese subjects had increased circulating concentrations of A-Ghr and decreased circulating concentrations of desacyl ghrelin than lean individuals. Furthermore, acylated ghrelin values were higher in obese individuals with impaired glucose tolerance and type 2 diabetes than in obese normoglycemic patients. No effect of glucose tolerance or diabetes was observed in circulating desacyl ghrelin concentrations. A highly significant positive correlation was observed between acylated ghrelin and BMI, WC, and HOMA, whereas desacyl ghrelin showed a strong negative correlation with these parameters.

To our knowledge, we are the first to investigate the relevance of ghrelin profiles, including A-Ghr and NA-Ghr, and the ratio of A/NA-Ghr in the context of IR in a large population of obese children as well as of normal weight children. The results of the present study show that both T-Ghr and NA-Ghr are inversely related with IR, while A-Ghr and the ratio of A/NA-Ghr are positively associated with IR, independently of anthropometric and metabolic variables. We have also demonstrated that obese children with MS have marked alterations in plasma ghrelin profile than obese children without MS; in particular, T-Ghr and NA-Ghr were lower, while the fraction of acylated circulating hormone relative to nonacylated circulating hormone was higher. Moreover, T-Ghr and NA-Ghr decreased, while A/NA-Ghr ratio increased monotonically with the number of MS components.

Interestingly, there was also an alteration of ghrelin secretion profiles in normal weight children with MS. In fact, T-Ghr and NA-Ghr were lower, while A-Ghr and the A/NA-Ghr ratio were higher in normal weight subjects with MS than in normal weight counterparts without MS. A positive correlation between A-Ghr and the A/NA-Ghr ratio and insulin levels as well as IR index was observed in these subjects. In agreement with previous findings in adults (17), NWMA children had a larger WC than healthy normal weight subjects despite similar BMI and total fat mass. Recent evidence in children shows that WC is more closely associated with

Table 3 Multivariate linear regression analyses of plasma ghrelin forms (as independent variables) and homeostasis model assessment of insulin resistance (HOMA-IR) (dependent variable) in the entire study population.

	Unstandardized coefficient, b^a (95% CI)	Unstandardized coefficient, b^b (95% CI)
Total ghrelin (ng/l)	-0.141 (-0.275 to -0.008)*	-0.133 (-0.269 to -0.003)*
Nonacylated ghrelin (ng/l)	-0.100 (-0.171 to -0.028)†	-0.092 (-0.166 to -0.018)*
Acylated ghrelin (ng/l)	0.168 (0.016–0.321)*	0.165 (0.012–0.317)*
Acylated to nonacylated ghrelin ratio (%)	0.104 (0.042–0.166)†	0.098 (0.035–0.161)†

Total ghrelin, acylated ghrelin, nonacylated ghrelin, acylated to nonacylated ghrelin ratio, and HOMA-IR were log transformed for the best fit.

* $P < 0.05$; † $P < 0.01$; ‡ $P < 0.001$.

^aAdjusted for age, gender, Tanner stage, and BMI.

^bAdjusted for age, gender, Tanner stage, and BMI and other components of metabolic syndrome including triglycerides, HDL cholesterol, and systolic BP. Similar results were observed when adjusting for waist circumference (instead of BMI).

visceral fat, whereas BMI is more associated with subcutaneous fat (42). Similarly, visceral fat (as measured by magnetic resonance imaging), but not BMI or waist–hip ratio, has been associated with fasting insulin and triglycerides in obese adolescent girls (43). Given that the visceral adipose tissue constitutes a source of ghrelin (44), it has been suggested that abdominal fat accumulation may contribute to the relative or absolute A-Ghr excess, which may in part account for the interaction between visceral fat and IR in MS patients (17, 41). However, despite adjustment for WC (as a surrogate and indirect index of visceral obesity), ghrelin forms remained independently associated with IR.

Our results of a differential association of A-Ghr and NA-Ghr with IR are supported by several *in vitro* and *in vivo* studies. Ghrelin forms had an opposite impact on glucose output in primary cultures of porcine hepatocytes (13). In addition, NA-Ghr was shown to counteract the influence of A-Ghr on insulin secretion and on glucose metabolism in young male volunteers (14). Furthermore, A-Ghr and NA-Ghr displayed correlations of opposite signs with variables regarding cholesterol metabolism and hepatic function, confirming the divergent effects of both forms on metabolic processes (41). The relation of A-Ghr to NA-Ghr may have a clinical significance in metabolic disorders. The current results suggest that the A/NA-Ghr balance may represent a fine-tuning mechanism that can modulate insulin action. Unbalanced plasma concentrations of ghrelin forms and a relative A-Ghr excess may negatively modulate insulin action in obese and normal weight children, thus contributing to the association of IR with MS. Factors regulating circulating A-Ghr levels are largely unknown. Putative mechanisms for elevated A-Ghr include increased ghrelin acylation and/or increased ghrelin synthesis and secretion. Increased availability of fatty acids may enhance hormone acylation in mice (45). Recently, Yang *et al.* (46) have identified a novel enzyme implicated in the *n*-octanoylation of ghrelin, namely ghrelin O-acyltransferase, which is expressed in the major ghrelin-secreting tissues, such as the stomach and the intestine. Recent evidence in rodents also suggests a direct role of dietary lipids in ghrelin acylation (47). Therefore, it seems plausible that obesity, in particular visceral adiposity, may influence the expression and/or activity of this acyltransferase, leading to elevated plasma A-Ghr concentrations even in the presence of normal total body fat mass.

In adults, low total ghrelin concentration has been associated with the single features of MS, including elevated BP, hypertriglyceridemia, high fasting blood glucose, and low HDL cholesterol (48–51). Yet, the relationship between ghrelin levels and metabolic abnormalities as a cluster *per se* has also been reported (51). Because adiposity influences all other features of the MS, it has been suggested that low ghrelin in MS could reflect only the obesity state. Langenberg *et al.*

(49) demonstrated that systolic BP and diastolic BP, fasting and postchallenge insulin, HOMA-IR, HDL cholesterol, and triglycerides were associated with ghrelin before and after adjustment for age and sex. Adjustment for BMI attenuated most associations; only fasting and postchallenge insulin and HDL cholesterol remained significantly associated with ghrelin. Furthermore, Ukkola *et al.* (51) reported that the association of HDL cholesterol and fasting blood glucose with plasma ghrelin levels remained significant after adjustment for BMI. Yet, ghrelin levels decreased with an increase in the number of metabolic abnormalities. Finally, in the study by Ukkola *et al.* low ghrelin was a significant predictor of MS as a cluster *per se* after adjustment for age and sex (51). Our results confirm and expand on the above-mentioned findings. We have found that single components of MS were independently and inversely associated with total ghrelin levels. We have also demonstrated that single components of MS were independently and positively associated with the A/NA-Ghr ratio.

In conclusion, our data indicate that relative A-Ghr excess is associated with IR independently of obesity as well as of other components of MS. Further longitudinal investigations will be needed to elucidate whether dysregulation of ghrelin secretion profiles in obese children as well as in normal weight children with metabolic abnormalities may influence the long-term metabolic and cardiovascular outcomes.

Declaration of interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

The authors' responsibilities were as follows: L Pacifico participated in the design of the study, data collection and analysis, and writing of the manuscript; E Poggiogalle and F Costantino participated in the collection and analysis of data; F Ferraro assisted with the data analysis and in drafting the manuscript; C Anania and F Chiarelli provided significant advice on all the aspects of the study; and C Chiesa participated in the design of the study, data analysis, and writing of the manuscript. All the authors participated in the critical review and in the final approval of the manuscript.

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