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Hormonal aspects in paediatric obesity

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1. INTRODUCTION

1.1 Obesity prevalence in childhood

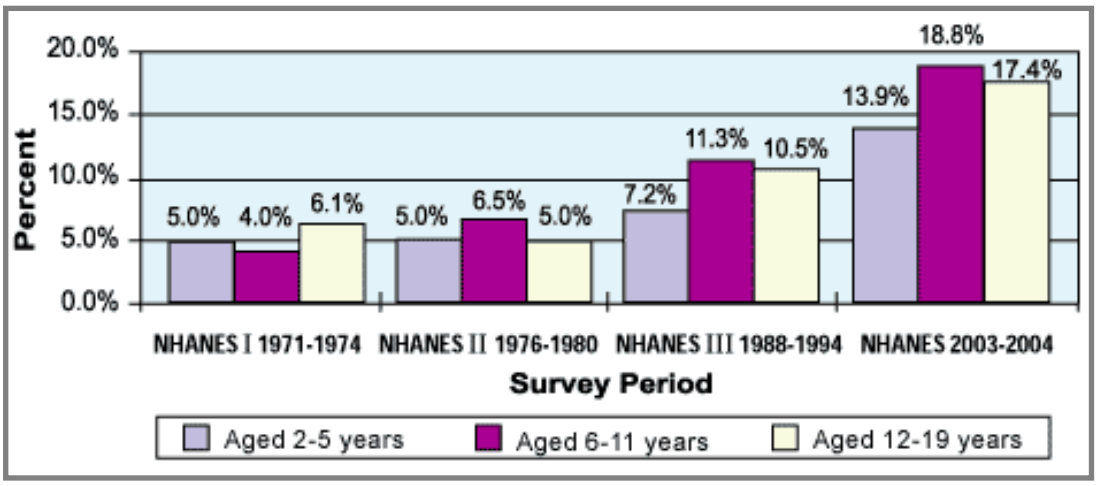
Overweight and obesity are a major public health concern both in adults and in children. In 1997, the World Health Organization has declared obesity a global epidemic (WHO, 2001), and the Healthy People 2010 has identified overweight and obesity an indicator of the health of a given population (Healthy People, 2000). In fact, childhood obesity is most strongly associated to insulin resistance with an increased prevalence of type 2 diabetes, dyslipidemia and hypertension at the pediatric age, developing to an increased cardiovascular mortality in adulthood (Maffeis et al., 2001).

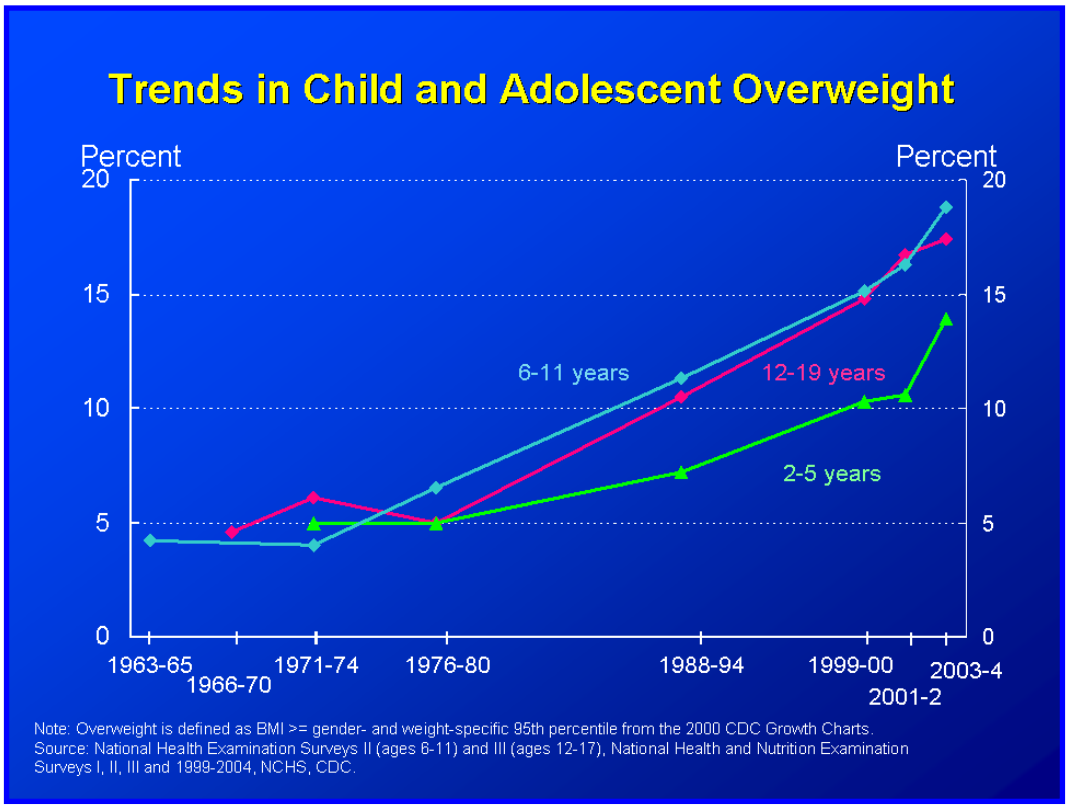
The prevalence of obesity in children and adolescents has increased over several decades in many industrialized countries (Wang Y. et al., 2006).

Epidemiological studies of National Health and Nutritional Examination Surveys (NHANES) representing United States Population demonstrated that the prevalence of obesity between 1971-1974 and 2003-2004 has been increased in pre-school children (2-5 yrs) from 5% to 13.9%, from 4% to 18.8% in the age 6-11 years, and from 6.1% to 17.4% in adolescent age (12-19 yrs) (**Figure 1**).

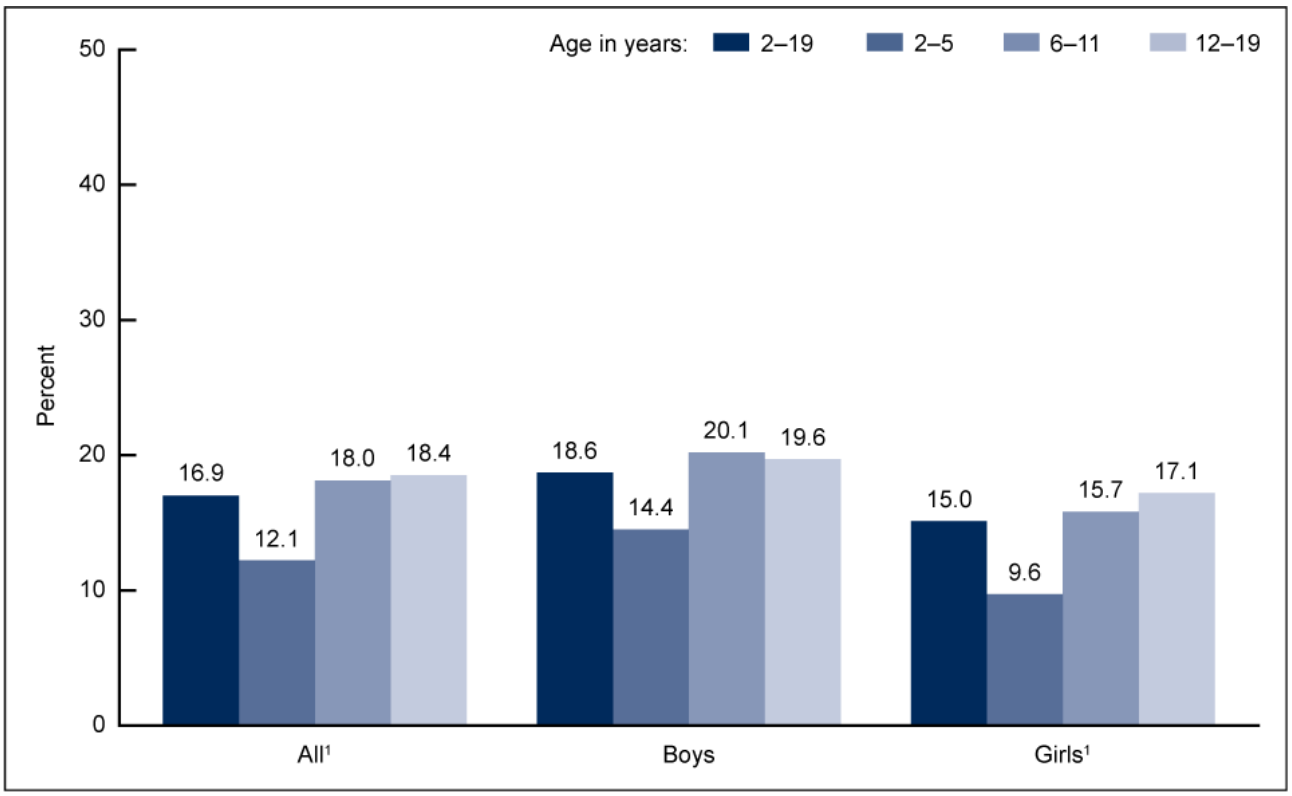
In the last period of observation it has been shown that an increase is present in particular ethnic groups like African American or Hispanic American in the scholar and adolescent age (National Centre for Health Statistics, 2003-2004) (Ogden CL. et al., 2008).

Figure 1. *Trends in child and adolescent overweight.*





(National Centre for Health Statistics, 2003-2004)



[1]Significant increasing linear trend by age ($p < 0.005$).
SOURCE: CDC/NCHS, National Health and Nutrition Examination Survey, 2009–2010.

In 2003 the study of Lostein et al. demonstrated that in Europe it is possible to detect two apparent trends. The first is the generally lower levels of overweight found among children in the countries of central and eastern Europe whose economies suffered varying degrees of recession during the period of economic and political transition in the 1990s. This has been particularly noticeable in Russia, in Czech Republic and in Poland; mainly in rural areas, and among children under age 10, the figure was even lower at 7% overweight. The second trend apparent in the data is for the prevalence of overweight to be higher among the southern countries of Europe, especially those outside of the former eastern bloc. The non-eastern bloc countries surrounding the Mediterranean show prevalence rates for overweight children in the range 20–40%, while those in northern areas show rates in the range 10–20% (Lobstein et al., 2003, **Figures 2-3**).

Figure 2. *Prevalence (percentage) of overweight children aged around 7–11 years (higher panel) and 14-17 (lower panel) using the cut-off points recommended by International Obesity TaskForce (overweight includes obese).*

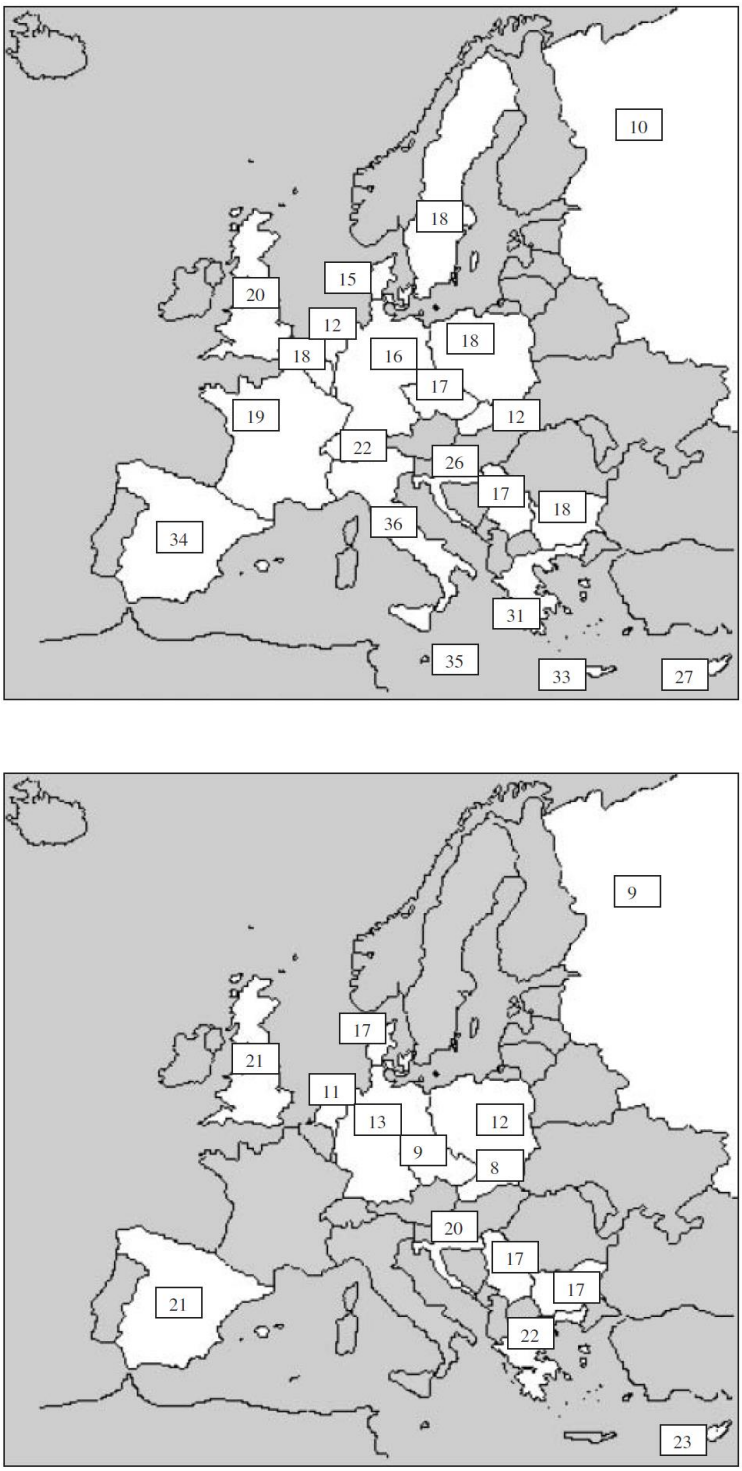


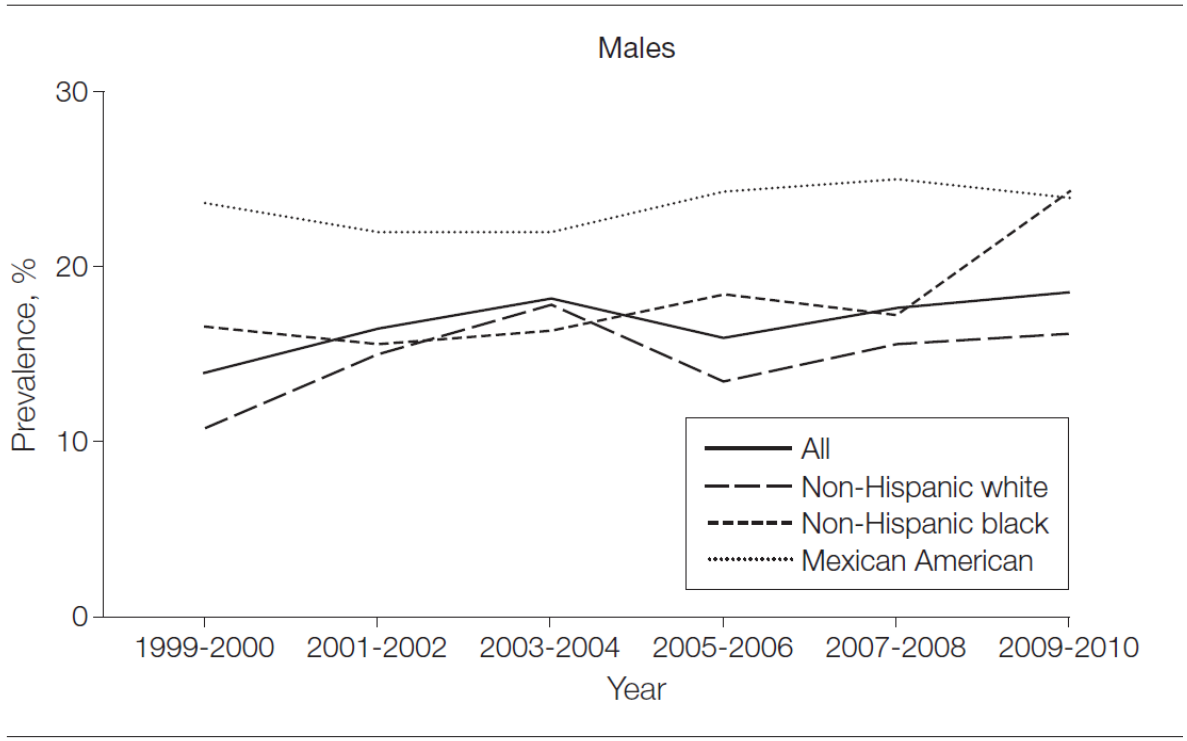
Figure 3. *Sources of data on the body mass indices of children in Europe.*

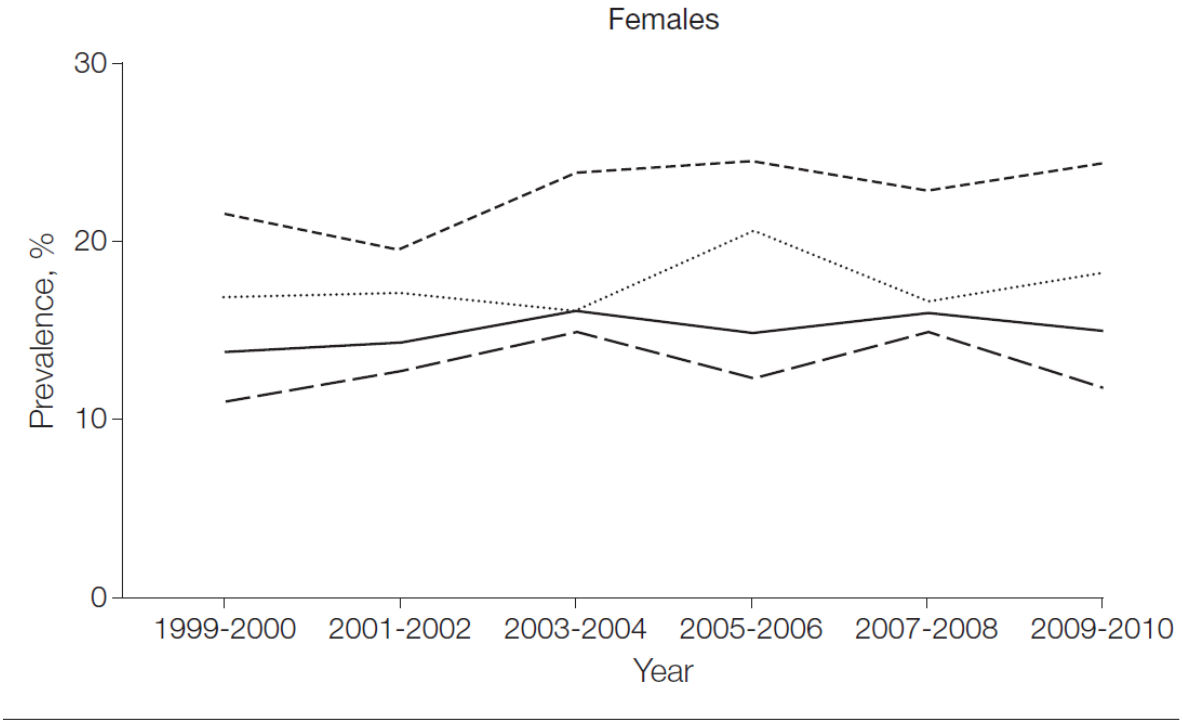
Country	Sample size	Age group (years)	Survey year(s)	Source
Belgium	1 026	6–12	1992	Guillaume *et al.* (10)
Bulgaria	6 655	7–17	1998	S. Petrova, K. Vatralova & L. Ivanova (personal communication)
Croatia (Zagreb)	6 419	7–19	1995–98	A. Kaic-Rak (personal communication)
Cyprus	2 467	6–17	1999–2000	Savva *et al.* (11)
Czech Rep	32 453	7–18	2001	J. Vignerová (personal communication)
Denmark	11 218	5–17	1996–97	Petersen *et al.* (12)
France	1 582	7–9	2000	Rolland-Cachera *et al.* (13)
Germany	32 429	1–17	1995	M. Wabitsch (personal communication)
Greece (Crete)	733	10–13	1998	Moschandreas (14)
Greece (Thessaloniki)	2 458	6–17	2000	Krassas *et al.* (15)
Italy	41 149	9	2001	M. Caroli, M. Vignolo, A. Luciano, C. Invitti & L. Censi (personal communication)
Malta	519	10	1992	Bellizzi *et al.* (16)
The Netherlands	14 377	0–21	1997	Fredriks *et al.* (17)
Poland	10 654	0–17	1996–99	Palczewska & Niedzwiecka (18); Mazur *et al.* (19)
Russia	2 688	6–18	1998	Wang *et al.* (1)
Slovakia	5 514	11–17	1995–99	K. Babinská (personal communication); A. Bederová (personal communication)
Spain	1 637	5–17	1998–2000	Majem *et al.* (20)
Sweden	6 700	9–11	2000–01	S. Mårild, K. Albertsson-Wickland, M. Bondestam, S. Ehnberg & A. Hollsing (personal communication)
Switzerland	595	6–13	1999	Zimmermann *et al.* (21)
UK	2 882	5–17	1998	Lobstein *et al.* (22)
Yugoslavia	48 528	2–18	1998	M. Pavlović & A. Kadvan (personal communication)

Starting from 2004-2006 the prevalence of childhood obesity in the United States remains unchanged at approximately 17% and the rapid increases in obesity prevalence previously seen have not continued in this decade and may be leveling off (Ogden CL. et al., 2008, Ogden CL et al., 2010).

Nevertheless NHANES has consistently reported significant differences in obesity prevalence by race/ethnicity and among adolescent males but not females of any age. This is consistent with previously published results showing an increase in prevalence of BMI for age at or above the 97th percentile among males but no change in females at any cutoff based on data through 2007-2008 (**Figure 4**, Ogden CL et al., 2010) (Flegal KM et al., 2010).

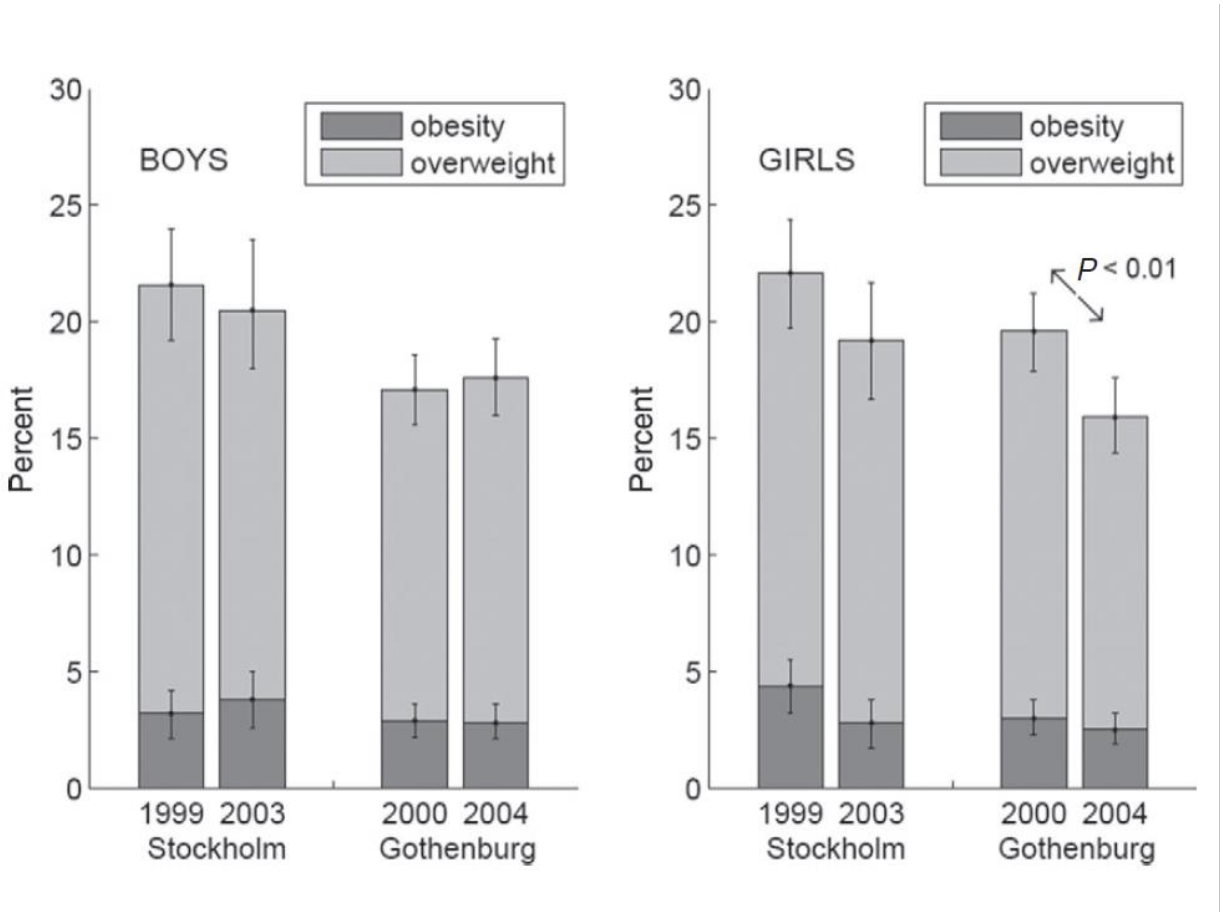
Figure 4. *Prevalence of Obesity in US Males and Females Aged 2 Through 19 Years.*





Also in Europe recent epidemiological studies demonstrated the achievement of a plateau in the prevalence of obesity (Great Britain, France, Sweden) (Olds T. et al., 2011, Lissner L. et al., *2010,* **Figure 5**).

Figure 5. *Percentages of fourth graders with obesity and overweight (+ obesity) with 95% confidence limits for 4-year changes in Stockholm and Gothenburg, among boys (left panel) and girls (right panel).*



More research is needed to understand why these changes may be occurring.

Concerning the Italian picture, a study conducted in 2010 including children and adolescents among 6-17 years of age, demonstrated a percentage of overweight and obesity of about 26% based on the cut-off proposed by the International Obesity Task Force; the study subdivided children in males and females and in three age categories (6-9 yrs, 10-13 yrs and 14-17 yrs) (see **Table 1**).

Table 1. *ISTAT study, 2010. Percentage of overweight and obesity in Italy.*

Population	***6-9 yrs***	***10-13 yrs***	***14-17 yrs***	***Mean***
Males	37,5%	29,1%	20,8%	28,9%
Females	37,1%	22,0%	11,3%	23,2%
Total	**37,3%**	**25,6%**	**16,2%**	**26,2%**

*(statistical information for health promotion, ISTAT, 2011**)***.

Interesting results come from studies on the Piedmont population, comparing school children living in Turin in 1977 and in Novara in 1999. After 20 years the percentage of obesity increased since 4% in males and 5% in females to 11,64% and 17,24%, respectively, in children of 7 years of age; while since 10% in females and 25% in males to 22,4 % and 28.2%, respectively in children of 11 years of age. These data confirm previous analysis demonstrating an important increase in pediatric overweight.

At the end of 2007, the Centre for Disease Control within the Italian Ministry of Health commissioned the creation of a national system to estimate the prevalence of childhood overweight and obesity by geographic area of Italy ('OKkio alla SALUTE' project) involving more than 45 000 third-grade students. This project demonstrated a high level of childhood obesity in the overall population, 23,6% of overweight and 12,3% of obesity, which was higher than that of most Western countries; furthermore there were substantial geographic differences, with the prevalence of obesity twice as

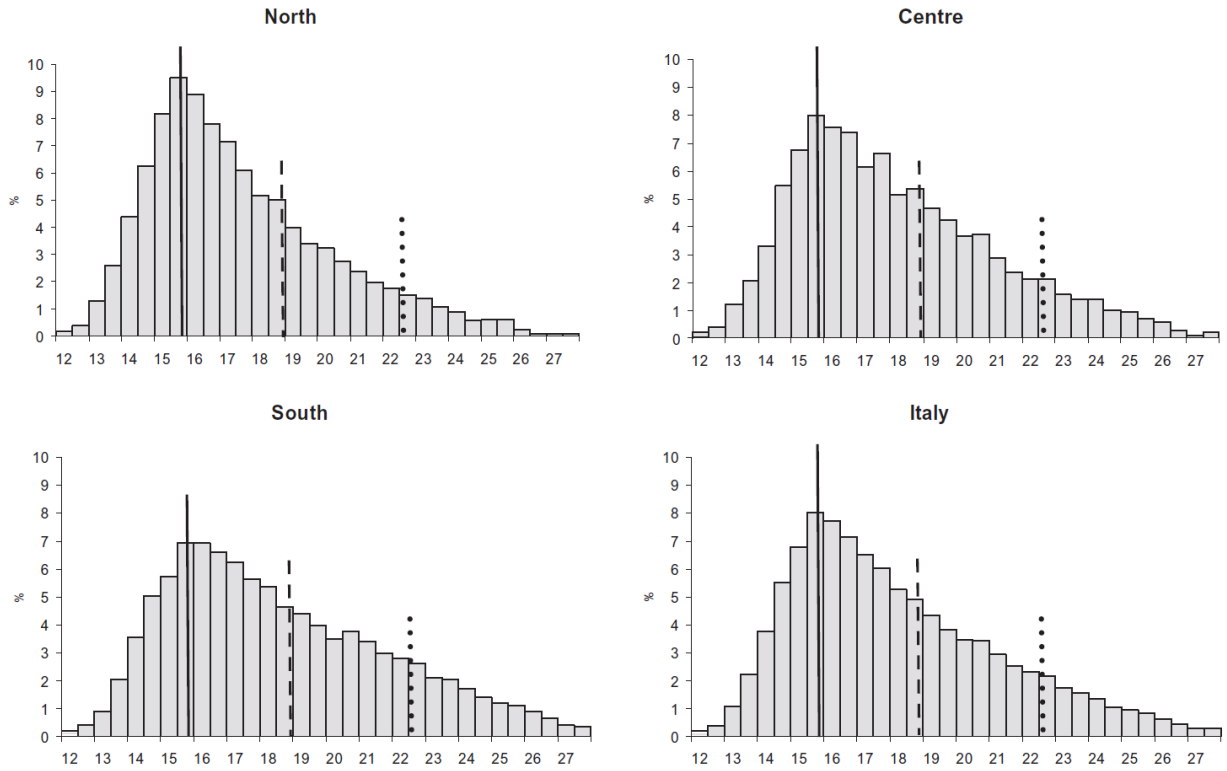
high in the south as in the north (49% in Campania and 23% in Valle D'Aosta) (Binkin N. et al., 2010) (**Table 2 and Figure 6**).

Table 2. *Nutritional status by geographic area, Italy, 2008*

Area	Normo or underweight		Overweight		Obese	
	%	95% CI	%	95% CI	%	95% CI
North	72.3	71.2–73.4	20.2	19.2–21.2	7.5	6.7–8.2
Centre	64.7	63.2–66.3	24.6	23.4–25.8	10.6	9.6–11.7
South	58.0	56.9–59.0	25.4	24.5–26.3	16.6	15.8–17.4
All	64.1	63.4–64.8	23.6	23.0–24.2	12.3	11.8–12.8

CI, confidence interval.

Figure 6. Distribution of body mass index by geographic area for children 8–9 years of age, Italy. Cut-offs refer to children 8 years and 10 months of age, the median age of the study population (solid line, International Obesity Task Force median; dashed line, cut-off for overweight; dotted line, cut-off for obesity).

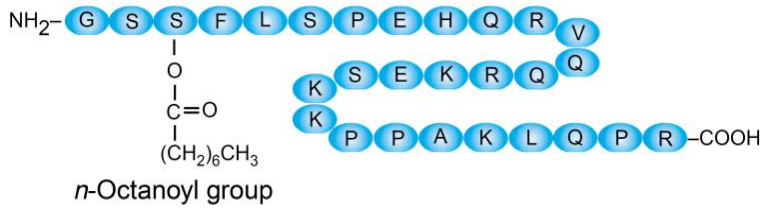


1.2 Ghrelin system in pediatric obesity

Ghrelin has an emerging role on appetite, glucose and lipid metabolism, and body composition and has provided an important strength to this research field opening new perspectives within neuroendocrinology and metabolism. In particular it is the only known appetite-stimulating hormone in humans and seems one of the principal factors involved in appetite, craving and regain weight after weight-loss (Adams CE. et al., 2011, Delhanty PJ. et al., 2012).

Ghrelin is a 28 amino-acid peptide predominantly produced by the stomach (Kojima M. et al., 1999). It has been discovered as the first natural ligand of the orphan GH Secretagogue Receptor (GHS-R) type 1a (Kojima M. et al., 1999, van Der Lely AJ. Et al., 2004, Kojima M. et al., 2005). Ghrelin presents a characteristic acylation with a medium fatty n-octanoic acid on the Ser3 residue (Kojima M. et al., 1999) (**Figure 7**). The n-octanoyl group seems essential for its binding to and activation of the GHS-R1a (Kojima M. et al., 1999, van Der Lely AJ. Et al., 2004).

Figure 7. Acylated ghrelin



The ghrelin human gene (*GHRL*) that is located on 3q25-36, encodes a molecule named pre-proghrelin of 117 aminoacids (Kojima M. et al., 1999, Kojima M. et al., 2005) from which derived by alternative splicing also other ghrelin forms, named des-Gln14ghrelin (Hosoda H. et al., 2000) and In1-ghrelin variant, which retains the *GHRL*

intron-1 sequence (Gahete MD. Et al., 2011); both variants can be acylated. Despite this background, unacylated ghrelin (UAG), that is devoid of the n-octanoil group at Ser3, is the most abundant circulating form (Kojima M. et al., 1999, Kojima M. et al., 2005, Korbonits M. et al., 2004). Increasing findings demonstrate that also UAG is a biologically active molecule. This evidence is consistent with the hypothesis of the existence of some GHS-R subtypes that are activated by ghrelin independently of its acylation (Caballero B. 2005).

The mechanism of acylation of the pre-proghrelin or of UAG is largely unknown. Yang and coworkers have identified the acyltransferase that octanoylates ghrelin. It has been named GOAT (Ghrelin O-Acyltransferase) (Yang J. et al., 2008). From the pre-proghrelin sequence, another ghrelin-associated peptide of 23 amino acids has been found, named obestatin (Zhang JV. Et al., 2005). The first studies have shown that obestatin possesses opposing action to ghrelin, inhibiting food intake, weight gain and jejunal movement, but the later ones do not fully confirm the previous reports (Hassouna R. et al., 2010).

Ghrelin emerged as one of the most powerful orexigenic and adipogenic agents known so far (van Der Lely AJ. Et al., 2004, Korbonits M. et al., 2004, Leite-Moreira AF. Et al., 2007, Cummings DE. et al., 2008, Tschop M. et al., 2000). In all, as a result of central and peripheral actions, acylated ghrelin (AG) administration in rodents causes weight gain that occurs even in absence of overfeeding (Wiedmer P. et al., 2007). AG influences energy balance involving NPY and AgRP in the arcuate nucleus as well as decreasing melanocortin tone and reducing the α- and β-melanocyte-stimulating hormone by neurons that produce pro-opiomelanocortin (van Der Lely AJ. Et al., 2004, Wiedmer P. et al., 2007, Shintani M. et al., 2001, Chen Y. Et al., 2004). Ghrelin regulation of energy homeostasis seems also mediated by efferent and afferent fibres of the vagal nerve (van Der Lely AJ. Et al., 2004, Wiedmer P. et al., 2007). The active

vaccination of mature rats with ghrelin immunoconjugates decreases feed efficiency, relative adiposity, and body weight gain (Zorrilla EP. Et al., 2006). More recently, by acting on GOAT the modification of fatty acid chain length enhances or reduces systemic and central chronic actions of AG on adiposity in rodents (Heppner KM. et al., 2012). On the other hand, the role of UAG in food intake is not fully clarified but it seems able to induce a negative energy balance by decreasing food intake and delaying gastric emptying via the hypothalamus (Delhanty PJ. et al., 2012).

In humans ghrelin secretion is pulsatile, with higher secretion night-time; it undergoes circadian variations with decreases after food ingestion, thus suggesting a metabolic control of it in vivo. Ghrelin could contribute to meal initiation or to nutrient type ingestion (van Der Lely AJ. Et al., 2004, Cummings DE. et al., 2006, Heppner KM. et al., 2012). The circulating levels of ghrelin are modulated by chronic and acute energy imbalance. In fact, ghrelin levels are negatively associated with body mass index; ghrelin secretion is increased in anorexia and cachexia, reduced in obesity and normalized by recovery of ideal body weight (van Der Lely AJ. Et al., 2004, Leite-Moreira AF. et al., 2007, Cummings DE. et al., 2008, Tschop M. et al., 2001). More recently, GOAT was detected in human circulation in healthy, obese and anorexic adults with a positive correlation with body mass index and a negative correlation with ghrelin levels, suggesting that GOAT counteracts the adaptive changes of ghrelin observed under these conditions (Goebel-Stengel M. et al., 2013). Whether an increase of ghrelin levels has been reported after weight loss induced by either diet and lifestyle modifications, this increase may help to promote regaining weight. Accordingly, GOAT inhibition attenuated food foraging, food intake, food hoarding, and hedonic in mice (Teubner BJ. Et al., 2013, Davis JF. Et al., 2012). Furthermore, the overall ghrelin profile is partially abnormal in adult obesity: there is absent or changed ghrelin elevation during fasting (Perreault M. et al., 2004), abolished or blunted increase during

the night or sleep deprivation (Yildiz BO. Et al., 2004, Vazquez RMI. Et al., 2006), and blunted suppression after a meal (English PJ. Et al., 2002). The only clinical exception to this picture seems to be Prader-Willi syndrome (PWS), a genetic disease characterized, among many other features, by severe obesity and hyperphagia. Interestingly, unlike essential obesity, patients with PWS show elevated ghrelin levels, both total and AG levels. Ghrelin hypersecretion has been hypothesized to participate in the development of at least some symptoms of PWS syndrome such as hyperphagia and weight excess (Yi CX. Et al., 2011).

As anticipated, circadian ghrelin secretion is profoundly modulated by acute variations in the energy balance and nutritional status. Though some stimulatory effects of short-term fasting on ghrelin secretion has been suggested by some Authors (van Der Lely AJ. Et al., 2004, Heppner KM. et al., 2012, Kim MS. Et al., 2003 Muller AF. Et al., 2002) but not definitively confirmed (Natalucci G. et al., 2005, Avram AM. Et al., 2005, Espelund U. et al., 2005), probably because the assay methods allowing only to evaluate total ghrelin levels. Notably, during fasting, AG decreases to nadir levels seen post-prandially and UAG remains near to peak levels seen pre-prandially, suggesting that long-term fasting inhibits acylation and that this one may be regulated independently by nutrient availability in the gut, or esterases which cleave the acyl group (Liu J. et al., 2008, Nass R. et al., 2008). In fact, the lipid group that is attached by GOAT is likely derived from free fatty acids in the lumen of the gut rather than circulation (Kirchner H. et al., 2009). Indeed, with prolonged fasting, AG levels are suppressed, whereas UAG is tonic secreted (Liu J. et al., 2008). However, the mechanisms mediating the metabolic control of ghrelin secretion are at present still matter of debate. Gastric secretion per se has been reported not to play a role, while interesting results derive from studies evaluating the effects of nutritional and metabolic determinants (van Der Lely AJ. Et al., 2004, Cummings DE. et al., 2008, Yi CX. Et al.,

2011, Prodam F. et al., 2006). The depth and duration of ghrelin decrease after a meals is related to the total amount of calories ingested and to the type of the macronutrients, in particular carbohydrates and proteins in spite of less effective suppression led by lipids (van Der Lely AJ. Et al., 2004, Leite-Moreira AF. et al., 2007, Prodam F. et al., 2006). It has also been shown that ingested medium-chain fatty acids are directly used for ghrelin acylation, thus theoretically modulating its biological activity (Heppner KM. et al., 2012, Nishi Y. et al., 2005). Consistent with its role in nutritional status, insulin and glucose seem among the major determinants of ghrelin secretion that, in turn, modulates insulin secretion and glucose metabolism as also been predicted by the negative correlation between ghrelin levels and body mass index (van Der Lely AJ. Et al., 2004, Leite-Moreira AF. et al., 2007, Cummings DE. et al., 2008, Tschop M. et al., 2001), and GOAT KO models (Zhao TJ. Et al., 2010).

Overall, published data suggest that ghrelin acts to optimize energy metabolism in period of food restriction as well as preparing the metabolism to percept and use fuel. Data derived by rodent models reveal an essential function of ghrelin, perhaps accounting for its evolutionary conservation - namely, maintenance of viability during periods of famine. Food intake, appetite and energy balance are strictly regulated during lifespan with critical changes in each specific period (infancy, adulthood, aging). There is increasing evidence, although not conclusive, that some of ghrelin changes may contribute to the regulation of food intake and weight also in children, starting from neonates.

1.3 Cortisol axis in paediatric obesity

Cortisol has been reported to have a role in obesity, hypertension, and the altered glucose and lipid profile in Cushing's syndrome, and some studies have suggested that moderately increased morning fasting cortisol may be associated with the presence of cardiovascular risk factors in adults (Whitworth JA. Et al., 1995, Pasquali R. et al., 2008, Sukhija R. et al., 2006).

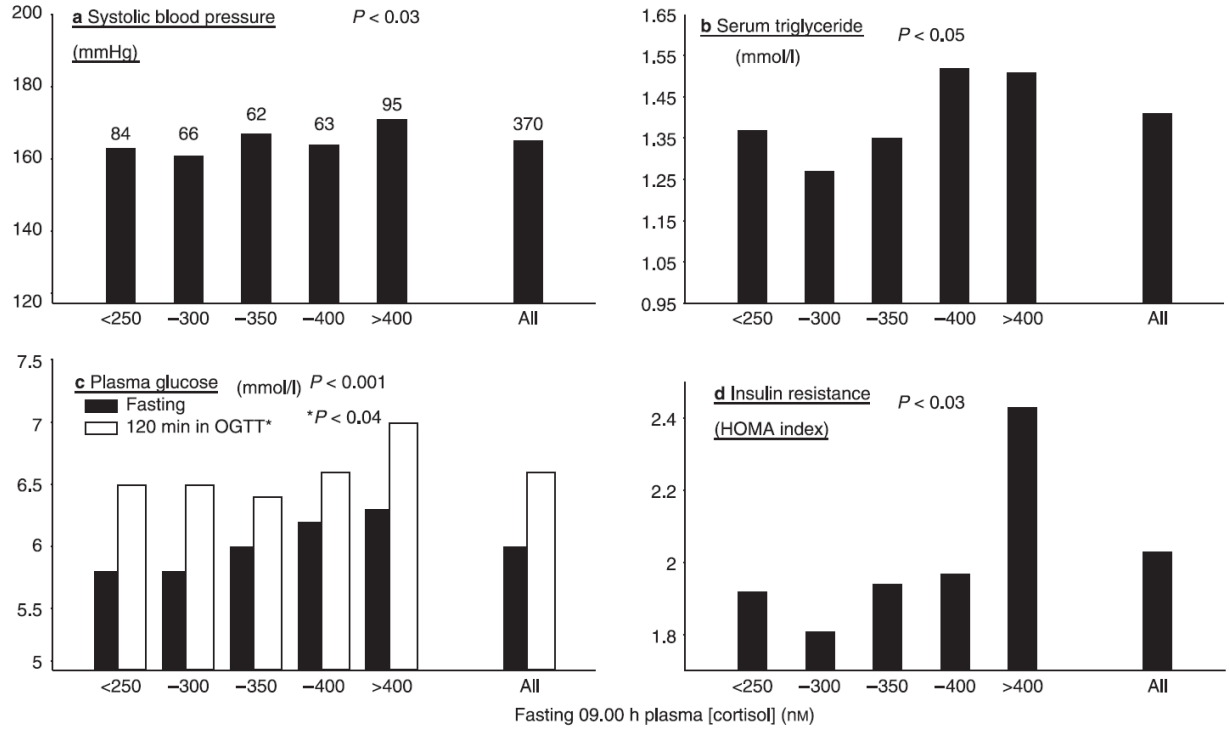
The metabolic syndrome (MetS) is a cluster of common abnormalities including hyperglycemia, abdominal obesity, reduced high-density lipoprotein cholesterol levels, and elevated triglycerides and blood pressure (Obunai K. et al., 2007, 2002 NCEP report). The components of MetS are associated with endothelial dysfunction and atherosclerosis and increase the risk for type 2 diabetes mellitus as well as vascular morbidity and mortality (Obunai K. et al., 2007, 2002 NCEP report, Lakka HM. Et al., 2002, Kolovou GD. Et al., 2007, Athyros VG. Et al., 2004). It is unclear whether a single primary abnormality triggers a cascade of diverse events that lead to the manifestation of the components of MetS.

Because the diagnostic features of MetS are shared by Cushing's syndrome (CS), which results from endogenous or exogenous hypercortisolism, it was proposed that cortisol contributes to the pathogenesis of both states although only mild hypercortisolism occurs in MetS in contrast with CS (Pasquali R. et al., 2006, Walker BR. Et al., 2006).

In adults some studies suggest that circulating cortisol concentrations are higher in patients with MetS compared with healthy subjects, in particular in patients with hypertension or impaired glucose tolerance (Sen Y. et al., 2008, Duclos M. et al., 2005, Weigensberg MJ. Et al., 2008, Phillips DI. Et al., 1998, Misra M. et al., 2008) (**Figure 8,** Walker BR. Et al., 2006**)**.

Furthermore subjects presenting visceral obesity possess an hyperactivity of HPA axis with a functional hypercortisolism (Pasquali R. et al., 2000).

Figure 8. Positive correlations between 09.00 h fasting plasma cortisol and features of the metabolic syndrome. Results are from 370 men aged 59–70 years studied in Hertfordshire, England. OGTT, Oral glucose tolerance test; HOMA, homeostasis model assessment.



Reinher and Andler found significant associations between the degree of cortisolemia and fasting insulin levels in obese children, and levels of both hormones decreased following weight loss (Reinher and Andler, 2004).

Abnormalities in the central regulation of the hypothalamic-pituitary-adrenal (HPA) axis due to stress may lead to a mild hypercortisolism in adults with obesity and metabolic syndrome (Walker BR. Et al., 2007, Anagnostis P. et al., 2009).

Two recent studies in overweight Latino youths with a family history of type 2 diabetes, confirmed higher fasting cortisol levels in those with lower insulin sensitivity (Walker BR. Et al., 2006) or metabolic syndrome, and an association with hypertension and high glucose levels (Sen Y. et al., 2008). However, a study in a small group of prepubertal children showed higher morning plasma cortisol levels in those with higher total cholesterol and triglycerides (Duclos M. et al., 2005).

These findings suggested that, in children, there are similar mechanisms to those reported in adults, but higher cortisol levels could be first a consequence rather than a cause of comorbidities in obesity (Reinher and Andler, 2004).

It is also to be emphasized that in CS, once the tumor is removed and consequently glucocorticoid excess, symptoms improve; in the MetS, weight loss reverses both hypercortisolism and phenotypic abnormalities (Obunai K. et al., 2007, 2002 NCEP report, Lakka HM. Et al., 2002, Kolovou GD. Et al., 2007, Athyros VG. Et al., 2004, Prodam F. et al., 2011) and improves insulin resistance (Prodam F. et al., 2011).

Despite the fact that cortisol levels are within the normal range, there is evidence of increased activity of cortisol in the periphery and dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis (Walker BR. Et al., 2006, Sen Y. et al., 2008).

The pathogenesis of the activation of HPA axis in metabolic syndrome remains unclear. It could be in part mediated by a pro-inflammatory state which characterize the obesity state. In fact it is known that cortisol secretion increase in response to inflammatory states and interleukin-6 levels, increased in overweight subjects, are negatively correlated to the cortisol–binding-globuline (CBG) levels (Bernier J. et al., 1998). It has also been demonstrated that CBG levels negatively correlate to BMI (Gagliardi L. et al., 2010). Therefore a reduction in CBG concentration in obese patients could be an explanation for the higher cortisol concentration in obesity. Recently it has been demonstrated that CBG is present also in the anterior pituitary and in supraoptic and

paraventricular nuclei, where it is suggested to have a role in HPA axis regulation (Henley DE. et al., 2011). Other cytokines produced in adipose tissue instead stimulates enzymatic activity of 11β-hydroxysteroid dehydrogenase1 (11βHSD1) which convert the metabolically inactive corticosterone in cortisol (Wake DJ. Et al., 2003). Higher levels of 11 βHSD1 mRNA have been demonstrated In obese subjects compared to that in normal weight subjects (Mariniello B. et al., 2006).

Furthermore in obese subjects higher leptin levels have been demonstrated. Leptin is a peptide produced by adipose tissue which possess an important role in appetite and metabolism regulation, but also induce an increase in glucocorticoid secretion (Nelson DL. Et al., 2006).

Therefore it seems that different pathophysiological mechanisms could contribute to a dysregulation of HPA axis in obesity state.

Although cortisol is associated with metabolic alterations, it appears that adrenocorticotropic hormone (ACTH) may directly contribute to comorbidities in obesity. It has been shown in vitro that ACTH interacts with adipocytes, promotes insulin resistance and is pro-inflammatory (Iwen KA. Et al., 2008). To date, however, the role of ACTH has not been determined in obese children.

2. EXPERIMENTAL RESULTS

2.1 Study 1

Title

Acylated and unacylated ghrelin levels in normal weight and obese children: influence of puberty and relationship with insulin, leptin and adiponectin levels.

Aim of the Study

In order to understand the biological implications of acylated (AG) and unacylated (UAG) ghrelin in the pediatric population, we evaluated AG and UAG levels in normal weight and obese children, prepubertal and pubertal. Furthermore we measured insulin, leptin and adiponectin levels in the same subjects.

Subjects and Methods

It has been evaluated a total 140 children followed by the Division of Pediatrics of our Hospital, “Azienda Ospedaliero Universitaria Maggiore della Carità” in Novara, Surgery of Pediatric Endocrinology and Auxology.

All subjects underwent a clinical evaluation by a trained research team. Pubertal stages were determined by physical examination, using the criteria of Marshall and Tanner (**Appendix 1**). Height was measured to the nearest 0.1 cm using a Harpenden stadiometer, and body weight with light clothing to the nearest 0.1 kg using a manual weighing scale. Body mass index (BMI) was calculated as body weight divided by squared height (kg/m2). Children were subdivided using Italian growth charts (Cacciari E. et al., 2006, **Appendix 2**). We considered obese children with BMI above the 95th percentile. Waist circumference was measured at the high point of the iliac crest around the abdomen and was recorded to the nearest 0.1 cm (**Appendix 3**). Systolic BP (SBP) and diastolic BP (DBP) were measured three times at 2-minute intervals using a mercury sphygmomanometer with an appropriate cuff size after participants were seated quietly for at least 15 minutes, with their right arm supported at the level of the heart and feet flat on the floor, prior to other physical evaluations, and at least 30 minutes after blood sampling, using a standard mercury sphygmomanometer. Mean values were used for the analyses. Hypertension was determined if BP values recorded on enrollment day and on blood samples day are always elevated. (**Appendix 4**).

Normal weight children presented to the clinic for an evaluation of growth, pubertal status, suspected thyroid disease, general health checkup, but no disease was confirmed at the end of the evaluations. Exclusion criteria were the presence of any psychiatric or organic diseases in particular neurological, endocrine (short stature), liver, and kidney abnormalities. Nobody was under pharmacological treatments. The

study protocol was approved by an Independent Ethical Committee and the informed consent had been obtained from each children' parents.

After a 12-hour overnight fast, children arrived at the clinical center at 7.30 AM and rested comfortably for half an hour prior to blood testing. At 8.00 AM, blood samples were taken for measurement of plasma AG, UAG, leptin, adiponectin, glucose, insulin, testosterone (in males) or estradiol (in females).

Human ghrelin (fm/ml) was measured by ELISA (DRG Instruments GmbH, Marburg, Germany). Acylated ghrelin: sensitivity: 1 fm/ml. Intra- and inter-assay CV ranges:3.5-3.8% and 2.6-3.9%. Unacylated ghrelin: sensitivity 10 fm/ml Intra e inter-assay CV ranges: 2.1-4.7 % and 4.2-7.2%.

Insulin (µUI/ml; 1µUI/ml = 7.175 pmol/l) was measured by a chemiluminescent enzyme-labelled immunometric assay (Diagnostic Products Corporation, Los Angeles, CA). Sensitivity: 2 µUI/ml, with an intraand inter-assay CV range: 2.5-8.3 and 4.4-8.6%.

Plasma glucose levels (mg/dl; 1 mg/dl:0,05551mMol/liter) were measured by the gluco-oxidase colorimetric method (GLUCOFIX, by Menarini Diagnostici, Florence, Italy).

Adiponectin was measured by an ELISA kit E09, (Mediagnost, Reutlingen, Germany). Sensitivity: 0.06 µg/ml, with an intra e inter-assay CV range: 4.7% e 6.7%.

Leptin was measured by Direct ELISA (Diagnostics Biochem Canada). Sensitivity: 0.5 ng/ml. Intra e inter-assay CV ranges: 7.4% e 8.7%.

Testosterone and estradiol were measured with Centaur instrument, Siemens kit, by chemiluminescent method. Testosterone: sensitivity 10 ng/dl, with an inter-assay: 6.2%; estradiol: sensitivity 7 pg/ml, with an inter-assay: 7.4%.

Insulin resistance was calculated using the formula of HOMA-IR = [fasting glucose (mg/dL)/18 ×fasting insulin (mUI/L)]/22.5. Beta cell function at fasting was calculated using the formula of HOMA-B =(20×fasting insulin)/(fasting glucose-3.5).

Insulin sensitivity fasting was calculated from the QUICKI index (1/[log fasting insulin + log fasting glucose]) (26).

Data are expressed as mean±SD or median and 25_{th}-75_{th} percentiles. For continuous variables, the variation between groups was compared by means on nonparametric Wilcoxon and Mann-Whitney U tests, where appropriate. A correlation analysis was performed using the Pearson's correlation test thought a logarithmic transformation of the parameters when necessary. A partial correlation analysis was performed to adjust for BMI, age, gender, and pubertal status. A stepwise regression model with two-tailed probability values and 95% confidence intervals for each significant parameter in the correlation analysis was used to measure the strength of association between variables.

Statistical significance was assumed for $p<0.05$. All statistical analyses were performed with SPSS for Windows version 15.0 (SPSS INC; Chicago, IL, USA).

Results

Hormonal parameters

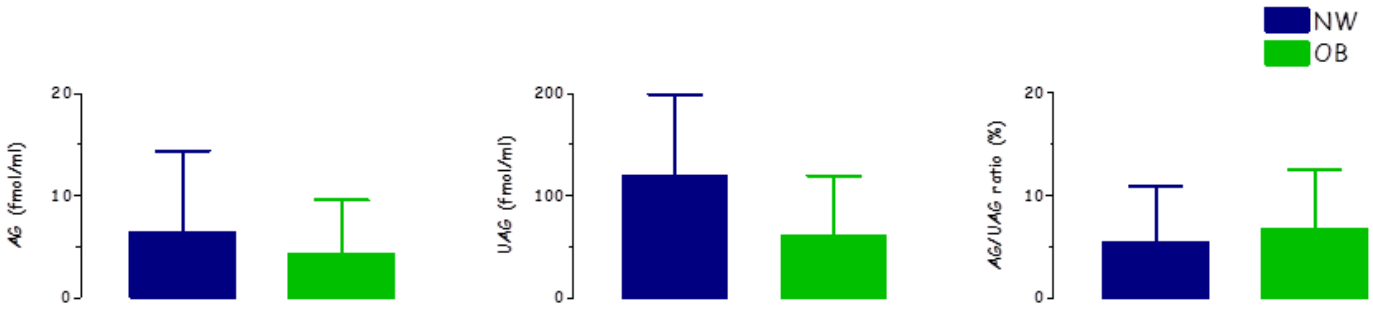
Eighty-two children (mean±SD) (age: 9.7 ± 4.1 yr) were normal weight (NW): 46 females and 36 males, 44 prepubertal children and 38 with pubertal stage from II to V. The remaining 58 children (age (mean±SD): 9.8±3.4 yr) were obese (OB): 30 females and 28 males, 28 prepubertal and 30 pubertal children. All auxological parameters of two groups are reported in **Table 3.**

Table 3. Auxological parameters of NW and OB children.

	NW			OB		
	All	PP	P	All	PP	P
n	82	44	38	58	28	30
M/F	36/46	26/36	10/10	28/30	21/19	7/11
Age (yr)	9.7±4.1	7.99±0.42 [d]	14.49±0.56 [d]	9.8±3.4	8.64±0.43 [d]	13.81±0.43 [d]
Weight (kg) c° weight	31.44±1.6 [a] 39±3.4	25.86±1.17 [d] 39.2±3.86	49.06±3.33 [d] 37.3±7.3	58.81±2.81 [a] 96.2±0.43	49.52±2.43 [d] 96.45±0.55	79.47±4.32 [d] 95.8±0.63
Height (cm) c° height	129.7±2.48 [a] 33±3.6	121.6±2.26 [d] 32.9±4.13	155.34±3.08 [d] 34.1±8.05	143.8±2.09 [a] 68.9±4.19	136.9±2.11 [d] 76.7±4.03	159.23±2.11 [d] 51.3±8.98
BMI (Kg/m^2) c° BMI	17.67±0.35 [a] 39±3.10	16.96±0.35 [d] 38.8±3.51	19.92±0.70 [d] 39.5±6.8	27.03±0.62 [a] 95.9±0.34	25.66±0.60 [d] 95.6±0.48	31.06±1.06 [d] 96.7±0.15
AG (fmol/ml)	6.5 (3.2-12) [b]	8.2 (3.9-14.9) [e]	4.6 (1.4-7.3) [e]	4.4 (1.4-8.1) [b]	4.8 (2.2-10.4) [e]	2.5 (0.0-5.4) [e]
UAG (fmol/ml)	120.1 (80.4-172.3) [a]	155 (111.1-201) [e]	97.2 (58.7-130) [e]	61.3 (45.9-98) [a]	94.7 (52.5-126) [e]	53.2 (37.6-64.2) [e]
AG/UAG ratio	0.05 (0.02-009)	0.06 (0.02-0.1)	0.05 (0.01-0.1)	(0.24-0.10)	0.07 (0.03-0.1)	0.07 (0.00-0.1)
Adiponectin	14.7 (10.2-19.9)[a]	16.1 (11.2- 20)[e]	10.7 (5.9-13.6)[e]	9.2 (6.6-11.3)[a]	9.9 (7.6-11.4)[e]	6.17 (5.3-9)[e]
Leptin	6.9 (4.7-11.9)[c]	6 (4.7-10.9)[d]	11.8 (3.3-16)[d]	36.2 (23.8-50)[c]	30.8 (22.2-41.2)[d]	46.4 (37.1-102.4)[d]
Insulin	5.3 (3.1-8.1)[c]	4.5 (2.6- 6.3)	10 (7.1-12.6)	12.9 (8.1-18.2)[c]	10.1 (6.6- 16)[e]	16 (10.4-25.8)[e]
HOMA	1.02 (0.62-1.76) [a]	0.66 (0.38-1) [d]	1.5 (1.03-2.23) [d]	2.5 (1.5-4.3) [a]	1.9 (1.1-3.3) [d]	3.3 (2.29-5.40)[d]
HOMAß	110.1 (60-178) [a]	72 (48-120) [d]	152 (93.8-249.0) [d]	212.2 (140-345) [a]	142.4 (97.2-177.6)[d]	305 (210-433) [d]
QUICKI	0.38 (0.35-0.41) [a]	0.41 (0.38-0.45) [d]	0.35 (0.33-0.38) [d]	0.33 (0.30-0.35) [a]	0.34 (0.31-0.37) [d]	0.31 (0.29-0.33) [d]

We observed that the median (IQR) of AG (6.5; 3.2-12.0 fmol/ml) in NW was higher ($p<0.02$) than in OB (4.4; 1.4-8.1 fmol/ml) (Fig.1). Likewise, the median of UAG (120.1; 80.4-172.3 fmol/ml) in NW was higher ($p<0.0001$) than in OB (61.3; 45.9-98 fmol/ml) (**Figure 9**), while the AG/UAG ratio was similar for both groups.

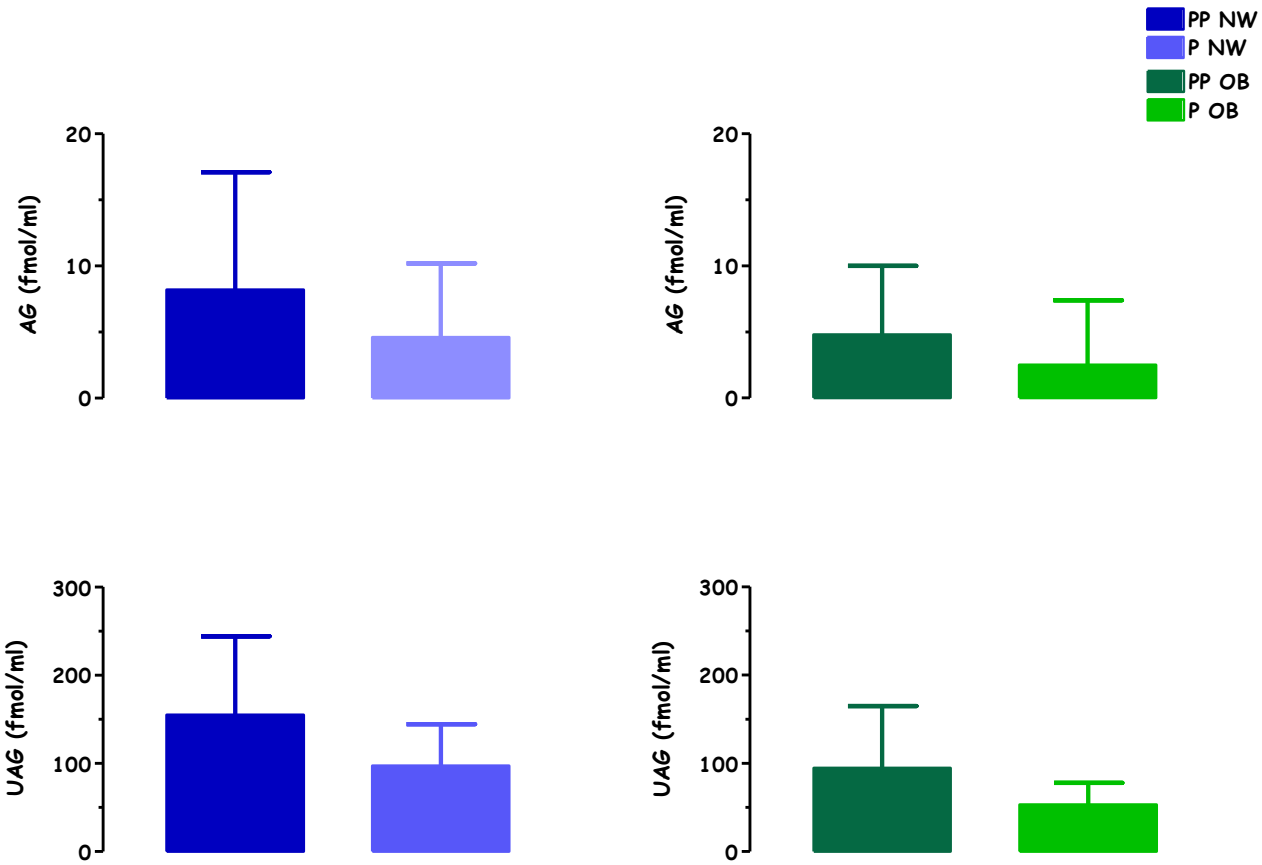
Figure 9. AG (fmol/ml), UAG (fmol/ml) levels and AG/UAG ratio in normal weight (NW) and obese (OB) children.



No differences in ghrelin levels were found between males and females.

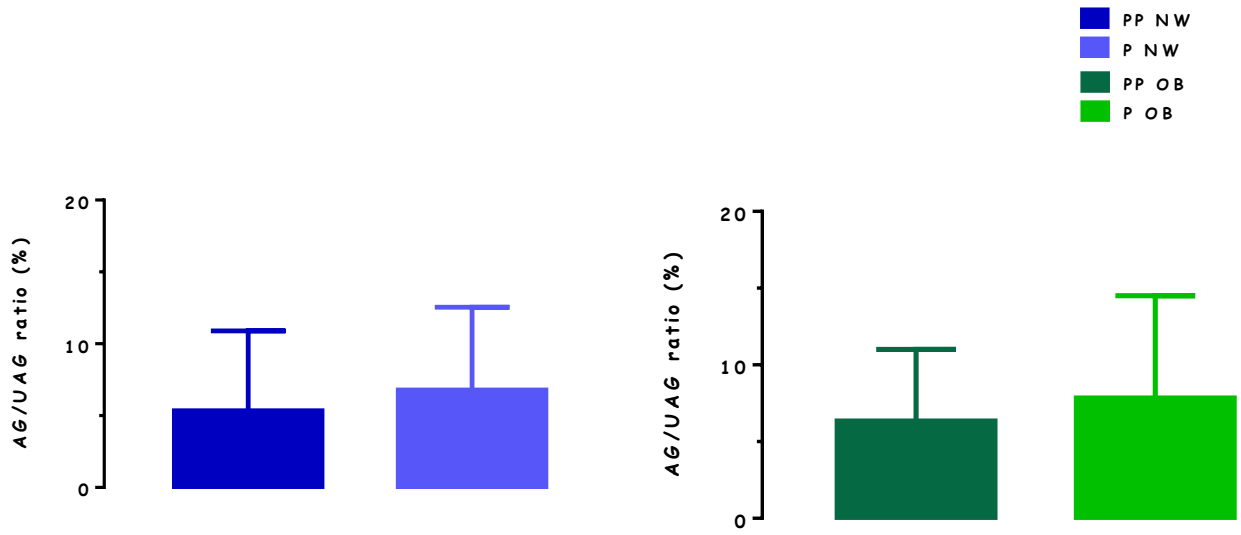
Interestingly in NW, AG and UAG were higher in prepubertal than in pubertal children (8.2 vs 4.6 fmol/ml and 155.0 vs 97.2 fmol/ml, $p<0.01$), with a similar profile observed in the OB group (4.8 vs 2.5 fmol/ml and 94.7 vs 53.2 fmol/ml, $p<0.01$) (**Figure 10**).

Figure 10. AG (fmol/ml) and UAG (fmol/ml) levels in normal weight (NW) and obese (OB) children, prepubertal (PP) and pubertal (P).



Therefore AG/UAG ratio were similar in prepubertal and in pubertal children, either in normal weight or in the obese group (**Figure 11**).

Figure 11. AG/UAG ratio in normal weight (NW) and obese (OB) children, prepubertal (PP) and pubertal (P).

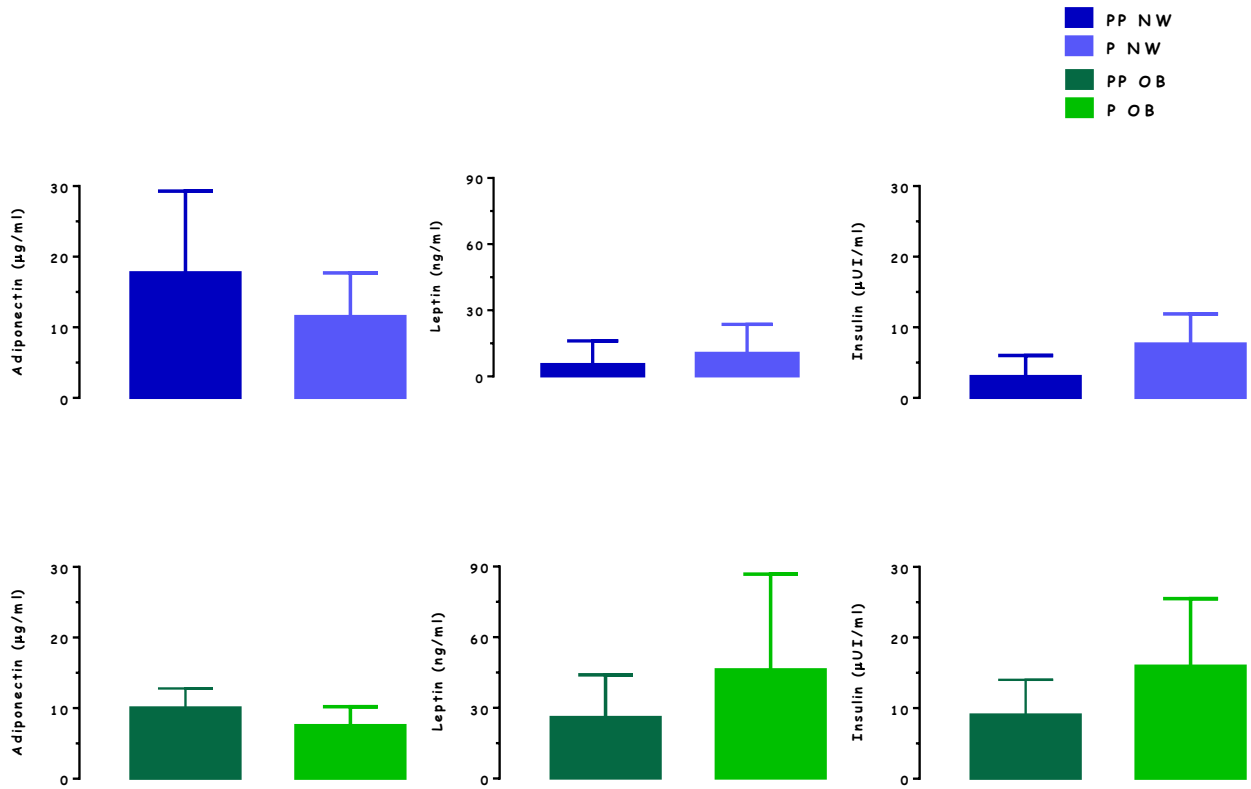


When metabolic parameters were evaluated, it was observed that adiponectin levels were higher ($p<0.0001$) in NW (14.7; 10.2-19.9 µg/ml) than in OB (9.2; 6.6-11.3 µg/ml), whereas in contrast leptin levels in NW (6.9; 4.7-11.9 ng/ml) were lower ($p<0.001$) than in the OB group (36.2; 23.8-50.0 ng/ml) (Fig.4). It was observed that insulin levels in the NW group (5.3; 3.1-8.1 µUI/ml) were lower ($p<0.001$) than in OB (12.9; 8.1-18.2 µUI/ml).

No gender differences were observed for adiponectin and insulin, while leptin levels in females (15.2; 7.2-38.8 µg/ml) were higher than in males (8.95; 4.3-32.6 µg/ml) ($p<0.01$).

During pubertal development in both NW and OB children, adiponectin levels were significantly lower ($p < 0.005$), while leptin levels were higher in pubertal when compared to prepubertal children ($p<0.0001$). Insulin levels were higher in OB pubertal than prepubertal ($p<0.01$), while in NW they increased during puberty, but this variation did not reach the statistical significance (**Figure 12**).

Figure 12. Adiponectin, leptin, insulin levels in normal weight (NW) and obese (OB) children, prepubertal (PP) and pubertal (P).



NW showed HOMA and HOMAβ indices lower than OB ($p<0.0001$) with a QUICKI index higher ($p<0.0001$).

Glucose levels maintained similar levels between groups. As expected, in both groups testosterone and estradiol levels increased with puberty ($p<0.0001$ and $p<0.01$, respectively).

HOMA and HOMAβ were higher and QUICKI index lower in puberal children than prepubertal in both groups ($p<0.0001$).

Correlation and regression analysis

When correlation analyses were performed in all the subjects together, it was found that both AG and UAG were negatively correlated with age, height, weight and BMI ($p<0.0001$) (**Table 4**).

Table 4. Pearson coefficients using correlation analysis for AG and UAG levels in all subjects. [a]:p<0.05; [b]:p<0.01; [c]:p<0.001; [d]:p<0.0001

	AG (FMOL/ML)	UAG (FMOL/ML)
	Pearson	
Age (years)	-0.314[d]	-0.526[d]
NW	-0.146	-0.520[d]
OB	-0.376[b]	-0.507[d]
Height (cm)	-0.402[d]	-0.668[d]
NW	-0,166	-0.641[d]
OB	-0.370[b]	-0.519[d]
Weight (Kg)	-0.378[d]	-0.615[d]
NW	-0.186	-0.627[d]
OB	-0.370[b]	-0.558[d]
BMI (kg/m^2)	-0.323[d]	-0.615[d]
NW	-0.163	-0.466[d]
OB	-0.384[b]	-0.603[d]
Insulin (µUI/ml)	-0.442[c]	-0.541[d]
NW	-0.435[c]	-0.677[d]
OB	-0.537[d]	-0.639[d]
Glicemia (mg/dl)	-0.07	-0.286[b]
NW	0.090	-0.273[a]
OB	-0.273	-0.171
Adiponectin (µg/ml)	0.173[a]	0.411[d]
NW	0.090	0.410[d]
OB	0.100	0.302[a]
Leptin (ng/ml)	-0.270[c]	-0.479[d]
NW	-0.240[b]	-0.456[d]
OB	-0.307[a]	-0.566[d]
Testosterone (ng/dl)	-0.230	-0.344[b]
NW	-0.051	-0.754[d]
OB	-0.416	-0.473[b]
Estradiol (pg/ml)	-0.214	-0.209[a]
NW	-0.216	-0.451[b]
OB	-0.308	-0.622[c]
HOMA	-0.496[d]	-0.744[d]
NW	-0.387[b]	-0.700[d]
OB	-0.544[d]	-0.619[d]
HOMAß	-0.374[d]	-0.483[d]
NW	-0.384[b]	-0.427[c]
OB	-0.297[b]	-0.336[b]
QUICKI	0.476[d]	0.739[d]
NW	0.366[b]	0.700[d]
OB	0.534[d]	0.624[d]
AG (pg/ml)	1	0.537[d]
NW	1	0.335[b]
OB	1	0.556[d]
UAG (pg/ml)	0.537[d]	1
NW	0.335[b]	1
OB	0.556[d]	1

We observed that AG was negatively correlated with leptin and insulin ($p<0.001$) and showed a weak positive correlation with adiponectin ($p<0.05$). UAG showed a negative correlation with leptin, testosterone, estradiol, glucose and insulin levels ($p<0.001$), with a stronger positive correlation with adiponectin levels ($p<0.0001$) (**Figures 13-14**).

Figure 13. Correlations between AG or UAG and BMI or insulin.

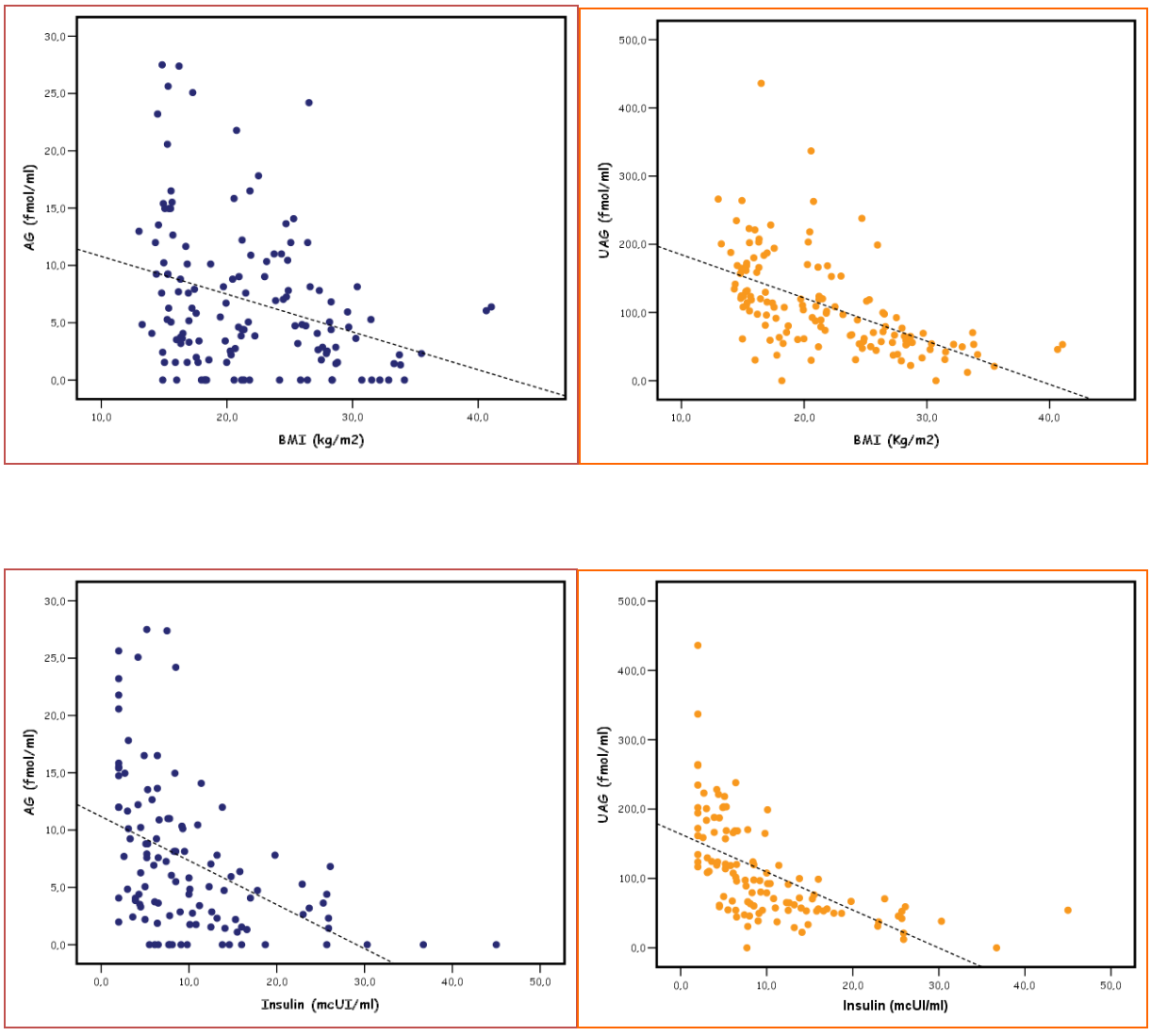
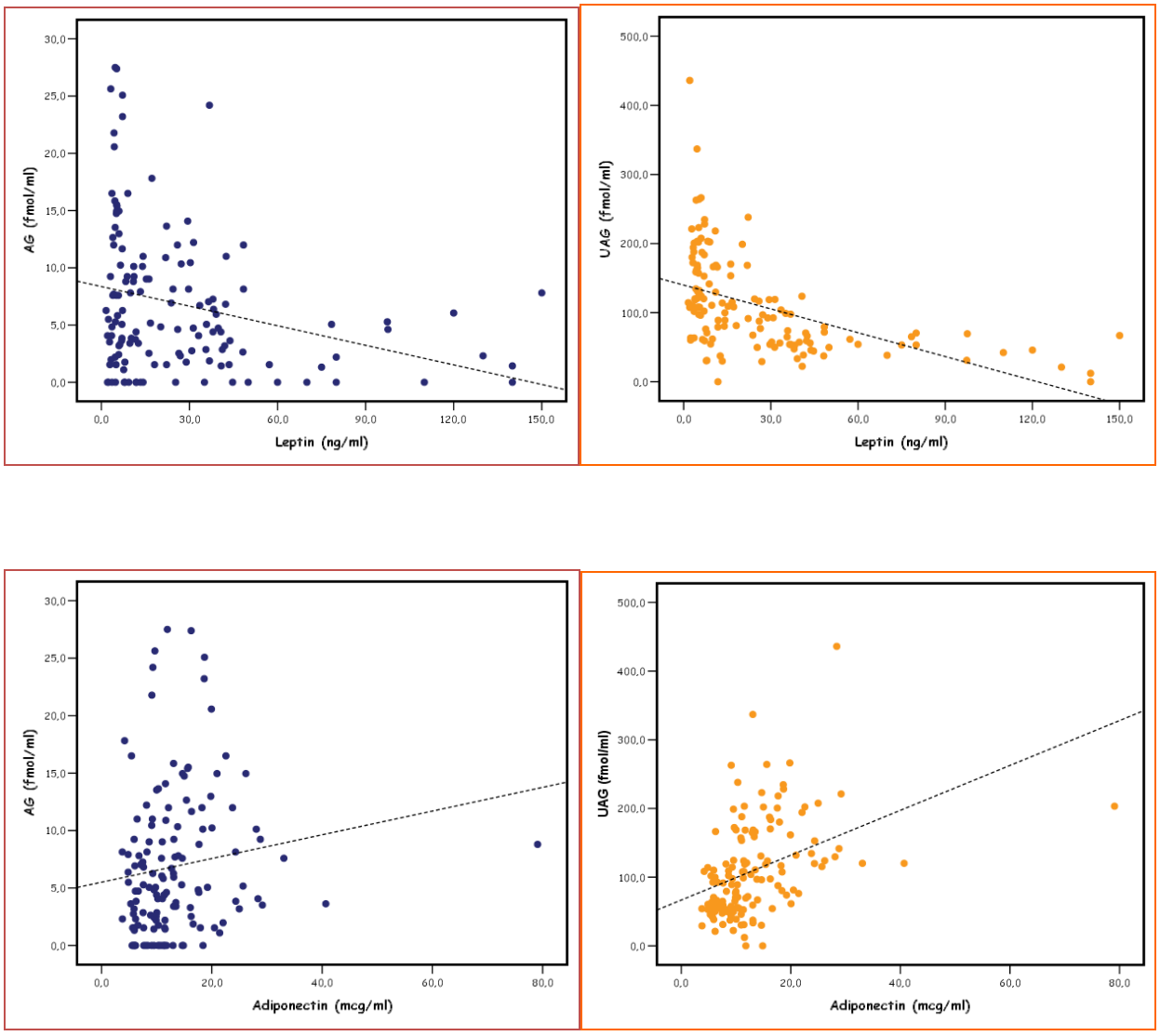


Figure 14. Correlations between AG or UAG and leptin or adiponectin.



AG and UAG were negatively correlated with HOMA and HOMAβ ($p<0.0001$), and positively with QUICKI ($p<0.0001$).

AG maintained the negative correlation with leptin and insulin ($p<0.0001$) when correcting for pubertal stage, and only with insulin ($p<0.001$) when corrections were made for pubertal stage and/or BMI. UAG showed a negative correlation with adiponectin, leptin and insulin ($p<0.0001$), when correcting for pubertal stage, whereas

correlations were maintained for leptin and insulin ($p<0.001$) when correcting for pubertal stage and/or BMI.

Analyzing the two groups separately, AG and UAG were negatively correlated with age, height, weight and BMI ($p<0.0001$) in OB children. UAG was correlated with anthropometric parameters ($p<0.01$) in NW, not AG. Moreover AG and UAG showed the same correlations with metabolic parameters and insulinemic indices, with exception of adiponectin. Only UAG was correlated with adiponectin in both OB ($p<0.02$) and NW ($p<0.0001$) children. Moreover, AG was negatively correlated with insulin ($p<0.05$) when corrected for pubertal stage, whereas UAG maintained its negative correlation with insulin and leptin ($p<0.0001$) in both groups. AG kept its correlation with insulin only in OB group, not in NW, and UAG with insulin and leptin ($p<0.001$) in OB group and with insulin ($p<0.0001$) in NW group when corrected for pubertal stage and/or BMI. In normal weight children auxological parameters resulted not correlated with AG levels.

Multiple regression analysis revealed that AG levels were negatively predicted (R2: 0.214; $p<0.001$) by insulin (standardized β: -0.462; unstandardized β: -0.379; IC 95% -0.516/-0.241), when corrected for pubertal stage or BMI. UAG levels were negatively predicted (R2: 0.407; $p<0.0001$) by BMI (β: -0.553; unstandardized β: -5.386; IC 95% -6.919/-3.853) and insulin (β: -0.271; unstandardized β: -1.999; IC 95% -3.381/-0.618) and positively by adiponectin (β: 0.260; unstandardized β: 2.467; IC 95% 0.881/4.053) when corrected for pubertal stage. Alternatively, UAG levels were negatively predicted (R2: 0.422; $p<0.0001$) by insulin (β: -0.571; unstandardized β: -5.150; IC 95% -6.549/-3.750) and leptin (β: -0.224; unstandardized β: -0.479; IC 95% -0.903/-0.056) and positively predicted by adiponectin (β: 0.277; unstandardized β: 2.640; IC 95% 1.153/4.127) when corrected for BMI.

Discussion

The present study demonstrates that obese children show AG and UAG levels lower than those in normal weight children; this reduction is similar between the two forms, with maintenance of the same AG/UAG ratio. We observed that ghrelin levels are lower during puberty in both groups, with no differences emerging for gender. Interestingly, when the two ghrelin forms were evaluated separately, a peculiar yet strong relationship between UAG levels and metabolic parameters has been observed, suggesting a significant role for UAG in metabolic functions.

To the present day, only few studies have reported AG and UAG levels in childhood, being mainly focused on adolescents and small cohorts of subjects (Harada T. et al., 2008, Mackelvie KJ. Et al., 2007). To our knowledge, this is the only larger study evaluating both forms of ghrelin in obese and healthy children and it clearly shows lower levels of both AG and UAG in the obese state.

Circulating ghrelin is comprised primarily of UAG (Van der Lely AJ. Et al., 2004, Soares JB. Et al., 2008), however, at present the regulation of the different forms of ghrelin is poorly understood. In adulthood it has been demonstrated that both forms of ghrelin are similarly inhibited by feeding, while in the fasting state a dissociation of the two forms is evident (Soares JB. Et al., 2008). During long-term fasting, AG levels decrease while UAG and total ghrelin remain unchanged (Soares JB. Et al., 2008, Liu J. et al., 2008). Available data in adulthood suggest that AG and UAG may have different and opposite effects on glucose homeostasis, where AG, most likely via GHS-R1a receptors, inhibits insulin secretion thus increasing glucose levels and exerting a direct effect on hepatocytes to modulate gluconeogenesis and glycogen synthesis (Van der Lely AJ. Et al., 2004). In contrast, UAG counteracts the AG effect on insulin secretion via as yet to be described receptors (Wiedmer P. et al., 2008).

In accordance with this data, in our study OB children show lower levels of both AG and

UAG when compared to NW subjects, either in prepubertal or in pubertal children maintaining the same AG/UAG ratio during childhood. In both groups AG, but more strongly UAG, correlated with adiponectin, leptin, insulin. Furthermore both forms are well related with insulinemic indices, negatively with HOMA and HOMAß and positively with QUICKI. Also when corrected for pubertal stage and BMI, AG levels were negatively predicted by insulin while UAG were predicted by insulin, leptin and adiponectin. The consensual reduction independent of pubertal stage of AG and UAG in obesity without modification of the AG/UAG ratio, as well as a negative correlation of both forms with insulin metabolism, further suggests that all the ghrelin secretion is strictly modulated by insulin lifespan. Analyzing the two groups separately both AG and UAG maintained their correlations with insulin levels and indices, while only UAG was correlated with adiponectin levels suggesting their interplay metabolic functions in children. In normal weight children auxological parameter resulted not correlated with AG levels, probably due to the little number of subjects or skewed distribution.

The regulation of AG and UAG circulating levels has not yet been clearly defined. It is thought that UAG could be produced directly from the ghrelin gene, therefore via a different pathway to the acyl form, or alternatively it could be derived by the deacylation of ghrelin (Soares JB. Et al., 2008, Liu J. et al., 2008).

Very recently GOAT, an enzyme catalyzing the addition of the octanoyl-group has been identified (Gualillo o. et al., 2008). It is not known at present whether the GOAT levels regulate changes in ghrelin acylation or, on the contrary, if GOAT itself depends on different metabolic conditions. Its discovery has introduced intriguing questions and new possibilities to understanding better the regulation of energy balance.

As such, the presently published data demonstrates that total ghrelin does not adequately reflect AG and UAG ghrelin levels, suggesting a role of ghrelin acylation in the modulation of energy intake (Harada T. et al., 2008). In this context, the measurement of AG/UAG ghrelin ratio could be a more useful tool to understanding ghrelin changes in different conditions. In our study, we observed that both forms of ghrelin did not show gender differences. Most studies are concordant with our results (Purnell JQ. Et al., 2003, Vilarrasa N. et al., 2005), but some have demonstrated that they are modulated by sex. For example, adult women studied in the late follicular phase showed higher levels of ghrelin with respect to men of a similar age and BMI (Van der Lely AJ. Et al., 2004). Studies evaluating total ghrelin levels in children are more homogeneous and confirm the present results (Bellone S. et al., 2004, Whatmore AJ. et al., 2003). Of particular relevance is the study by Ghizzoni *et al*. which demonstrated in prepubertal children that within a 24 hour period, ghrelin is secreted in a pulsatile manner and with a circadian rhythm, showing no differences for gender (Ghizzoni L. et al., 2004).

In the present study, AG and UAG ghrelin levels are lower during pubertal development, reflecting the pattern for total ghrelin as previously observed in normal weight children during puberty (Soriano-Guillen L. et al., 2004, Whatmore AJ. et al., 2003). When correcting for pubertal stage, AG maintains the negative correlation exclusively with insulin, while UAG showed a negative correlation with adiponectin, leptin and insulin.

To date, the physiological mechanisms for the regulation of ghrelin secretion is not yet fully understood. More certainly the gonadal system appears to play a role, with GHS receptors identified in the ovary and testis, with Leydig cells shown to synthesize ghrelin (Gil-Campos M. et al., 2003). Studies in polycystic ovary syndrome, a condition of hyperandrogenism, and in hypogonadal males support the hypothesis of a role of

androgens on ghrelin secretion (Pagotto U. et al., 2002, Pagotto U. et al., 2003). Lebenthal *et al.* also studied total ghrelin levels before and after priming with sexual steroids (Lebenthal Y. et al., 2006) demonstrating, in male subjects, a clear reduction in circulating ghrelin levels while no changes were observed in females. In line with these findings, we observed in the present study that both ghrelin forms were similarly lower during pubertal development and their levels negatively correlated with circulating testosterone and estradiol levels. On the other hand this different hormonal pattern could be due to changes in body composition, characteristic of puberty. Body proportions and fat distribution change over pubertal period due to differences in endocrine status, genetic factors, ethnicity and the environment with males assuming an android and females a gynecoid shape, respectively. In fact, during puberty, males gain greater amounts of lean and skeletal mass whereas females mainly acquire fat mass (Loomba-Albrecht LA. et al., 2009).

During puberty we also observed lower adiponectin levels and higher leptin levels in both groups. Insulin levels are higher only in obese children, not in normal weight ones with glucose maintaining similar levels. This effect is probably due to an increase in body fat mass that is correlated with the insulin secretion.The regulation of body weight and energy homeostasis is a complex system that involves signals converging on the central nervous system. The hypothalamic nucleus such as the arcuate nucleus, ventral tegmental area and the substantia nigra are the primary sites where peripheral signals are integrated for the control of weight balance (Van der Lely AJ. Et al., 2004, Gil-Campos M. et al., 2003). It has been demonstrated that ghrelin as well as other anorectic peptides signal to the same system exerting opposite functions. A negative relationship between leptin and ghrelin has been shown in several studies (Tolle V. et al., 2003, Weigle DS. Et al., 2003, Loomba-Albrecht LA. et al., 2009), with leptin being able to inhibit gastric ghrelin secretion (Kalra SP. Et al., 2005). To date, the existence

of a negative feed-back between these two hormones is still matter of debate. In addition there is evidence that adiponectin levels are lower in obese subjects and increase with weight loss while being directly correlated to insulin sensitivity (Svarbrick MM. et al., 2008). This correlation is lost after matching for pubertal development suggesting a different role for puberty on these two peptides. In the same population, adiponectin was positively correlated with AG and UAG levels. When correcting for puberty and BMI, we observed that AG was negatively correlated with insulin, while UAG was predicted primarily by leptin in OB and insulin in NW, suggesting diverse roles for the two forms of ghrelin in metabolism and gonadal maturation. The loss of correlation between UAG and leptin at puberty in NW but not in OB suggests that puberty is a key modulator of ghrelin, more specifically UAG with the data confirming the role of leptin as a trigger of gonadal maturation with implication of leptin- and insulin-resistance in obesity (Chan JL. Et al., 2001). The positive correlation between UAG and adiponectin, independent of puberty, strongly suggests a common metabolic regulation.

In conclusion the present study demonstrates in a large cohort of subjects that AG and UAG levels in obese children are lower than those in normal weight ones, with the same ratio between the two forms maintained. While no gender differences were observed, the levels of both ghrelin forms were comparable lower during puberty, suggesting the same inhibitory influence by sexual hormones on AG and UAG. Importantly, a separate evaluation of AG and UAG demonstrated a peculiar strong relationship between UAG levels and metabolic parameters suggesting a role for UAG in metabolic functions. Further studies focusing on the feeding state are needed to increase knowledge of the role of ghrelin acylation on energy homeostasis. Importantly, we feel that for clinical purposes the measurement of AG/UAG ratio should be a more useful tool in understanding the variation of ghrelin under different conditions.

2.2 Study 2

Title

Acylated/unacylated ghrelin ratio in cord blood: correlation with anthropometric and metabolic parameters and pediatric lifespan comparison

Aim of the Study

We hypothesize that, at birth, AGA NN show ghrelin levels similar to prepubertal children, with the same AG/UAG ratio. To understand the lifespan regulation and the biological implications of the two ghrelin forms at the neonatal age, we evaluated AG and UAG levels at birth compared with those of NW and OB children, both prepubertal and pubertal.

Subjects and Methods

We studied three groups of consecutive Caucasian subjects: neonates (NN), normal weight children (NW), and obese (OB) children according to Italian growth charts (Cacciari E. et al., 2006, **Appendix 2**). Group of newborn was composed of adequate for gestational age (AGA) Caucasian NN. AGA was defined as a birth weight from the 10th to the 90th percentile for gestational age according to Italian charts (**Appendix 5**). All babies were born after uncomplicated pregnancies by vaginal or cesarean delivery and were otherwise healthy. All the mothers were healthy and in particular none of the mothers had gestational diabetes. None of the babies showed signs of distress at delivery. Birth weight and length were recorded at birth by the attending nurse.

This neonatal population has been compared to the pediatric population described in study 1. Group NW included 82 children (46 females and 36 males) born AGA. Of these 44 were prepubertal and 38 in a pubertal stage from II to V according to Tanner scale (**Appendix 1**). NW subjects have their weight included between the 3rd and 75th percentile of Italian charts (**Appendix 2**). Group OB was composed of 58 children (30 females and 28 males), 28 of which were prepubertal and 30 pubertal. All OB children were born AGA. All NW and OB children were randomly enrolled according to the clinical criteria at Division of Pediatrics, University of Piemonte Orientale, Novara, Italy. Exclusion criteria were the presence of any psychiatric or organic diseases in particular neurological, endocrine (short stature), liver, and kidney abnormalities.

None of the children were under pharmacological treatments. The study protocol was approved by an Independent Ethics Committee and the informed consent was obtained from each child’s parents.

Auxological measurements on children have been described in study 1. In cord blood at birth in NN, and at 0830–0900 h following an overnight fast in NW and OB plasma, we measured AG, UAG, and insulin. The AG/UAG ratio was calculated. In NN, at

delivery, the cord was immediately clamped and venous blood samples were drawn by catheterization.

Human ghrelin (fmol/ml) was measured in acidified plasma stored at K80 *8*C using ELISA kits from DRG Instruments GmbH, Marburg, Germany. AG: sensitivity: 1 fmol/ml intra- and inter-assay coefficient of variation (CV) ranges: 3.5–3.8 and 2.6–3.9% and UAG: sensitivity: 10 fmol/ml intra- and inter-assay CV ranges: 2.1–4.7 and 4.2–7.2%.

Insulin (mUI/ml; 1 mUI/ml*Z*7.175 pmol/l) was measured by chemiluminescent enzyme-labeled immunometric assay (Diagnostic Products Corporation, Los Angeles, CA, USA). Sensitivity: 2 mUI/ml intra- and inter-assay CV ranges: 2.5–8.3 and 4.4–8.6%.

Data are expressed as mean ± S.E.M. Distributions of continuous variables were examined for skewness and were logarithmically transformed, where appropriate.

Differences between the groups were assessed by the Student's t-test or the one-way ANOVA with post-hoc analysis by the Bonferroni test. A correlation analysis was performed by the Pearson's correlation test. Statistical significance was assumed for P!0.05. All statistical analyses were performed with SPSS for Windows version 15.0 (SPSS, Inc., Chicago, IL, USA).

Results

Group of newborn was composed of 82 Caucasian NN (40 males and 42 females), born to term (37–41 weeks of gestation) and adequate for gestational age with a normal ponderal index. Thirty-eight were born by vaginal delivery and 44 from cesarean delivery.

All auxological parameters of the three groups are reported in **Table 5**.

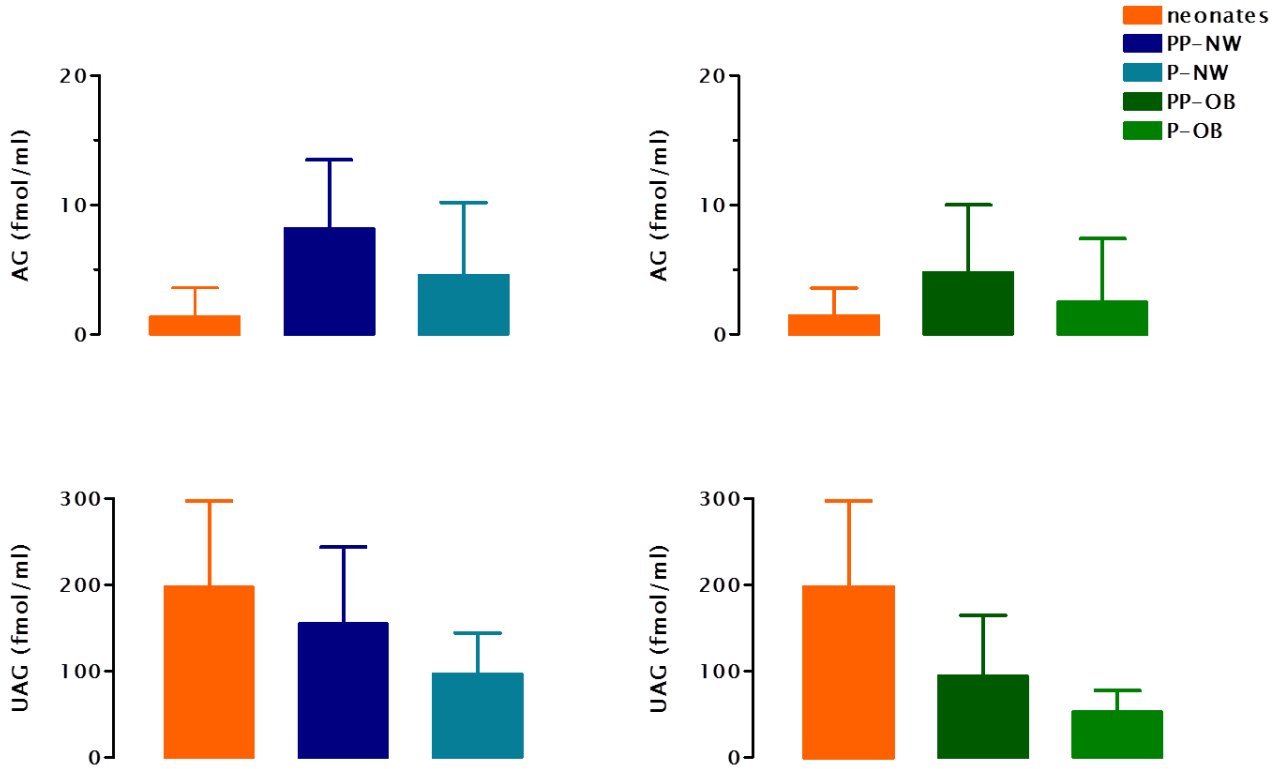
Table 5. Clinical parameters of NN, NW and OB children.

	NN	NW			OB		
		All	PP	P	All	PP	P
n	82	82	44	38	58	28	30
M/F	40/42	36/46	26/18	10/28	28/30	21/7	7/23
EU/TC	37/45	–	–	–	–	–	–
GA (week)	38.9±0.18	–	–	–	–	–	–
PI (kg/m^3)	2.6±0.3	–	–	–	–	–	–
Age (years)		9.7±0.47	7.99±0.42*	14.49±0.56*	9.8±0.4	8.64±0.43*	13.81±0.43*
Weight (kg)	3.28±0.06	31.44±1.6†	25.86±1.17*	49.06±3.33*	58.81±2.81†	49.52±2.43*	79.47±4.32*
Per. weight	50.15±2.88	39±3.4	39.2±3.86	37.3±7.3	96.2±0.43	96.45±0.55	95.8±0.63
Height (cm)	50±0.31	129.7±2.48†	121.6±2.26*	155.34±3.08*	143.8±2.09†	136.9±2.11*	159.23±2.11*
Per. height	49.8±0.31	33±3.6	32.9±4.13	34.1±8.05	68.9±4.19	76.7±4.03	51.3±8.98
BMI (kg/m^2)	–	17.67±0.35†	16.96±0.35*	19.92±0.70*	27.03±0.62†	25.66±0.60*	31.06±1.06*
Per. BMI		39±3.10	38.8±3.51	39.5±6.8	95.9±0.34	95.6±0.48	96.7±0.15

Per., percentile; EU, eutocical delivery; TC, cesarean delivery; GA, gestational age; PI, ponderal index; PP, prepubertal; P, pubertal. *$P<0.0001$ NW and OB PP vs P; †$P<0.0001$ NW vs OB.

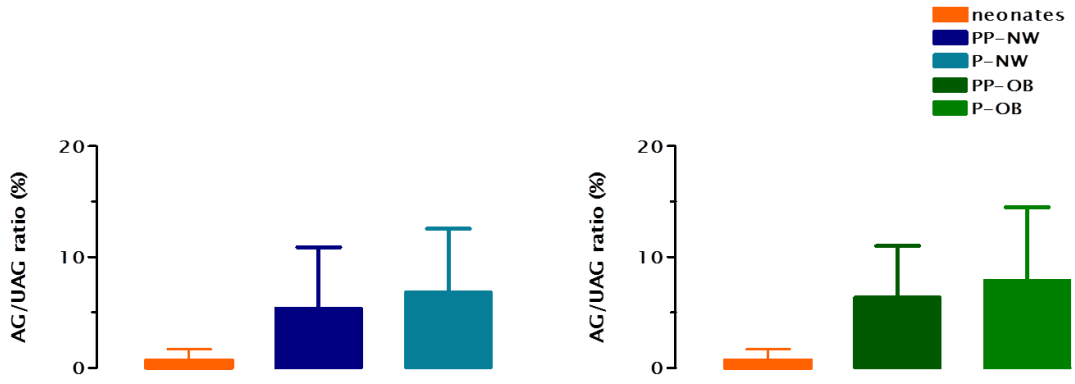
AG levels (mean±S.E.M.) were lower in NN compared with both NW (1.68±0.24 vs 8.43±0.87 fmol/ml; P<0.0001) and OB children (1.68±0.24 vs 5.30±0.68 fmol/ml; P<0.0001; Fig. 1). AG levels were particularly lower in NN than in prepubertal NW and OB children (9.77±1.06 and 6.23±0.73 fmol/ml, respectively; P<0.007). UAG levels were higher in NN (213.2±9.1 fmol/ml) compared with NW (135.9±8.7 fmol/ml; P<0.0001) and OB children (79.5±7.6 fmol/ml; P<0.0001; **Figure 15 and Table 5**).

Figure 15. Acylated and Unacylated ghrelin levels in neonates and in normal weight (NW) and obese (OB) children, prepubertal (PP) and pubertal (P)



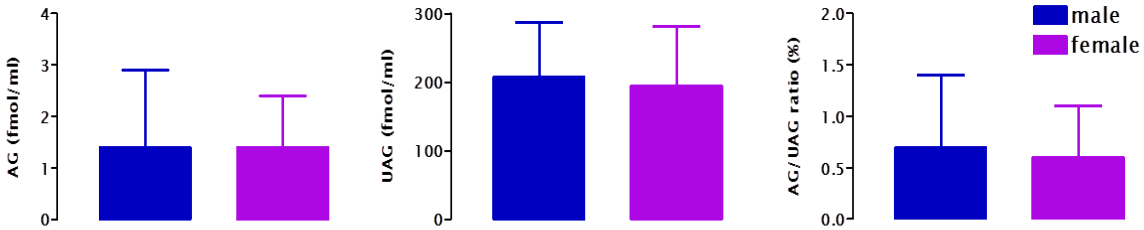
Furthermore, AG/UAG ratio was lower in NN than in NW (0.01±0.0 vs 0.07±0.01; P<0.0001) and OB children (0.01±0.0 vs 0.07±0.01; P<0.0001). AG/UAG ratio was similar between NW and OB (**Figure 16**).

Figure 16. AG/UAG ratio in neonates and in normal weight (NW) and obese (OB) children, prepubertal (PP) and pubertal (P).



No gender differences have been found (**Figure 17**).

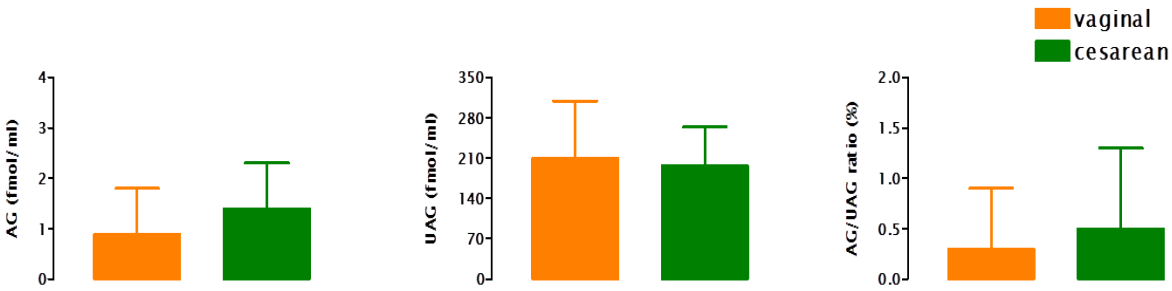
Figure 17. Acylated, Unacylated ghrelin levels and AG/UAG ratio in males and females neonates.



NN showed insulin levels (6.40±0.76 mUI/ml) similar to NW (6.26±0.51 mUI/ml) and lower than OB children (14.4±G1.24 mUI/ml; P<0.0001).

AG, UAG levels and the AG/UAG ratio were not different in NN according to the type of delivery (**Figure 18**).

Figure 18. Acylated, Unacylated ghrelin levels and AG/UAG ratio in neonates born from cesarean or vaginal delivery.



No gender differences were detected in each of the three groups.

At birth UAG was positively correlated with AG (Pearson: 0.425; P<0.0001) and negatively with insulin (-0.253; P<0.02). No association was found between UAG and anthropometric parameters. AG did not demonstrate any associations with anthropometric or hormonal parameters, with the exception of UAG. In NW and OB, UAG was positively correlated with AG (0.537; P<0.0001) and negatively with insulin and BMI (-0.566 and -0.541; P<0.0001). Similarly, AG was positively correlated with UAG and negatively with insulin and BMI (-0.442 and -0.323; P<0.0001).

In a model composed of all three groups, UAG was negatively correlated with weight and insulin (β: -0.661 and -0.489, respectively; P<0.0001) and AG was weakly associated in a negative manner exclusively with insulin (β: -0.214; P<0.003).

Discussion

Our study is mainly focused on a physiological investigation of the two forms of ghrelin, AG and UAG, in healthy AGA newborns compared with later in life. The results demonstrate that in full-term NN, the venous cord blood at birth presents a very different profile of the two ghrelin forms compared with that found in children. NN show lower AG and higher UAG levels than NW and OB children, independent of pubertal status. As a consequence, the AG/UAG ratio in cord blood of NN is lower compared with that found in NW and OB children.

To date, most authors have studied total ghrelin independent of the two forms in NN and children (Soriano-Guillen L. et al., 2004, Ng PC. Et al., 2005, Chanoine JP. Et al., 2003, Chanoine JP. Et al., 2002, Kitamura S. et al., 2003, Farquhar J. et al., 2003, Whatmore AJ. Et al., 2003), with a few studies published regarding the ghrelin isoforms, particularly in newborns.

It has been clearly demonstrated that total ghrelin levels are similar in female and male newborns (Soriano-Guillen L. et al., 2004, Martos-Moreno GA. Et al., 2009, Pirazzoli P. et al., 2005, Bellone S. et al., 2003) and are higher in SGA compared with AGA newborns (Onal EE. Et al., 2004, Kitamura S. et al., 2003, Farquhar J. et al., 2003), while controversial data exists regarding correlations between ghrelin levels and gestational age or auxological parameters (Martos-Moreno GA. Et al., 2009, Farquhar J. et al., 2003, Chiesa C. et al., 2088, Bellone S. et al., 2004). Soriano-Guillen et al. (Soriano-Guillen L. et al., 2004) demonstrated that

total ghrelin levels in newborns were similar between full term and preterm, increasing during early postnatal life and decreasing thereafter during puberty with a negative correlation between ghrelin, age, and Tanner stages.

Only a few studies have shown that AG is present in fetal and neonatal circulation (Martos-Moreno GA. Et al., 2009, Pirazzoli P. et al., 2005, Bellone S. et al., 2004)

equally between preterm and SGA newborns, and full term and AGA, without differences with respect to gender. Moreover, no correlations were found between AG and auxological parameters (Martos-Moreno GA. Et al., 2009, Pirazzoli P. et al., 2005, Lanyi E. et al., 2004). Recently, Mendez- Ramirez et al. (Mendez-Ramırez F. et al., 2009) measured UAG levels in AGA and SGA newborns at the age of 1 week of life, showing that UAG was higher in SGA compared with AGA NN. To date, no authors have studied UAG levels in cord blood. In our study, we opted to use an assay based on a double-antibody sandwich technique where a monoclonal antibody specific to the C-terminus of ghrelin is coated onto the multiwell plate and detection is performed by an acetylcholinesterase labeled antibody specific to the N-terminus of ghrelin, therefore sandwiching AG when present. This ELISA kit has been demonstrated to have greater assay specificity, particularly with respect to nutritional states (Prudom C. et al., 2010). Using the same assay, we have previously discussed data related to AG and UAG in prepubertal and pubertal NW and OB children (Bellone S. et al., 2004, study 1). In this study, our data demonstrates that the AG/UAG ratio is very different in the venous cord blood of NN compared with later in life, demonstrating lower AG and higher UAG levels than NW and OB children. Interestingly, AG/UAG ghrelin ratio is lower in NN than in children, considering the prepubertal age and pubertal age. This is supported by studies in rat embryos, where elevated plasma concentrations of UAG and lower AG were demonstrated, with a circulating AG/UAG ratio that increased from fetal day 20 to postnatal days (Chanoine JP. Et al., 2004). A possible hypothesis is that UAG levels could be higher at birth, reflecting the fetal state, due to the immaturity of the GOAT system that turns UAG into the AG form. This enzyme has recently been discovered to be responsible for ghrelin octanoylation, but its physiological role and regulation is at present unclear, particularly in the fetal state and childhood. Furthermore, the placenta has been demonstrated to express very low levels of the GOAT transcript (Young J. et

al., 2008, Gutierrez JA, 2008). Some authors have described the regulation of UAG and AG with respect to metabolic impairments in adulthood. Rodriguez et al. (Rodriguez A. et al., 2009) demonstrated that OB subjects with respect to lean individuals had increased levels of AG and decreased UAG. Barazzoni et al. (Barazzoni R. et al., 2007) demonstrated that AG/UAG ratio in patients with metabolic syndrome was increased and positively correlated with insulin resistance indexes compared with non-OB subjects. Pacifico et al. (Pacifico L. et al., 2009) showed lower UAG levels and higher AG/UAG ratio in patients with metabolic syndrome than in those without metabolic syndrome. Therefore, at present, the available information seems to suggest that pathological conditions may likely influence ghrelin form levels and their ratio.

In the literature, acute AG administration in adult subjects induced a rapid increase in glucose and insulin levels with AG related to insulin resistance. On the contrary, UAG prevented AG effects when co-administered with AG and its levels have been found to be negatively associated with insulin levels and insulin resistance (Broglio F. et al., 2004, Van der Lely AJ. Et al., 2009). Also in our study UAG levels and insulin showed a negative correlation, suggesting a major metabolic implication of UAG rather than AG in the neonatal period. Taking into account data in the literature together with our data, we can speculate that the peculiar state of ghrelin secretion in venous cord blood and the negative correlation between UAG and insulin levels, is focused to improve insulin sensitivity in the fetal state. Therefore, at birth, UAG could have a different role with respect to AG. Our data strengthens the importance of the different AG/UAG ratio, proposing a role in metabolic function and fetal growth. Accordingly, NN showed insulin levels similar to NW and lower than in OB children. Insulin levels primarily contribute to neonatal growth as insulin is one of its major hormone regulators promoting lipogenesis, glycogenesis, and protein synthesis (Fant ME & Weisoly D, 2001).

There is a high degree of controversy regarding the relationship between ghrelin and anthropometric parameters. A negative association between UAG and birth weight has been demonstrated by Mendez-Ramirez et al. (Mendez-Ramırez F. et al., 2009) suggesting that diminished body weight induces different adaptive signals. A recent study by Martos-Moreno et al. (Martos-Moreno GA. Et al., 2009) assessing both preterm and term newborns, failed, like us, to demonstrate any association between AG and anthropometric indices, including ponderal index. Our study is in line with the majority of studies failing to find an association at birth, even if it has to be considered that our population includes only AGA NN. Moreover, both forms of ghrelin were independent of gender. The data in the literature are concordant with these results in NN (Martos-Moreno GA. Et al., 2009, Pirazzoli P. et al., 2005). The type of delivery does not influence ghrelin levels in our study nor in the literature (Bellone S. et al., 2004, study 1, Mendez-Ramırez F. et al., 2009, Bellone S. et al., 2004).

In conclusion, our study demonstrated that in physiological conditions, NN show higher UAG and lower AG levels compared with children in later life, resulting in a lower AG/UAG ratio. This hormonal pattern and the negative correlation between UAG and insulin levels would suggest a different metabolic function at birth. These peculiarities could be related to rapid hormonal and metabolic changes that could influence weight gain in early postnatal life. As such, it is important that further studies be performed to clarify the exact role of different ghrelin forms in fetal and postnatal life.

2.3 Study 3

Title

Unacylated, acylated ghrelin and obestatin levels are differently inhibited by oral glucose load in pediatric obesity: Association with insulin sensitivity and metabolic alterations.

Aim of the Study

The first aim of this study was to detail post-OGTT AG, UAG and OBST dynamics in obese children and adolescents. A further aim was to explore if insulin resistance and metabolic alterations clustering in MS could be related to the fasting and glucose-induced regulation of the three peptides.

Subjects and Methods

From November 2008 to December 2010, 30 prepubertal and 40 pubertal pediatric subjects with primary obesity and a body mass index (BMI) equal or higher than 97th percentile were consecutively enrolled. They were sedentary (engaging in less than 1 or 2 h per week of mild physical activity at school). Obesity linked to genetic syndromes or organic dysfunctions like craniopharingiomas were excluded. Exclusion criteria also included the presence of type 1 and 2 diabetes, renal dysfunction, liver steatosis and other conditions known to influence body composition and energy balance (insulin and glucocorticoid treatments, endocrine diseases including sleep apnea syndrome). A group of 22 age-matched lean controls were also recruited. They engaged 6 h per week of moderate or vigorous physical activity. Physical activity was recorded by a register and was not instrumentally measured. All subjects regularly went to school and their socio-cultural environment was similar with a medium-high social extraction. Subjects underwent a complete clinical and auxological evaluation by a trainee research team using the Italian growth charts (Cacciari E. et al., 2006, **Appendix 2**).

Auxological measurements on children have been described in study 1. Patients were divided into prepubertal (stage 1) and pubertal (stages 2e5) subjects. The waist-to-height ratio was calculated by dividing waist circumference (cm) by height (cm) and used as another surrogate measure of central fat distribution. Systolic and diastolic blood pressure were measured three times on the left arm and after 15 min at rest in the supine position by using a standard mercury sphygmomanometer; the average was recorded and stratified according to pediatric percentiles of National High Blood Pressure Education Program Working Group on High Blood Pressure in Children and Adolescents (Pediatrics 2004, **Appendix 4**). Children and adolescents underwent an evaluation of metabolic alterations clustering in MS by using the modified NCEP-ATP III criteria of Cruz and Goran (Cruz ML. & Goran MI., 2004, **Appendix 5**). Impaired fasting

glucose and impaired glucose tolerance were defined according to MS and American Diabetes Association classifications. Accordingly, MS was defined by the presence of 3 or more of the following 5 criteria: 1) waist circumference _ 90th percentile for age and gender; 2) triglycerides (TG) _ 90th percentile for age and gender; 3) HDLcholesterol _ 10th percentile for age and gender; 4) impaired fasting glucose or glucose tolerance; 5) blood pressure _ 90th percentile for age and gender. Waist circumference percentiles were defined according to sex and age (McCarthy HD. et al., 2001, **Appendix 3**) Triglycerides and HDLcholesterol percentiles were considered in accordance to distribution based on American Academy of Pediatrics cut-off values (Daniels SR. et al., 2008).

After a 12-h overnight fast, blood samples for AG, UAG, OBST, total cholesterol, HDL-cholesterol, triglycerides, GH and IGF-I were measured. LDL-cholesterol was determined using the Friedwald formula. All subjects underwent an oral glucose tolerance test (OGTT, 1.75 g of glucose solution per kg, maximum 75 g). Blood samples were drawn for the determination of glucose and insulin every 30 min and of AG, UAG, OBST every 60 min from 00 to 1200 min. The energy intake and food requirements were defined for each subject starting from breakfast and ending at bedtime with direct questions to both children and parents and using validated food frequency questionnaires before performing tests. To assess food consumption, foods were divided according to the classic basic food groups of the Italian food pyramid elaborated by the Italian Institute of Research on Food and Nutrition. A balanced diet (50-60% of carbohydrates; 15-20% of proteins; 30% of total fats of which saturated less than 7%) was suggested in the two weeks before the study; the daily dietary intake was calculated mirroring that registered at the moment of recruitment to avoid weight and hormonal changes. The area under the curve (AUC) for parameters after OGTT was calculated according to the trapezoidal rule. The stimulus for insulin secretion related to

the increment of plasma glucose as insulinogenic index was calculated as the change in insulin concentration from 0 to 30 min (Ins30) and from 0 to 120 min (Ins120). Insulin resistance was calculated using the formula of HOMA-IR = (fasting glucose x fasting insulin/22.5). Beta cell function at fasting was calculated using the formula of HOMAβ =(20 x fasting insulin)/(fasting glucose-3.5). Insulin sensitivity during OGTT was calculated from the Matsuda index. The disposition index, which reflects the capacity of pancreatic islets to compensate for lower insulin sensitivity, was defined as the product of the Matsuda Index and Ins 30 (DI30) or Ins120 (DI120) (De Fronzo RA. Et al., 2010). All beta-cell function measures yielded similar results. The study protocol was approved by the Local Ethical Committee and informed consent was obtained by all infant's parents before the evaluations.

Human AG and UAG (pg/ml) were measured by ELISA kits (BioVendor - Laboratori Medicina GmbH, Heidelberg Germany) AG: sensitivity: 0.2-0.6 pg/ml. Intra and inter-assay coefficient of variation ranges: 11.8-13.2%. UAG: sensitivity 0.3-0.8 pg/ml. Intra and inter-assay coefficient of variation ranges: 10.3-10.9%. OBST (ng/ml) was measured by EIA kit (Peninsula Laboratories, LLC, CAF USA). Sensitivity: 0.02-25 ng/ml. Insulin (mUI/ml; 1mUI/ ml = 7.175 pmol/l) was measured by chemiluminescent enzymelabelled immunometric assay (Diagnostic Products Corporation, Los Angeles, CA). Sensitivity: 2 mUI/ml. Intra- and inter-assay coefficient of variation: 2.5-8.3 and 4.4-8.6%. Plasma glucose levels (mg/dl; 1 mg/dl:0,05551 mMol/liter) were measured by the gluco-oxidase colorimetric method (GLUCOFIX, by Menarini Diagnostici, Florence, Italy). Total cholesterol (mg/dl; 1 mg/dl: 0.0259 mMol/l), HDL-cholesterol (mg/dl; 1 mg/dl: 0.0259 mMol/l), triglycerides (mg/dl; 1 mg/dl: 0.0113 mMol/l) were evaluated using standardized methods in the hospital's chemistry laboratory. Total cholesterol concentration was measured by esterase and oxidase conversion (Advia 1650, Bayer Diagnostics, Newbury, UK); coefficient of variation 1.9%. Triglycerides and HDL-

cholesterol concentrations were measured by enzymatic determination (Advia 1650, Bayer Diagnostics, Newbury, UK); CV 1.7%. GH and IGF-I were measured with commercial kits. Categorical variables were expressed as frequencies or percentages. For continuous variables, non-Gaussian data were log transformed before analyses and all the data were expressed as absolute or delta mean ± SEM or percentiles to assist with interpretation. For continuous variables, the variation between groups was compared by means on nonparametric Wilcoxon and Manne -Whitney U tests.

The stepwise regression model with two-tailed probability values and 95% confidence intervals was used to measure the strength of the association between variables. Statistical significance was assumed for $p < 0.05$. All statistical analyses were performed with SPSS for Windows version 17.0 (SPSS INC; Chicago, IL, USA).

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Results

Clinical and biochemical fasting characteristics

Seven prepubertal and 3 pubertal obese subjects were excluded because they were unable to complete the OGTT due to emotional issues. Complete anthropometrical and biochemical fasting data of the enclosed subjects are summarized in **Table 6**.

Table 6. Clinical and biochemical characteristics of all subjects according to puberty.

	Obese			NW		
Variables	All	Prepubertal	Pubertal	All	Prepubertal	Pubertal
Subjects	60	23	37	22	5	17
Gender (M/F)	44/16	21/2	23/14	13/5	3/2	14/3
Age (yrs)	11.0 ± 0.4	8.5 ± 0.3	12.5 ± 0.4***	13.4 ± 0.8†	8.7 ± 0.7	14.7 ± 0.6†***
Tanner		I (23)	II (17); III-IV (9); V(11)		I (5)	II(5); III-IV(6); V(6)
BMISDS (Kg/m^2)	2.28 ± 0.07	2.21 ± 0.09	2.31 ± 0.10	−0.49 ± 0.18††	0.12 ± 0.36†††	−0.64 ± 0.19†††*
WC (cm)	94.1 ± 1.8	86.8 ± 2.3	98.7 ± 2.3***	76.8 ± 3.5†††	66.0 ± 1.1††	78.3 ± 3.6††**
WHr	0.60 ± 0.02	0.61 ± 0.03	0.60 ± 0.03	0.46 ± 0.01†††	0.45 ± 0.03†	0.47 ± 0.01†††
T-c (mg/dl)	142.4 ± 3.3	146.4 ± 5.8	139.8 ± 3.9	135.4 ± 5.6	120.5 ± 17.5	137.5 ± 5.7*
HDL-c (mg/dl)	38.6 ± 1.2	40.3 ± 1.7	37.5 ± 1.6*	48.6 ± 2.2†††	44.0 ± 3.0	49.2 ± 2.3†††*
TG (mg/dl)	85.5 ± 5.6	82.8 ± 9.4	83.9 ± 6.6	53.6 ± 4.3†††	52.0 ± 8.0	53.7 ± 4.7†††
LDL-c (mg/dl)	84.7 ± 2.7	88.5 ± 1.7	82.3 ± 3.2	76.2 ± 5.1†	66.0 ± 12.7	77.6 ± 5.3*
PAS (mmHg)	132.2 ± 2.0	126.4 ± 3.2	135.8 ± 2.5**	115.7 ± 3.1†††	113.4 ± 3.2†	114.6 ± 3.1†††
PAD (mmHg)	87.3 ± 1.5	82.5 ± 2.4	90.3 ± 1.9**	71.8 ± 3.0†††	72.5 ± 1.4†	71.9 ± 3.1†††
GLC (mg/dl)	88.3 ± 0.9	87.7 ± 1.4	88.6 ± 1.3	85.1 ± 2.0	89.5 ± 4.5	84.5 ± 2.1
Insulin (μUI/ml)	16.1 ± 1.4	12.0 ± 1.4	18.8 ± 2.1**	8.4 ± 1.0†††	6.5 ± 0.6†	8.6 ± 1.1†††**
HOMA-IR	3.6 ± 0.3	2.6 ± 0.3	4.2 ± 0.5**	1.8 ± 0.2††	1.4 ± 0.1†	1.8 ± 0.2†††*
HOMA-B (%)	235.0 ± 18.7	186.1 ± 26.4	266.3 ± 24.7***	148.9 ± 21.2††	92.3 ± 23.8†	156.4 ± 22.5††*
Matsuda index	4.4 ± 0.5	5.6 ± 1.2	3.6 ± 0.5**	5.7 ± 0.7††	7.2 ± 0.2†	5.6 ± 0.7††*
Ins30 (μUI/mg*L^{-1})	2.4 ± 0.2	2.2 ± 0.2	2.6 ± 0.3	3.0 ± 1.2	2.1 ± 1.7	3.0 ± 1.2
DI30 (μUI/mg*L^{-1})	8.8 ± 1.0	10.6 ± 1.9	7.6 ± 1.1*	13.8 ± 4.4	15.4 ± 2.3	13.6 ± 4.5†
Ins120 (μUI/mg*L^{-1})	2.9 ± 0.4	2.7 ± 0.7	3.0 ± 0.5	−0.5 ± 1.0†††	−2.2 ± 1.1†	−0.3 ± 1.0†††*
DI120 (μUI/mg*L^{-1})	9.6 ± 2.9	12.9 ± 4.9	7.3 ± 3.1	−3.5 ± 5.3†††	−7.1 ± 6.9†	−2.0 ± 5.2†*
AG (pg/ml)	8.4 ± 0.4	9.0 ± 0.6	7.8 ± 0.3*	11.1 ± 3.8†	10.8 ± 2.5	11.1 ± 4.0†
UAG (pg/ml)	31.3 ± 1.8	33.7 ± 1.9	28.7 ± 1.7*	38.6 ± 6.6†	37.1 ± 8.0†	35.5 ± 4.5†*
OBST (ng/ml)	0.526 ± 0.077	0.653 ± 0.121	0.439 ± 0.081*	0.299 ± 0.094†	0.072 ± 0.032†	0.365 ± 0.118*

Data are expressed as mean ± SEM. AG, acylated ghrelin; BMISDS, body mass index standard deviation score; DI, disposition index; F, female; M, male; NW: normal weight subjects; GLC, glycemia; HDL-c, HDL-cholesterol; Ins, insulinogenic index; LDL-c, LDL-cholesterol; OBST, obestatin; PAS and PAD, arterial systolic and diastolic blood pressure; T-c: total cholesterol; TG, triglycerides; UAG, unacylated ghrelin; WC, waist circumference; WHr, waist-to-height ratio. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ prepubertal vs pubertal subjects. †$p < 0.05$; ††$p < 0.01$; †††$p < 0.001$ obese vs normal weight subjects.

Of the obese subjects, 73.3% satisfied NCEP criteria. The most represented NCEP criteria, with the exclusion of waist circumference, were a systolic or diastolic blood pressure higher than the 90th percentile (93.3%) and HDL-cholesterol lower than the 10th percentile (66.6%). The subjects were mainly euglycemic with only 18.3% (11/60) of them with dysglycemia. Lipid profile, percentiles of lipids and blood pressure, BMISDS and waist-to-height ratio were similar between prepubertal and pubertal individuals (Table 4). One normal weight control subject also satisfied NCEP criteria. MS. Subjects with metabolic alterations clustering in MS, including a normal weight individual, presented higher BMISDS and waist-to-height ratio ($p < 0.001$) (**Table 7**).

Table 7. Metabolic syndrome (MS) criteria distribution and fasting and post-OGTT AG, UAG, OBST levels according to MS diagnosis.

MS diagnosis				
	Obese		NW	
Variables	no	yes	no	yes
Subjects	16	44***	21	1
Puberty (PP/P)	7/9	16/28**	5/16	0/1
WC≥90th percentile	0	60***	3	1
TG≥90th percentile	20	40***	0	0
HDL≤10th percentile	36	24***	0	1
PAS/PAD≥90th percentile	4	56***	2	1
IFG	54	6***	0	0
IGT	52	8***	0	0
AG (pg/ml)	7.8 ± 1.4	8.6 ± 0.8	11.3 ± 3.1	5.8
UAG (pg/ml)	34.8 ± 2.4	30.1 ± 1.6*	36.6 ± 3.8	38.1
OBST (ng/ml)	0.849 ± 0.459	0.398 ± 0.101	0.306 ± 0.096	0.137
AG/UAG ratio	0.21 ± 0.85	0.40 ± 0.05	0.32 ± 0.08	0.15
Δ_{60}AG (pg/ml)	−1.7 ± 0.4	−1.6 ± 0.4	−1.7 ± 2.3	−2.3
Δ_{60}UAG (pg/ml)	−16.0 ± 2.0	−14.6 ± 2.1	−15.2 ± 2.0	−13.3
Δ_{60}OBST (ng/ml)	−0.586 ± 0.173	−0.061 ± 0.040*	−0.015 ± 0.087	−0.024

Data are expressed as categorical absolute numbers or mean ± SEM. AG, acylated ghrelin; HDL-c, HDL-cholesterol; IFG, impaired fasting glucose; IGT, impaired glucose tolerance; NW: normal weight subjects; OBST, obestatin; PAS and PAD, arterial systolic and diastolic blood pressure; PP, prepubertal; P, pubertal; TG, triglycerides; UAG, unacylated ghrelin; WC, waist circumference. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ subjects with MS vs those without MS.

3.2. Hormonal parameters and OGTT study

3.2.1. Glucose and insulin

Both obese and control pubertal subjects presented higher fasting insulin levels ($p < 0.01$) and insulin resistance measured as HOMA-IR ($p < 0.01$), but compensated with an increased beta-cell function defined with HOMA-β ($p < 0.007$). Ins120 and DI120 were lower in controls than in obese subjects ($p < 0.001$). Glucose and insulin levels

increased after OGTT ($p < 0.0001$). Glucose levels were similar between prepubertal and pubertal obese individuals while insulin at each time point and its AUC (9187.8 ± 1148.9 vs 4927.7 ± 1152.9 mUI/ml*h; $p < 0.04$) were higher in pubertal subjects. Furthermore, insulin variation at 120 min was higher (67.5 ± 11.4 vs 44.9 ± 8.8 mUI/ml; $p < 0.04$) while Matsuda index (3.6 ± 0.5 vs 5.6 ± 1.2; $p < 0.03$) and DI30 (7.6 ±1.1 vs 10.6 ± 1.9 mUI/mg*L_1; $p < 0.05$) were lower in pubertal individuals without variation in Ins30, Ins120 and DI120. The same patterns were observed in control subjects.

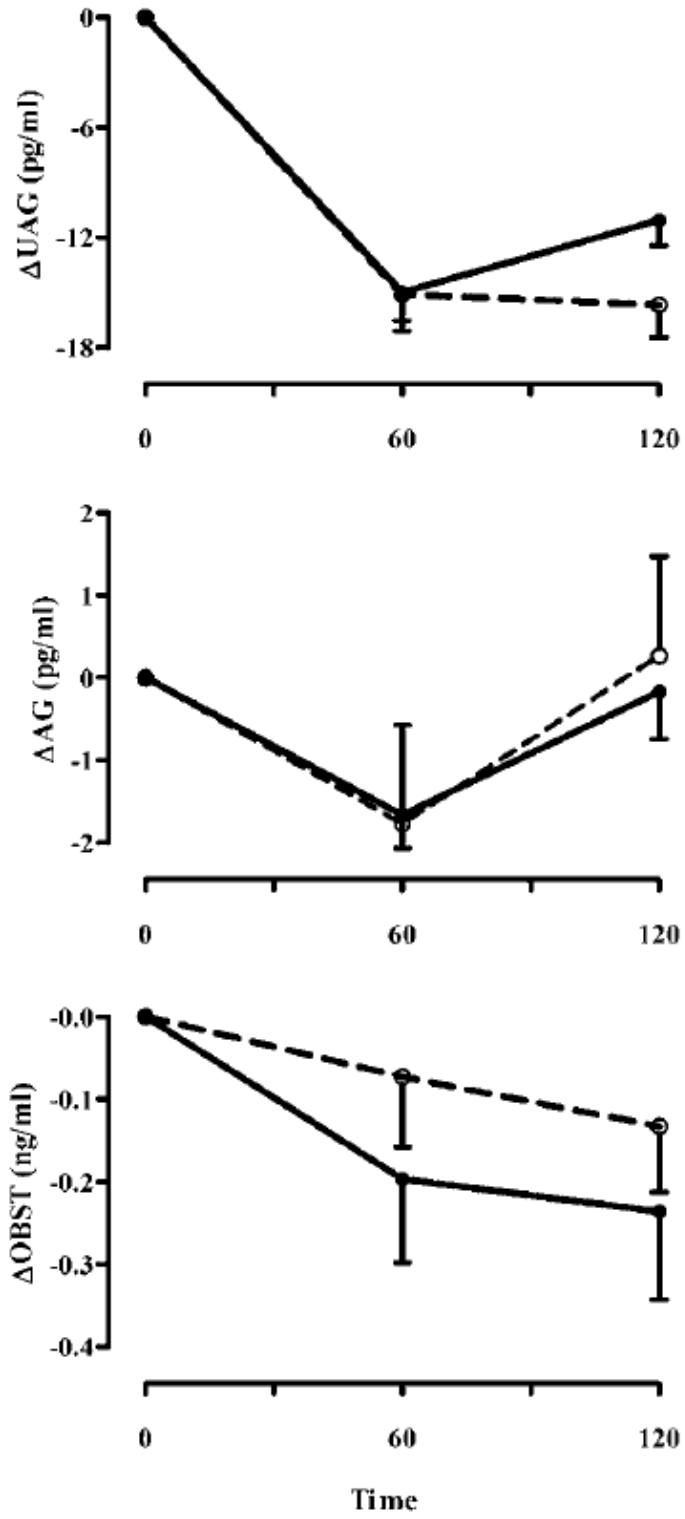
MS. Obese subjects with metabolic alterations had higher insulin levels and insulin resistance ($p < 0.0001$) compensating with an increased beta-cell function ($p < 0.0001$). They also had a lower Matsuda index (4.2 ± 0.7 vs 5.1 ± 0.5; $p < 0.01$) and DI30 (8.2 ±1.1 vs 10.7 ± 1.6 mUI/mg*L^{-1}; $p < 0.04$), higher insulin variation at 120 min (69.8 ± 9.4 vs 29.3 ± 7.6 mUI/ml $p < 0.04$) and Ins120 (3.3 ± 0.5 vs 1.6 ± 0.4 mUI/mg*L^{-1}; $p < 0.02$), but compensated with similar DI120.

3.2.2. UAG

Fasting UAG levels were higher in normal weight than obese subjects ($p < 0.05$) and lower in normal weight and obese pubertal than prepubertal subjects ($p < 0.05$), without gender differences. UAG decreased for the entire OGTT session ($p < 0.0001$) with a maximum inhibition at 60 min (-15.0 ± 1.5 pg/ml, $p < 0.0001$) (**Figure 18**). Pubertal obese subjects presented lower total UAG (DAUC: 1090.5 ± 171.0 vs 1363.3 ± 187.6 pg/ml*h; $p < 0.05$) and lower inhibition of UAG concentrations at 60 min (-13.1 ± 2.1 vs -17.8 ± 2.2 pg/ml; $p < 0.009$), with respect to prepubertal individuals (**Figure 18**).

The same dynamic was shown in control subjects. Normal weight children also presented a higher inhibition at 120 min with respect to the obese group (-15.6 ± 1.7 vs -11.1 ± 1.4 pg/ml; $p < 0.04$) (**Figure 19**).

Figure 19. AG, UAG and OBST levels during OGTT in prepubertal and pubertal normal weight (white circle and dotted line) and obese (dark circle and solid line) subjects.

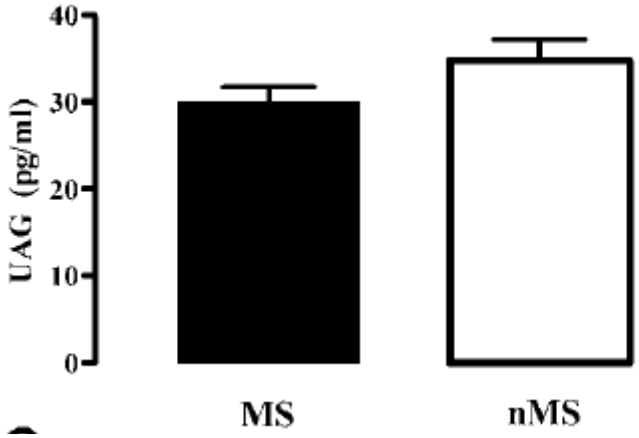


When correcting the UAG variation at OGTT in control and obese subjects for puberty, sex. BMISDS, waist-to-height ratio, waist circumference or insulin sensitivity indexes, the models were not significant. HOMA-IR was the only predictor which explained the

30.1% of the UAG variation ($p < 0.01$). Evaluating absolute values, AUC of UAG was negatively associated with insulin AUC and positively with Matsuda index ($p < 0.04$ for both). The Matsuda index explained with obesity the 46.1% of post-OGTT AUC of UAG ($p < 0.04$). GH and IGF-I levels did not correlate with UAG and did not influence each model. MS.

Obese subjects with metabolic alterations that cluster in MS, had lower fasting UAG ($p < 0.01$) (**Figure 20**), but similar UAG dynamics after OGTT. However, UAG levels did not show a trend among metabolic components of MS.

Figure 20. Fasting UAG levels in obese subjects with metabolic sybdrome (MS); black bar) and withot metabolic syndrome (nMS; white bar).



When correcting fasting UAG for MS diagnosis, puberty, sex, BMISDS, waist-to-height ratio, waist circumference and HOMA-β, the models were not significant. However, when correcting for MS, puberty, sex and HOMA-IR, the Matsuda index or fasting insulin UAG levels remained lower (5.5-0.67 fold), but the predictors were only indices of insulin resistance and not MS. The model did not change if all subjects or exclusively obese subjects were included.

3.2.3. AG

Fasting AG levels were higher in normal weight than obese subjects ($p < 0.05$), with lower levels in obese pubertal than in prepubertal subjects ($p < 0.05$), without gender differences. AG/UAG tended to be higher in obese children without reaching significance. AG levels decreased at 60 min after OGTT (-1.6 ± 0.4 pg/ml, $p < 0.001$; and -1.7 ± 1.2 pg/ml, $p < 0.05$) and subsequently increased at 120 (-0.2 ± 0.5 pg/ml; $p < 0.0001$ and 0.2 ± 1.7 pg/ml; $p < 0.01$) returning to basal concentrations in both obese and control subjects (**Figure 19**). The AG dynamic was similar in prepubertal and pubertal subjects.

Correlation analyses revealed that the AG variation, particularly at 60 min, and the AG nadir were associated with the waist-to height ratio (r: 0.384; $p < 0.004$) and Ins120 (r: -0.245; $p < 0.01$), which were maintained when correcting for puberty and BMISDS. Regression analyses performed with significant factors and adjusted for sex, puberty and BMISDS, revealed that waist-to height ratio explained 34.4% of the AG variation at OGTT ($p < 0.005$). AG secretion associated with insulin only when evaluated as an absolute value, including the effect of fasting concentration in all subjects or exclusively obese subjects. Accordingly, the AUC of AG was explained for 49.5% positively by Ins120 and negatively by insulin AUC ($p < 0.001$) in adjusted models. GH and IGF-I levels did not correlate with AG and did not influence each model.

MS. Subjects with and without metabolic alterations had similar fasting and post-OGTT AG levels with no trends observed among the components.

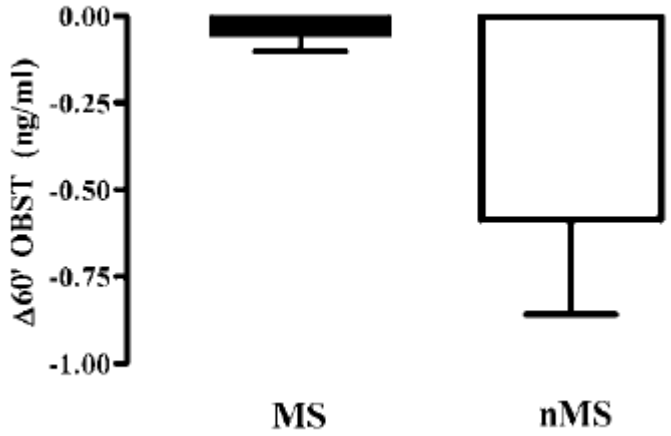
3.2.4. OBST

Fasting OBST levels were higher in obese than in controls with lower levels in obese pubertal than prepubertal subjects ($p < 0.05$). OBST decreased for the entire OGTT session ($p < 0.01$) with maximum inhibition at 120 min in both obese and control subjects (-0.236 ± 0.107 ng/ml, $p < 0.0001$; and -0.133 ± 0.076 ng/ml; $p < 0.01$) (**Figure**

19) and no differences with respect to puberty. Correlation analyses revealed that the OBST variation at 120 min and OBST nadir were associated with absolute and delta glucose variation at 120 min after OGTT (r:0.379; $p < 0.006$) in obese children, without being modified by sex or puberty. Evaluating absolute values, AUC of OBST was associated with glucose variations at 120 min (r:-0.332; $p < 0.006$), AUC of glucose (r:-0.438; $p < 0.0001$) and Ins120 (r:-0.376; $p < 0.002$) in all subjects. The AUC of glucose and Ins120 explained 55.1% of the AUC of OBST ($p < 0.0001$) in adjusted models. GH and IGF-I levels did not correlate with OBST and did not influence each model.

MS. Subjects satisfying NCEP criteria had a lower inhibition of OBST at 60 min (-0.061 ±0.040 vs -0.586 ± 0.272 ng/ml $p < 0.05$) (**Figure 21**). Fasting OBST levels did not show a trend among components of MS.

Figure 21. Variation of Obestatin levels respect to basal at 60 min post-OGTT in subjects with metabolic syndrome (MS; black bar) and whitout metabolic syndrome (nMS; white bar).



In a model composed by all metabolic alterations and controlled by puberty and sex, 46.7% of the OBST variation at 60 min in obese subjects was explained by HDL-cholesterol being lower than the 10th percentile and blood pressure higher than 90th percentile ($p < 0.0001$). Alternatively, 26.4% of the OBST variation was explained by HDL-cholesterol being lower than 10th percentile in the entire group ($p < 0.0001$). Including the insulin variation at 120 min and Ins120 to the model, the best predictors were Ins120 and HDL-cholesterol which explained 59.9% of the OBST variation ($p < 0.0001$) for obese, but not for normal weight subjects (**Table 8**).

Table 8. Relative variation of Obestatin at 60 min (Δ_{60}OBST) during OGTT during the metabolic (MS) syndrome criteria in adjusted regression stepwise models.

Dependent Variable	Effect	β (95% CI)	*p*-value
Model 1	Intercept	−2.672 (−4.258, −1.086)	<0.001
Δ_{60}OBST (ng/ml)	HDL-c 10th percentile	0.473 (0.070, 0.876)	<0.02
	PAS/PAD 90th percentile	0.871 (0.074, 1.668)	<0.03
Model 2	Intercept	−0.723 (−1.293,−0.152)	<0.01
Δ_{60}OBST (ng/ml)	HDL-c 10th percentile	0.353 (0.005, 0.702)	<0.04

CI, confidence interval; HDL-c, HDL-cholesterol; Ins, insulinogenic index; OBST, obestatin; PAS and PAD, arterial systolic and diastolic blood pressure; TG, triglycerides; WC, waist circumference. Model 1: adjusted for puberty, sex, all MS criteria (WC, PAS/PAD, TG $\geq 90^{th}$ percentile, HDL-c$\leq 10^{th}$ percentile or dusglycemia; no = 1; yes = 2) in obese children. Model 2: adjusted for all variables in Model 1 in normal weight and obese children.

Discussion

Previous studies have evaluated the total ghrelin response to OGTT both in adulthood (Wiedmer P. et al., 2007, Barber TM. Et al., 2008, Prodam F. et al., 2008) and in childhood (Baldelli R. et al., 2006, Paik KH. Et al., 2006, Lanyi E. et al., 2007) showing an inhibition. However, this is the first study aiming to investigate the contemporary dynamics of AG, UAG and OBST after OGTT administration in pediatric obesity with respect to insulin resistance, indices of beta-cell function and metabolic alterations that cluster in MS. The results of the present study show that UAG, AG and OBST are all inhibited by glucose load but with particular patterns and each associated with different factors. Specifically, post-OGTT UAG levels are associated with insulin sensitivity, while conversely, AG associates with compensatory insulin secretion and the insulinogenic index. OBST is associated with glucose and compensatory insulin secretion. Metabolic alterations that cluster with MS are not associated with UAG and AG, whereas HDL-cholesterol correlates with glucose-induced OBST secretion.

It has yet to be demonstrated that measuring total ghrelin levels has limited value. Only a few studies have reported AG and UAG levels in pediatric subjects and they are mainly focused on adolescents (Harada T. et al., 2008, Lanyi E. et al., 2007, Mackelvie KJ. et al. 2007). Interestingly, the peptides are modulated by puberty at fasting with decreasing levels associated with insulin resistance according to data evaluating total ghrelin secretion (Van der Lely AJ. Et al., 2004, Wiedmer P. et al., 2007, Bacha F. et al., 2005).

Puberty does not seem to associate with a different glucose- induced response, with the exception of UAG. However, these data need to be confirmed in larger populations that allow an evaluation according to the individual Tanner stages.

4.1. UAG

The fasting UAG concentration was lower in children and adolescent obese and in those with a cluster of MS components. This inhibition was mainly influenced by the insulin resistance state and not by the components of MS. Being that UAG is the major circulating form (Van der Lely AJ. Et al., 2004, Wiedmer P. et al., 2007), these results are supported by all studies evaluating total ghrelin levels which repeatedly confirmed a lower fasting concentration in conditions of insulin resistance (Van der Lely AJ. Et al., 2004, Wiedmer P. et al., 2007, Barber TM. Et al., 2008, Langenberg C. et al., 2005). However, after OGTT UAG concentrations were further inhibited in the prepubertal state and in normal weight subjects being dependent on insulin sensitivity. The lower UAG suppression in pubertal and obese subjects with respect to prepubertal and normal weight subjects, occurs independently of weight or fat distribution and indirect measures of adiposity confirming that insulin is a key factor in the regulation of UAG secretion. Evaluation of insulin resistance across Tanner stages will be helpful in getting a clearer picture. A lower fall in UAG secretion may influence the regulation of insulin secretion over time, although this remains to be elucidated in longitudinal studies.

4.2. AG

In this study fasting AG secretion was lower in obese subjects and presented a similar inhibition at 60 min post-OGTT in both obese and control children being followed by a rebound to basal levels. These data confirm those of another two studies conducted in smaller groups (Paik KH. et al., 2006, Lanyi E. et al., 2007) However, Lanyi and co-workers observed that AG levels rapidly surged at 120 min in obese adolescents, but not controls, suggesting that the relative increase of AG in obese children at 120 min after OGTT might induce early hunger and contribute to the maintenance of obesity (Lanyi E. et al., 2007). In contrast, we evaluated total absolute AG levels, which included the effects of fasting and rebound values, as well as delta post-OGTT AG

secretion. All data correlated with insulin, in agreement with other authors (Park WH. et al., 2007), as well as the insulinogenic index at 1200 min. These data suggest that insulin has a major role also in the AG glucose-induced regulation. The role of insulin is in agreement with the lower levels in pubertal individuals. The association with the insulinogenic index at 1200 , which expresses a late compensatory insulin secretion to glucose, suggests an interplay between the precocious surge of AG, insulinand glucose. Whether a progressive decrease in insulin secretion allows AG levels to rebound or the increase in AG concentrations inhibits the later glucose-induced insulin secretion, demonstrated by an AG inhibitory action on the first phase of insulin secretion (Wiedmer P. et al., 2007, Dezaky K. et al., 2008, Broglio F. et al., 2008) and an inhibition of insulin on ghrelin (Van der Lely AJ. Et al., 2004, Wiedmer P. et al., 2007, Barber TM. Et al., 2008, Purnell JQ. Et al., 2003) cannot be explained in a cross-sectional study but it requires interventional models.

4.3. OBST

Fasting OBST levels were higher in obese than normal weight subjects in this study. OBST regulation in obesity is still a matter of debate with higher (21,22 Vicennati V. et al., 2007, Reinehr T. et al., 2008) lower (Guo ZF. Et al., 2007, Anderwald-Stadler M. et al., 2007, Balagopal PB. Et al., 2010) or unchanged levels (Zou CC. et al., 2009) in both children and adults. Based on available data, it is difficult to explain these disparate findings which seem mainly to depend on values in the obese groups, age and ethnicities. Candidate regulators to explain these debatable data include several parameters of glucose and lipid metabolism. Our results also demonstrated that glucose induced OBST inhibition is blunted in subjects with metabolic alterations clustering in MS. Firstly lower HDL-cholesterol and secondly higher blood pressure levels are the major contributors more of a presence of the MS cluster. HDL-cholesterol may seem an unlikely variable associated with ghrelinergic system, however many

studies have confirmed these findings for both plasma ghrelin concentrations (Purnell JQ. Et al., 2003, Langenberg C. et al., 2005) and ghrelin gene polymorphisms (Choi HJ. Et al., 2006).

Moreover, HDL-cholesterol transports both AG and mainly UAG through its N- and C-terminal sequence (Holmes E. et al., 2009). The possibility of OBST bound to lipoproteins has not been investigated and should be further clarified. The association between OBST dynamics and blood pressure is in line with similar preliminary results at fasting in humans and needs further investigation to elucidate its physiological significance (Tang SQ. et al., 2008, Anderwald-Stadler M. et al., 2007). It cannot be excluded that different findings for OBST levels in the published papers on obesity are mainly due to the uninvestigated HDL-cholesterol or blood pressure levels. However, after OGTT, OBST reached a nadir at 120 min with a progressive and delayed inhibition in both normal weight and obese children. Interestingly, higher glucose levels at the end of the testing session and, a higher insulinogenic index are both associated with a blunted inhibition of this peptide in all the groups. These results are in agreement with a previous observation in adults that OBST levels are reduced in insulin-resistant subjects at fasting but are refractory to inhibition in the same individuals during hyperinsulinemic euglycemic clamp with respect to those that are insulin-sensitive.20 On this basis, insulin and mainly whole body insulin sensitivity, as expressed by the insulinogenic index, seem to be regulators of the OBST secretion. Whether an excess of insulin may be responsible for increased OBST levels in obesity still remains unknown.

4.4. Limitations

Our study has a few potential limitations. First, it was a crosssectional study thus the data needs to be confirmed in a longitudinal study. Second, AG is not so stable and degradation can occur. So, unless the stability of AG in the samples is preserved, it

may not represent the physiological regulation. However, fluctuations and dynamics remain a good representation as the bias should be the same at each time point. Third, pubertal stagewas divided only into prepubertal and pubertal and not the five stages of Tanner because a significant sample size could not be reached for each stage.

Despite the fact that all stages were included, minimal changes among each of these cannot be excluded, in particular regarding OBST regulation. Future investigations considering the Tanner stages could better explain the role of insulin resistance in the system. Fourth, physical activity was recorded by a register and was not instrumentally measured. Because, control subjects performed 6 h per week of moderate or vigorous physical activity while obese children engaged in less than 2 h of mild physical activity in school hours, a role of physical activity in the regulation of the system cannot be excluded, considering the data on acute exercise on the system (Mackelvie KJ. et al. 2007).

5. Conclusions

In conclusion, the present study demonstrates that UAG, AG and OBST have different glucose-induced dynamic patterns in obese pediatric subjects. Compensatory increases in insulin secretion pointing to insulin resistance and indices of insulin sensitivity are the major contributors associated with the system. Metabolic alterations, mainly HDL-cholesterol, are associated with the OBST secretion. Further investigations to be performed will explain whether these secretory profiles could be features of adulthood, as well as their physiological significance.

2.4 Study 4

Title

High-end normal adrenocorticotropic hormone and cortisol levels are associated with specific cardiovascular risk factors in pediatric obesity: a cross-sectional study

Aim of the Study

This study recruited a large cohort of overweight and obese pediatric subjects to determine the following:

1. to establish whether an association between cardiovascular risk factors and morning cortisol levels is present in obese Caucasian children and adolescents;
2. to evaluate whether ACTH is associated with cardiovascular risk factors in this population; and
3. to establish whether ACTH and cortisol levels are higher in those with specific cardiovascular risk factors.

Subjects and Methods

Study design and population

This was a cross-sectional study. We consecutively recruited 450 children and adolescents, aged 4 to 18 years, referred to the Pediatric Endocrine Service of our Hospital from January 2008 to October 2011 for obesity. The Hospital covers an area of North-East Piedmont with a population of approximately 500,000. The sampling rate was based on the age structure of the community and of the general pediatric population referred to the Service. Subjects were eligible if they were generally healthy, overweight or obese and not on a weight-loss diet (no engagement in any program to lose weight before the enrollment). Exclusion criteria were the known presence of diabetes or high blood pressure (BP), the use of drugs which influence glucose or lipid metabolism, specific causes of endocrine or genetic obesity, low birth weight, distress during blood sampling or a difficult phlebotomy (more than 5 minutes). The protocol was conducted in accordance with the declaration of Helsinki and was approved by the Local Inter-Hospital Ethic Committee (Maggiore Hospital Ethical Committee). Informed consent was obtained from all parents prior to the evaluations after careful explanations were given to each patient.

Anthropometric and biochemical measurements.

All subjects underwent a clinical evaluation by a trained research team. Pubertal stages were determined by physical examination, using the criteria of Marshall and Tanner (**Appendix 1**).

Height was measured to the nearest 0.1 cm using a Harpenden stadiometer, and body weight with light clothing to the nearest 0.1 kg using a manual weighing scale. Body mass index (BMI) was calculated as body weight divided by squared height (kg/m2).

The BMI standard deviation score (BMISDS) was calculated by the least median squares method (**Appendix 7**).

Waist circumference was measured at the high point of the iliac crest around the abdomen and was recorded to the nearest 0.1 cm.

Systolic BP (SBP) and diastolic BP (DBP) were measured three times at 2-minute intervals using a mercury sphygmomanometer with an appropriate cuff size after participants were seated quietly for at least 15 minutes, with their right arm supported at the level of the heart and feet flat on the floor, prior to other physical evaluations, and at least 30 minutes after blood sampling, using a standard mercury sphygmomanometer. Mean values were used for the analyses. Hypertension was determined if BP values recorded on enrollment day and on blood samples day are always elevated.

After a 12-hour overnight fast, children arrived at the clinical center at 7.30 AM and rested comfortably for half an hour prior to blood testing. At 8.00 AM, blood samples were taken for measurement of ACTH, cortisol, glucose, insulin, high-density lipoprotein (HDL)-cholesterol and triglycerides. ACTH and cortisol samples were drawn first.

Subjects also underwent an oral glucose tolerance test (OGTT; 1.75 g of glucose solution per kg, maximum 75 g). Plasma samples were immediately separated and stored at-80°C. Children were screened for symptoms suggestive of Cushing's syndrome, and a 1 mg overnight dexamethasone suppression test and urinary free cortisol measurement were performed in case of suspicion. Signs of Cushing's syndrome were a low height percentile and high weight percentile as suggested by the guidelines of the Endocrine Society (Nieman LK et al., 2008). We also screened children for a height below that expected from parental height or a previous episode of severe hypertension. Children with positive screenings were excluded.

Insulin resistance was calculated using the homoeostasis model assessment of insulin resistance (HOMA-IR). Low-density lipoprotein (LDL)-cholesterol was calculated by the Friedwald formula. ACTH and cortisol levels were measured by an Immulite 2000 Medical System (Medical Systems S.p.A., Via Rio Torbido 40, Genova, Italy) (sensitivity: <12.0 pmol/l and <27.59 nmol/l, respectively). Other assays and formulas are as previously described in study 3.

Definitions

Subjects were classified as overweight (BMI: 75th to 94th percentile) or obese (BMI: ≥95th percentile) according to Italian growth charts (Cacciari E. et al., 2006, **Appendix 2**). Children and adolescents underwent an evaluation for cardiovascular risk factors identified in the classification of MetS and by using cutoff values of the modified National Cholesterol Education Program-Adult Treatment Panel (NCEP-ATP) III criteria as follows: (1) triglycerides ≥90th percentile for age and sex; (2) HDL-cholesterol ≤10th percentile for age and sex; and (3) impaired fasting glucose or glucose tolerance. High LDL-cholesterol was defined as ≥90th percentile for age and sex. Because of differences in the literature, hypertension was defined according to two specific cut-offs: (1) >95th percentile as suggested by the National High Blood Pressure Education Program (NHBPEP) Working Group of American Academy of Pediatrics (AAP) (Pediatrics 2004, **Appendix 4**); and (2) >90th percentile as suggested by definitions of pediatric MetS (Cruz ML. & Goran MI., 2004, Cook S. et al., 2003, De Feranti SD., 2004). Triglyceride, LDL-cholesterol and HDL-cholesterol cut-off levels for age and sex were those used in the Lipid Research Clinic Pediatric Prevalence Study (Daniels SR. et al., 2008). Impaired fasting glucose and impaired glucose tolerance were defined according to MetS and American Diabetes Association classifications as fasting plasma glucose of ≥5.6 to 6.9 nmol/l, and as 2-h post-OGTT glucose of ≥7.8 to 11.0 nmol/l, respectively (Cruz ML. & Goran MI., 2004, **Appendix 6**). Both SBP and DBP values

were stratified according to percentiles of the NHBPEP Working Group (Daniels SR. et al., 2008).

Statistical analysis

All data are expressed as mean ± standard deviation (SD), absolute values or percentages. A sample of 84 individuals has been estimated to be sufficient to demonstrate a difference of 27.59 µg/dl in cortisol with a SD of 2 with 90% power and a significance level of 95% using the Student's t test. Distributions of continuous variables were examined for skewness and were logarithmically transformed as appropriate. Correlation of ACTH and cortisol with continuous values of SBP, DBP, triglycerides, HDL-cholesterol and LDL-cholesterol, glucose, insulin, and HOMA-IR were examined using Pearson correlation coefficients. Partial correlation was used to correct for covariates. Analysis of covariance was used to determine differences in subjects with and without cardiovascular risk factors. Covariates were sex, age, pubertal stage, and BMI; BMISDS was used when the analysis was not contemporarily corrected for age. Analysis of covariance was also used to determine ACTH and cortisol differences among age and pubertal subgroups, with BMISDS (or BMI), HOMA-IR and sex as covariates.

ACTH and cortisol were also categorized into tertiles. Multiple logistic regression was used to determine the association of tertiles of ACTH and cortisol with the odds ratio (OR, 95% CI) of each cardiovascular risk factor.

Tertiles of ACTH and cortisol were included as independent variables, with the first tertile as the reference group. Statistical significance was assumed at $P < 0.05$. The statistical analysis was performed with SPSS for Windows V.17.0 (SPSS Inc., Chicago, IL, USA).

Results

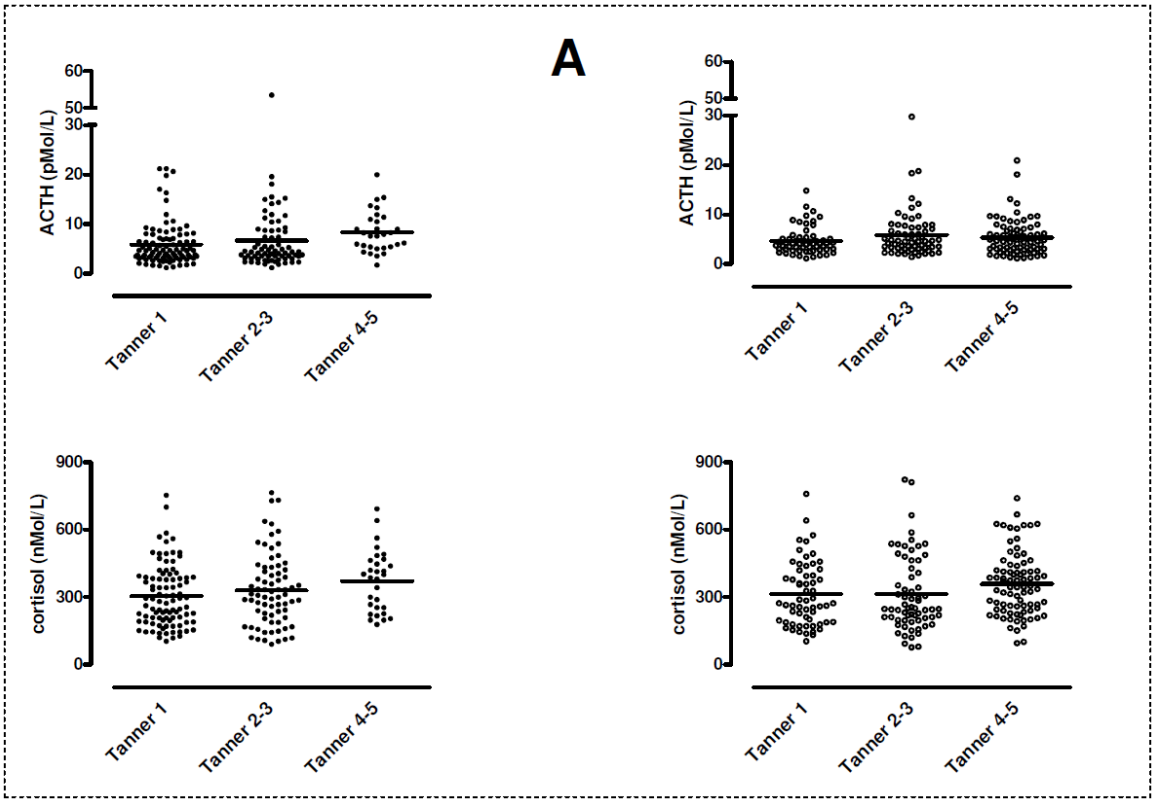
Anthropometric and metabolic phenotypes of all groups.

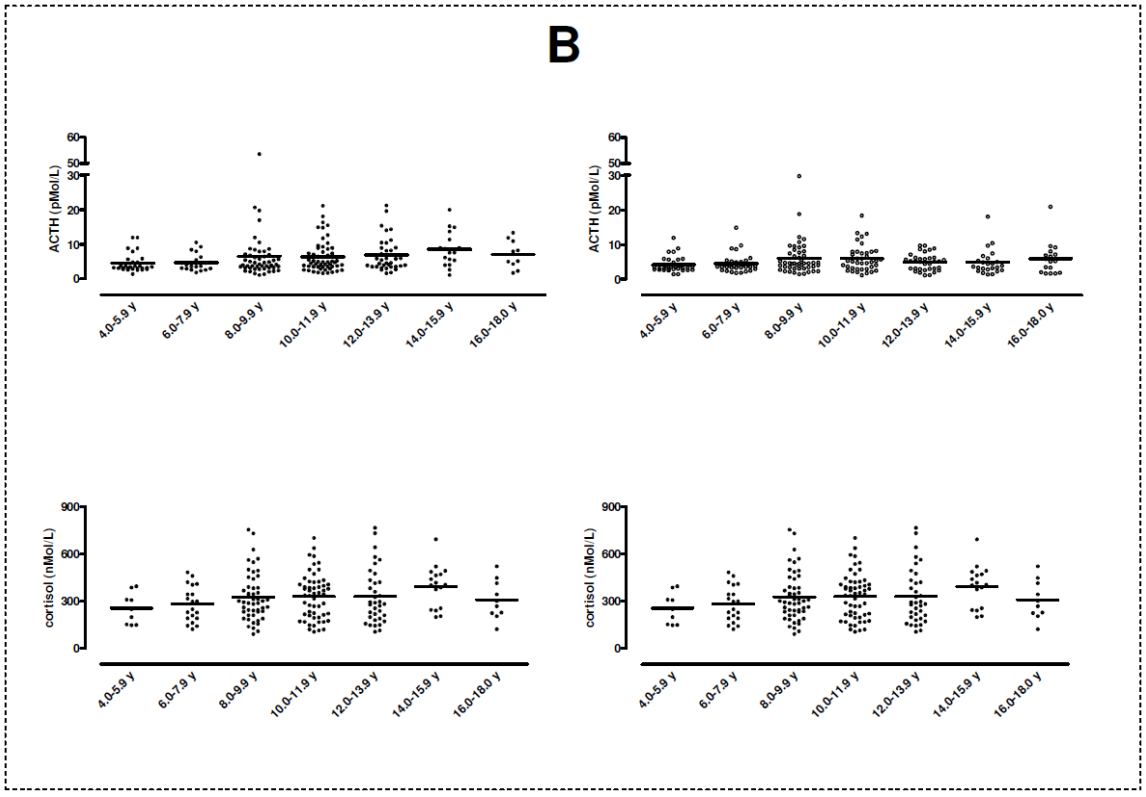
The final dataset included 406 participants, aged 4 to 18 years (198 male, 208 female). A total of 29 subjects were excluded because they did not satisfy inclusion criteria (15 with difficult blood sampling, 5 with a low birth weight, 4 with hypothyroidism in thyroiditis, and 5 treated with glucocorticoids in the last 6 months). Other exclusions were two subjects diagnosed with late-onset congenital adrenal hyperplasia, eight with distress during BP monitoring, and five who refused the dexamethasone test. A total of 31 out of 406 subjects had the dexamethasone test and showed correct inhibition of cortisol levels (all <27.59 μg/dl), so Cushing's syndrome was excluded. We performed 24-h urine sampling for free cortisol measurement, but this was incomplete in 20 out of 31 patients.

ACTH levels were higher in Tanner 4 to 5 stage participants ($P < 0.001$) and in 14.0 to 15.9-year-olds ($P < 0.01$) in males than in females, and this was also the case in the analysis corrected for BMISDS (or BMI) and HOMA-IR (**Figure 23**).

Cortisol levels were higher in Tanner 4 to 5 stage participants than in Tanner 1 stage participants for the whole group ($P < 0.02$), and in females ($P < 0.02$); this was also the case in the corrected analysis for BMISDS and HOMA-IR (**Figure 23**).

Figure 23. Tanner dependent (A) and age dependent (B) adrenocoticotropic hormone ACTH (pmol/l) and cortisol (nm/l) levels in 406 overweight and obese children and adolescents (filled circles, males, left panel; open circles, females, right panel).





A total of 97 (24.0%) subjects were overweight and 309 (76.0%) were obese.

Of the 406 subjects included, 23 (5.7%) had impaired fasting glucose, 10 (2.5%) had impaired glucose tolerance and 5 (1.2%) had both. None were diabetic. A total of 91 (22.4%) subjects had triglycerides ≥90th percentile, 216 (53.2%) had HDL-cholesterol ≤10th percentile and 25 (6.2%) had LDL-cholesterol ≥90th percentile for age and sex. Hypertension according to NCEP-ATP criteria was diagnosed in 339 (83.4%) subjects and in 274 (67.4%), according to AAP criteria. Only 1 subject presented with all cardiovascular risk factors, while 63 (15.5%) had none.

All clinical and biochemical characteristics are shown in **Tables 9**-**10**.

Table 9. Biochemical and clinical characteristics of subjects.

	All	Tanner 1 to 2	Tanner 2 to 3	Tanner 4 to 5
Subjects	406	154	140	112
M	198	93	75	30
F	208	61	65	82
Age, years	10.7 ± 3.1	8.4 ± 2.3	10.4 ± 2.0	14.2 ± 2.0
M	10.6 ± 2.8	9.0 ± 2.2	11.1 ± 2.1	14.6 ± 1.7
F	10.8 ± 3.4	7.6 ± 2.3	9.6 ± 1.5	14.0 ± 2.1
BMI, kg/m^2	27.2 ± 4.8	25.2 ± 3.6	26.3 ± 4.0	31.1 ± 4.8
M	27.5 ± 4.8	26.1 ± 3.9	27.1 ± 4.1	32.7 ± 5.9
F	27.0 ± 4.8	23.9 ± 2.7	25.4 ± 3.8	30.6 ± 4.3
BMISDS	1.99 ± 0.59	1.95 ± 0.51	1.81 ± 0.53	2.26 ± 0.65
M	1.99 ± 0.57	1.98 ± 0.44	1.82 ± 0.55	2.40 ± 0.78
F	1.99 ± 0.60	1.90 ± 0.61	1.78 ± 0.50	2.21 ± 0.60
WC, cm	89.1 ± 13.2	82.5 ± 11.5	88.5 ± 11.1	100.1 ± 11.7
M	89.9 ± 13.2	84.8 ± 11.8	91.4 ± 11.2	104.4 ± 11.6
F	88.4 ± 13.3	79.0 ± 9.1	85.2 ± 10.2	98.7 ± 11.5
SBP, mmHg	127.1 ± 17.1	121.1 ± 12.6	125.6 ± 14.2	137.3 ± 21.1
M	128.7 ± 16.8	122.0 ± 13.1	129.2 ± 13.9	148.4 ± 19.0
F	125.6 ± 17.3	119.7 ± 11.8	121.4 ± 13.5	133.3 ± 20.4
DBP, mmHg	82.3 ± 10.9	78.7 ± 9.6	81.6 ± 10.6	88.0 ± 10.6
M	83.6 ± 10.8	80.1 ± 9.7	84.1 ± 10.3	93.4 ± 9.4
F	81.0 ± 10.8	76.4 ± 9.1	78.8 ± 10.3	86.0 ± 10.4
SBP percentile	90.4 ± 12.5	89.3 ± 12.2	89.7 ± 14.3	93.0 ± 10.1
M	91.0 ± 12.2	88.9 ± 12.5	91.6 ± 12.9	96.0 ± 6.2
F	89.9 ± 12.8	89.9 ± 10.7	87.4 ± 15.5	91.9 ± 11.0
DBP percentile	91.2 ± 11.5	90.5 ± 11.0	90.0 ± 12.9	93.7 ± 9.8
M	91.8 ± 11.4	91.0 ± 11.2	90.9 ± 12.8	96.8 ± 5.0
F	90.6 ± 11.6	89.9 ± 10.7	88.9 ± 13.0	92.6 ± 10.9
HDL, mmol/l	1.08 ± 0.24	1.15 ± 0.25	1.06 ± 0.23	1.00 ± 0.22
M	1.06 ± 0.23	1.13 ± 0.23	1.05 ± 0.22	0.91 ± 0.20
F	1.09 ± 0.25	1.18 ± 0.27	1.08 ± 0.25	1.04 ± 0.22
LDL, mmol/l	2.23 ± 0.59	2.36 ± 0.63	2.14 ± 0.54	2.18 ± 0.56
M	2.23 ± 0.63	2.37 ± 0.68	2.10 ± 0.53	2.16 ± 0.66
F	2.23 ± 0.54	2.35 ± 0.54	2.18 ± 0.55	2.19 ± 0.52
TG, mmol/l	0.87 ± 0.47	0.84 ± 0.46	0.84 ± 0.42	0.96 ± 0.54
M	0.88 ± 0.52	0.86 ± 0.47	0.83 ± 0.34	1.10 ± 0.89
F	0.86 ± 0.42	0.81 ± 0.44	0.85 ± 0.49	0.91 ± 0.32
Glc0', mmol/l	4.80 ± 0.47	4.73 ± 0.51	4.79 ± 0.45	4.91 ± 0.39
M	4.89 ± 0.44	4.86 ± 0.46	4.85 ± 0.41	5.06 ± 0.39
F	4.72 ± 0.48	4.55 ± 0.54	4.72 ± 0.49	4.86 ± 0.38
Glc120', mmol/l	6.05 ± 1.01	5.99 ± 0.90	6.11 ± 0.99	6.04 ± 1.13
M	6.09 ± 0.94	6.02 ± 0.91	6.02 ± 0.98	6.38 ± 0.91
F	6.00 ± 1.08	5.93 ± 0.89	6.25 ± 1.01	5.90 ± 1.18
Ins0', pmol/l	97.3 ± 62.8	80.3 ± 50.3	101.1 ± 61.0	115.7 ± 73.9
M	92.6 ± 50.7	79.0 ± 44.6	100.2 ± 46.4	114.0 ± 66.3
F	101.8 ± 72.4	82.3 ± 58.0	102.2 ± 75.6	116.4 ± 77.0
HOMA-IR	2.9 ± 2.0	2.4 ± 1.6	3.0 ± 2.0	3.5 ± 2.4
M	2.8 ± 1.6	2.4 ± 1.4	3.0 ± 1.5	3.6 ± 2.2
F	3.0 ± 2.3	2.4 ± 1.8	3.0 ± 2.4	3.5 ± 2.4
ACTH, pmol/l	5.90 ± 4.70	5.37 ± 3.99	6.26 ± 5.88	6.17 ± 3.86
M	6.53 ± 5.49	5.79 ± 4.39	6.74 ± 5.99	8.20 ± 4.10
F	5.30 ± 3.72	4.73 ± 3.21	5.70 ± 4.40	5.40 ± 3.40
Cortisol, nmol/l	327.9 ± 148.9	311.9 ± 138.2	320.3 ± 161.2	359.4 ± 140.8
M	324.8 ± 146.7	308.1 ± 135.6	327.8 ± 161.0	369.2 ± 137.2
F	330.8 ± 151.3	317.8 ± 143.0	313.6 ± 163.4	355.8 ± 142.8

Data are expressed as mean ± SD. BMI = body mass index; DBP = diastolic blood pressure; F = female; Glc0' = fasting glucose; Glc120' = post-challenge glucose; HDL = high-density lipoprotein; HOMA-IR = homeostatic model assessment insulin resistance; Ins0' = fasting insulin; LDL = low-density lipoprotein; M = male; SBP = systolic blood pressure; TG = triglycerides; WC = waist circumference.

Table 10. Distribution of cardiovascular risk stratified for weight and sex.

	All	OW	OB
Subjects	406	97 (24.0%)	309 (76.0%)
M	198	47	151
F	208	50	158
Hypertension (>90° percentile)	339 (83.4%)	71 (17.4%)	268 (66.0%)
M	173	36	137
F	166	35	131
Hypertension (>95° percentile)	274 (67.4%)	49 (12.0%)	225 (55.4%)
M	141	25	116
F	133	24	109
HDL <10° percentile	216 (53.2%)	39 (9.6%)	177 (43.6%)
M	123	23	100
F	93	16	77
TG >90° percentile	91 (22.4%)	15 (3.6%)	76 (18.8%)
M	61	11	50
F	30	4	26
LDL >90° percentile	25 (6.2%)	8 (1.9%)	17 (4.3%)
M	17	5	12
F	8	3	5
Dysglycemia	38 (9.3%)	7 (1.7%)	31 (5.4%)
M	21	4	17
F	17	3	14

Data are expressed as absolute values or percentages. HDL = high-density lipoprotein; LDL = low-density lipoprotein; OB = obese subjects; OW = overweight subjects; TG = triglycerides.

Associations between ACTH, cortisol and metabolic parameters.

In the unadjusted analyses, ACTH and cortisol levels were positively associated with SBP, DBP, triglycerides, fasting glucose and HOMA-IR. ACTH, but not cortisol, was positively associated with a higher BMI and insulin levels. Cortisol, but not ACTH, was positively associated with LDL-cholesterol levels (Table 3). Adjustment for confounding factors did not change any association for ACTH and revealed a further association

with 2-h post-OGTT glucose. However, the association between cortisol and HOMA-IR was lost after adjustment (**Table11**).

Table 11. Partial correlation for adrenocorticotropic hormone (ACTH pmol/l) and cortisol (nmol/l) with cardiovascular risk factors.

Model and factor	ACTH		Cortisol	
	r	*P* value	r	*P* value
Model 1				
SBP, mmHg	0.098	0.052	**0.121**	**<0.01**
DBP, mmHg	**0.105**	**<0.03**	**0.119**	**<0.01**
HDL, mmol/l	-0.016	0.753	0.040	0.432
TG, mmol/l	**0.144**	**<0.004**	**0.107**	**<0.03**
LDL, mmol/l	-0.002	0.961	**0.123**	**<0.01**
Glc0′, mmol/l	**0.248**	**<0.0001**	**0.110**	**<0.02**
Glc120′, mmol/l	**0.144**	**<0.02**	-0.020	0.712
Ins0′, pmol/l	**0.122**	**<0.01**	0.050	0.327
HOMA-IR	**0.152**	**<0.003**	0.065	0.200
Model 2				
SBP, mmHg	**0.100**	**<0.05**	**0.112**	**<0.03**
DBP, mmHg	**0.106**	**<0.04**	**0.124**	**<0.01**
HDL, mmol/l	-0.013	0.796	0.048	0.353
TG, mmol/l	**0.128**	**<0.01**	**0.102**	**<0.04**
LDL, mmol/l	-0.019	0.712	**0.116**	**<0.02**
Glc0′, mmol/l	**0.218**	**<0.0001**	**0.117**	**<0.02**
Glc120′, mmol/l	**0.128**	**<0.04**	-0.030	0.638

Values in bold represent significant results. Partial correlation was adjusted for gender, age, Tanner stage, and BMI in model 1 and for gender, age, Tanner stage, BMI, and insulin resistance in model 2. Log transformation was used for skewed variables. DBP = diastolic blood pressure; Glc0′ = fasting glucose; Glc120′ = post-challenge glucose; HDL = high-density lipoprotein; HOMA-IR = homeostatic model assessment insulin resistance; Ins0′ = fasting insulin; LDL = low-density lipoprotein; SDB = systolic blood pressure; TG = triglycerides.

ACTH and cortisol levels and cardiovascular risk factors.

In the unadjusted analyses, ACTH levels were higher in those with triglycerides ≥90th percentile (P <0.003) and LDL-cholesterol ≥90th percentile (P <0.04). Higher ACTH levels were also observed in those with impaired fasting glucose or glucose tolerance (P <0.001) and BP ≥95th percentile (P <0.009), but not in those with HDL-cholesterol ≤10th percentile and BP ≥90th percentile. Cortisol levels were higher in individuals with LDL-cholesterol ≥90th percentile (P <0.006) and BP ≥95th percentile (P <0.02).

In adjusted models, ACTH levels remained higher in those with triglycerides ≥90th percentile (P <0.02) and impaired fasting glucose or glucose tolerance (P <0.001).

Cortisol levels remained high in those with BP ≥95th percentile and LDL-cholesterol ≥90th percentile. Higher ACTH levels (third tertile >5.92 pmol/l), although within the normal range, increased the odds of hypertension (>95th percentile), higher triglycerides, impaired fasting or post-OGTT glucose tolerance in the univariate analysis. After adjusting for confounding factors, only the odds of higher triglycerides (OR 2.118, 95% CI 1.139 to 3.939), impaired fasting or post-OGTT glucose (OR 2.548, 95% CI 1.003 to 6.475) remained significant. Higher cortisol levels (third tertile, >383.5 nmol/l), although within the normal range, increased the odds of hypertension (>95th percentile; OR 1.593, 95% CI 1.002 to 3.133) and higher LDL-cholesterol (OR 3.546, 95% CI 1.095 to 11.490) in both univariate and multivariate analyses (**Table 12**).

Table 12. Plasma adrenocorticotropic hormone (ACTH pmol/l) and cortisol (nmol/l) tertiles and obesity comorbidities in logistic regression.

Factor	Tertile	ACTH			Cortisol		
		OR	95% CI	*P* value	OR	95% CI	*P* value
Hypertension	I	1.000			1.000		
	II	1.088	0.661 to 1.821	0.719	1.071	0.646 to 1.778	0.789
	III	**1.907**	**1.107 to 3.286**	**<0.02**	**1.721**	**1.008 to 2.913**	**<0.04**
HDL <10° percentile	I	1.000			1.000		
	II	1.433	0.884 to 2.323	0.145	0.866	0.549 to 1.429	0.619
	III	1.439	0.886 to 2.338	0.141	1.993	0.610 to 1.614	0.976
TG >90° percentile	I	1.000			1.000		
	II	1.182	0.631 to 2.212	0.601	0.981	0.545 to 1.768	0.950
	III	**2.367**	**1.319 to 4.248**	**<0.004**	1.410	0.798 to 1.491	0.237
LDL >90° percentile	I	1.000			1.000		
	II	2.181	0.655 to 7.263	0.204	2.520	0.676 to 7.490	0.186
	III	3.098	0.973 to 9.866	0.056	**3.223**	**1.012 to 10.265**	**<0.04**
Dysglycemia	I	1.000			1.000		
	II	1.659	0.632 to 4.356	0.304	1.161	0.484 to 2.788	0.738
	III	**2.959**	**1.198 to 7.307**	**<0.01**	1.699	0.740 to 3.900	0.211
Hypertension[a]	I	1.000			1.000		
	II	1.033	0.603 to 1.769	0.906	1.042	0.607 to 1.790	0.882
	III	1.668	0.978 to 2.966	0.072	**1.593**	**1.002 to 3.133**	**<0.04**
HDL <10° percentile[a]	I	1.000			1.000		
	II	1.287	0.771 to 2.148	0.335	0.817	0.489 to 1.365	0.440
	III	1.090	0.649 to 1.832	0.745	0.868	0.514 to 1.467	0.598
TG >90° percentile[a]	I	1.000			1.000		
	III	1.050	0.546 to 2.019	0.884	1.075	0.578 to 2.000	0.819
	III	**2.118**	**1.139 to 3.939**	**<0.01**	1.559	0.851 to 2.856	0.150
LDL >90° percentile[a]	I	1.000			1.000		
	II	2.279	0.678 to 7.665	0.183	2.286	0.678 to 7.725	0.183
	III	3.179	0.975 to10.365	0.055	**3.546**	**1.095 to 11.490**	**<0.03**
Dysglycemia[a]	I	1.000			1.000		
	II	1.582	0.593 to 4.217	0.359	1.040	0.421 to 2.570	0.932
	III	**2.548**	**1.003 to 6.475**	**<0.04**	1.516	0.641 to 3.585	0.344

Values in bold represent significant results. Hypertension was considered as >95° percentile. [a]Adjusted for gender, age, Tanner stage, and BMI. ACTH tertiles were subdivided as: I = <3.54 pmol/l; II = 3.54 to 5.92 pmol/l; III = >5.92 pmol/l. Cortisol tertiles were subdivided as: I = <240.0 nmol/l; II = 240.0 to 383.5 nmol/l; III = >383.5nmol/l. BMI = body mass index; CI = confidence interval; HDL = high-density lipoprotein; LDL = low-density lipoprotein; OR = odds ratio; TG = triglycerides.

Discussion

A series of studies in adults and a few studies in selected groups of adolescents, have shown alterations in cortisol in individuals with cardiovascular risk factors (Anagnostis P. et al. 2009, Reinehr T. et al. 2004, Barat P. et al., 2007). In adults, abdominal obesity, high triglyceride and low HDL-cholesterol levels, hypertension, hyperglycemia, MetS and chronic stress have all been characterized by hyperactivity of the HPA axis leading to a functional hypercortisolism. It has also been suggested that inhibiting cortisol action could provide a novel approach for these conditions (Anagnostis P. et al. 2009).

In the present study, although ACTH and cortisol levels were within the normal ranges, we observed higher ACTH and cortisol levels in obese children and adolescents with specific cardiovascular risk factors. In particular, ACTH levels were higher in those with higher glucose and triglyceride levels, while cortisol levels were higher in those with hypertension and higher LDL-cholesterol, thereby increasing the risk for these metabolic disturbances. The first aim of our study was to determine whether cortisol and ACTH were associated with cardiovascular risk factors in obese Caucasian children and adolescents.

We showed that ACTH and cortisol were directly associated with glucose, triglycerides, and BP independently of sex, age, puberty, BMI and insulin resistance. Moreover, cortisol was also associated with LDL-cholesterol. These data suggest that the link between HPA and comorbidities in obesity is present in very young children and that elevated ACTH and cortisol levels, although within normal ranges, are already associated with cardiovascular risk factors. The data regarding cortisol are in agreement with the study of Weigensberg and coworkers who demonstrated that cortisol is higher in obese Latino youths with MetS, independent of the degree of obesity and insulin sensitivity (Weigensber MJ. Et al., 2008). Many studies evaluating

cortisol in pediatric obesity have demonstrated an association between cortisol and insulin resistance, leading to hypothesis that a relationship between cortisol and metabolic disturbances would be mediated by insulin sensitivity (Reinehr T. et al. 2004, Adam TC. Et al., 2012, Barat P. et al., 2007). However, both our data and those from Latino youths suggest that the relationship is complex and not only due to insulin resistance. It is well known that Hispanic people have a higher prevalence of type 2 diabetes and cardiovascular diseases (CooK S. et al., 2003, Weiss R. et al., 2004, Duncan GE. Et al., 2004, Tfayli H. et al., 2009), thus such a selected sample of subjects could have quite a different phenotype linked to their genetic susceptibility. Despite this, however, our data and that of others have shown that the association with cardiovascular risk factors remains positive in non-specific populations of obese children and adolescents (Reinehr T. et al. 2004, Barat P. et al., 2007). The lack of association between BMI and cortisol was unexpected, particularly because an association was present for ACTH. However, a number of studies have failed to show an association (Weigensber MJ. Et al., 2008, Pasquali R. et al., 2000, Rosmond R 1998, Knutsson U. et al., 1998). Similar to the lack of an association between BMI and plasma cortisol in the obese population, a lack of association between BMI or body fat levels and urinary free cortisol and free cortisone (in 24-h urine) has also been demonstrated in non-obese children (Dimitriou T. et al., 2003). One explanation could be the homogenous population in terms of weight in our and other studies. However, it could be that ACTH is a better biomarker in childhood in relation to obesity and associated cardiovascular risk factors. Accordingly, major glucocorticoid metabolites in 24-h urine samples (reflecting ACTH-driven adrenocortical activity or cortisol secretion) were significantly associated with body fat in non-obese children (Dimitriou T. et al., 2003). The latter findings, together with our cross-sectional study, suggest that adrenocortical activity (driven by ACTH) is related to body composition during growth

whether children are lean/normal weight (Dimitriou T. et al., 2003) or obese. However, in this context it is important that higher body fat levels do not always imply higher total blood cortisol although ACTH is increased. ACTH can be increased and total circulating cortisol concomitantly reduced (Jessop DS. Et al., 2003). However, also in children reduced cortisol blood levels are not uncommon in case of elevated body fat levels (Chalew SA. Et al., 1991, Soros A. et al., 2008). Moreover, our paper and that of Reinehr and Andler (Reinehr T. et al. 2004) indicate that children's total cortisol plasma levels are not necessarily reduced if body fat is higher. However, really elevated cortisol concentrations in obese children appear to emerge only if a marked insulin resistance is also present (Reinehr T. et al. 2004). Interestingly, we also observed changes in ACTH and cortisol levels at the last years and at the end of puberty. Healthy adults have higher blood cortisol levels than children. Adults also have clearly higher 24-h excretion rates of free cortisol and free cortisone after correction for body surface area than children. However 24-h excretion of free cortisol and free cortisone in healthy children up to an age of about 14 years is constant after body surface area correction (Dimitriou T. et al., 2003, Wudy SA. Et al., 2007). We showed in our obese cohort that cortisol rose in Tanner 4 to 5 stage participants, particularly in females. Conversely, ACTH was higher in males with respect to females in the same pubertal stage and at the age of 14.0 to 15.9 years in presence of still unmodified cortisol levels. These data, with respect to what age ACTH and cortisol start to increase, have to be considered in future works on cortisol in obese adolescents. We also demonstrated that ACTH levels were associated with metabolic alterations in pediatric obesity, and that some associations were stronger with respect to those of cortisol, in particular insulin resistance. The characteristics of this association suggest that higher ACTH levels could better reflect the interplay between obesity and the HPA axis, and that cortisol-binding globulin (CBG) may be important. Higher CBG levels reduce the rate of cortisol

clearance, and thus reflect cortisol levels in plasma (Gagliardi L. et al., 2010). In a large population study, CBG levels were negatively correlated with BMI, BP and insulin resistance, perhaps indicating suppression of CBG synthesis, or a CBG gene polymorphism in obesity (Fernandez-Real JM. Et al., 2002). Since stress-induced cortisol pulses are elevated in obesity, lower CBG levels may enhance the glucocorticoid action on tissues and also increase the cortisol clearance. Because CBG is costained with ACTH in corticotrophs and is colocalized with vasopressin in the hypothalamus (Gagliardi L. et al., 2010), lower CBG levels may result in higher ACTH levels in obesity by regulating the HPA stress response. However, ACTH levels also represent expression of the negative feedback loop formed by corticotropin-releasing hormone and cortisol, which is influenced by genetic differences in the glucocorticoid receptor (Witchel SF. Et al. 2006). The highest tertiles of the normal ranges of ACTH and cortisol levels in this study were associated with an increased risk of higher triglyceride and LDL-cholesterol levels, respectively. The specific associations for ACTH and cortisol were of interest. The association between cortisol and LDL-cholesterol could be a consequence of multifactorial mechanisms, including direct and indirect effects on lipolysis, free fatty acid production and turnover, and very-low-density lipoprotein synthesis and fatty acid accumulation in the liver (Anagnostis P. et al. 2009, Arnaldi G. et al., 2010). The association between ACTH and triglycerides may be secondary to the strong association between ACTH and insulin resistance in our study. Moreover, ACTH has been shown to increase apolipoprotein E levels in humans, a key protein in determining triglyceride metabolism (Berg AL. et al., 2006). However, a higher ACTH-driven adrenocortical activity could have consequences for the hepatic fat and triglyceride metabolism likely through a higher hepatic glucocorticoid metabolism (Anagnostis P. et al. 2009, Arnaldi G. et al., 2010). Higher ACTH levels, although within the normal range, were also associated with fasting and post-challenge glucose, and

ACTH levels were strong predictors of hyperglycemia. These data are in line with findings in obese Latino youths (Adam TC. Et al., 2012, Weigensber MJ. Et al., 2008), suggesting that changes in the HPA in altered glucose conditions are present also in a broader population. The relationship between glucose and cortisol is in line with glucocorticoid effects on hepatic gluconeogenesis, insulin secretion and resistance (Anagnostis P. et al. 2009, Lambillotte C. et al., 1997). However, only ACTH increased the risk of high glucose levels. These findings are concordant with the evidence that lower daily cortisol levels and normal CBG concentrations have been shown in childhood obesity as an age-dependent mechanism to prevent type 2 diabetes (Soros A. et al., 2008). Because ACTH-driven adrenocortical activity or cortisol secretion has been shown associated with body fat in childhood, as previously discussed (Dimitriou T. et al., 2003), ACTH might also associate with higher glucose levels before overt disease. In agreement with this hypothesis is that none of the subjects in the present study had overt type 2 diabetes, but they had impaired fasting glucose or glucose tolerance. We also found higher cortisol levels, although within the normal range, in subjects with higher BP, reflecting the data in Latino youths (Weigensber MJ. Et al., 2008). It is interesting to note that when a cut-off was imposed, cortisol levels were significantly higher only with respect to the 95th percentile of BP, whereas there was no significant association with the 90th percentile, which is suggested as pathological in the MetS definition (Cruz ML. et al., 2004), and is prehypertensive according to the NHBPEP Working Group definition (Pediatrics 2004). The lack of an association between cortisol and hypertension using the lower BP cut-off suggests that cortisol levels may be increased only in overt disease. HPA alterations are thus likely to be a consequence of obesity comorbidities, as is also suggested by normalization of cortisol levels after weight reduction (Reinehr T. et al. 2004).

There are limitations in the present study. First is the cross-sectional design, in which we could not determine whether slightly higher ACTH and cortisol levels were a consequence rather than a cause of cardiovascular risk factors in pediatric obesity. Longitudinal studies might clarify this aspect. The second limitation was the inability to define the length of exposure to HPA alterations. The third limitation was evaluation of the HPA without the evaluation of urinary free cortisol. It is difficult to collect daily urine samples properly in pediatric cases, in particular in younger children. In fact, urinary samples were incomplete in most of the children who also took the dexamethasone test for exclusion of Cushing syndrome. However, a single morning fasting cortisol measurement has been shown to be associated with chronic stress and metabolic disturbances (Pervanidou P. et al., 2012). The fourth limitation was the lack of precise data on socioeconomic status due to the refusal of many parents. Socioeconomic status has been found to affect chronic stress and cortisol levels and its role needs to be explored further. The fifth limitation was the absence of true body fat measurements through radiological techniques. However, BMI is a good surrogate for body fat in obesity in large epidemiological sample sizes (Freedman DS. Et al., 2009). The final limitation was the lack of a control group. It would, however, be difficult to choose a good control group for our purpose. Our population was followed in a tertiary care center, and a healthy population of schoolchildren would not be completely comparable in terms of chronic stress. Conversely, the strength of the study was the large sample size, the measurement of post-challenge glucose levels, and the evaluation of many confounding factors.

Conclusions

In summary, we have shown that obese children and adolescents with cardiovascular risk factors have higher ACTH and cortisol levels, although still within the normal range. These findings have led to the hypothesis that the HPA is involved in obesity

comorbidities early in life and in a broad population. Higher ACTH levels are specifically associated with higher triglyceride levels and hyperglycemia, whereas higher cortisol levels are specifically associated with hypertension and high LDL-cholesterol levels. These specific associations suggest complex mechanisms between the HPA axis and metabolic impairments in obesity.

3. Conclusions

Overweight and obesity are a major public health concern both in adults and in children as stated by the World Health Organization. In fact, childhood obesity is most strongly associated to insulin resistance with an increased prevalence of type 2 diabetes, dyslipidemia and hypertension at the pediatric age, developing to an increased cardiovascular mortality in adulthood. The prevalence of obesity in children and adolescents has increased over several decades in many industrialized countries (Wang Y. et al., 2006). The southern countries of Europe, surrounding the Mediterranean, show prevalence rates for overweight children in the range 20–40%, while those in northern areas show rates in the range 10–20% (Lobstein et al., 2003). In Italy, the “OKkio alla SALUTE” project demonstrated a high level of childhood obesity in the overall population, 23,6% of overweight and 12,3% of obesity, which was higher than that of most Western countries; furthermore there were substantial geographic differences, with the prevalence of obesity twice as high in the south as in the north.

Among many potential factors, derangement of multiple hormone systems have increasingly been considered for their potential importance in the pathophysiology of obesity and metabolic alterations.

Ghrelin has an emerging role on appetite, glucose and lipid metabolism, and body composition and has provided an important strength to this research field opening new perspectives within neuroendocrinology and metabolism. In particular it is the only known appetite-stimulating hormone in humans and seems one of the principal factors involved in appetite, craving and regain weight after weight-loss (Adams CE. et al., 2011, Delhanty PJ. et al., 2012). As anticipated, circadian ghrelin secretion is profoundly modulated by acute variations in the energy balance and nutritional status. However, the mechanisms mediating the metabolic control of ghrelin secretion are at present still matter of debate. Overall, published data suggest that ghrelin acts to optimize energy metabolism in period of food restriction as well as preparing the

metabolism to percept and use fuel. There is increasing evidence that some of ghrelin changes may contribute to the regulation of food intake and weight also in children, starting from neonates, but these data are not conclusive. Our first three studies investigate the role of ghrelin system in paediatric age. The first study demonstrates that obese children show AG and UAG levels lower than those in normal weight children; this reduction is similar between the two forms, with maintenance of the same AG/UAG ratio. We observed that ghrelin levels are lower during puberty in both groups, with no differences emerging for gender. Interestingly, when the two ghrelin forms were evaluated separately, a peculiar yet strong relationship between UAG levels and metabolic parameters has been observed, suggesting a significant role for UAG in metabolic functions. The second study is mainly focused on a physiological investigation of the two forms of ghrelin, AG and UAG, in healthy AGA newborns compared with later in life. The results demonstrate that in full-term NN, the venous cord blood at birth presents a very different profile of the two ghrelin forms compared with that found in children. NN show lower AG and higher UAG levels than NW and OB children, independent of pubertal status. As a consequence, the AG/UAG ratio in cord blood of NN is lower compared with that found in NW and OB children. Further studies should be performed to clarify the exact role of different ghrelin forms in fetal and postnatal life.

Our third study aim to investigate the contemporary dynamics of AG, UAG and OBST after OGTT administration in pediatric obesity with respect to insulin resistance, indices of beta-cell function and metabolic alterations that cluster in MS. The results show that UAG, AG and OBST are all inhibited by glucose load but with particular patterns and each associated with different factors. Specifically, post-OGTT UAG levels are associated with insulin sensitivity, while conversely, AG associates with compensatory insulin secretion and the insulinogenic index. OBST is associated with glucose and

compensatory insulin secretion. Metabolic alterations that cluster with MS are not associated with UAG and AG, whereas HDL-cholesterol correlates with glucose-induced OBST secretion. Further investigations to be performed will explain whether these secretory profiles could be features of adulthood, as well as their physiological significance.

Also cortisol has been reported to have a role in obesity, hypertension, and the altered glucose and lipid profile in Cushing's syndrome, and some studies have suggested that moderately increased morning fasting cortisol may be associated with the presence of cardiovascular risk factors in adults (Whitworth JA. Et al., 1995, Pasquali R. et al., 2008, Sukhija R. et al., 2006). A series of studies in adults and a few studies in selected groups of adolescents, have shown alterations in cortisol in individuals with cardiovascular risk factors (Anagnostis P. et al. 2009, Reinehr T. et al. 2004, Barat P. et al., 2007). In our fourth study we have shown that obese children and adolescents with cardiovascular risk factors have higher ACTH and cortisol levels, although still within the normal range. These findings have led to the hypothesis that the HPA is involved in obesity comorbidities early in life and in a broad population. Higher ACTH levels are specifically associated with higher triglyceride levels and hyperglycemia, whereas higher cortisol levels are specifically associated with hypertension and high LDL-cholesterol levels. These specific associations suggest complex mechanisms between the HPA axis and metabolic impairments in obesity.

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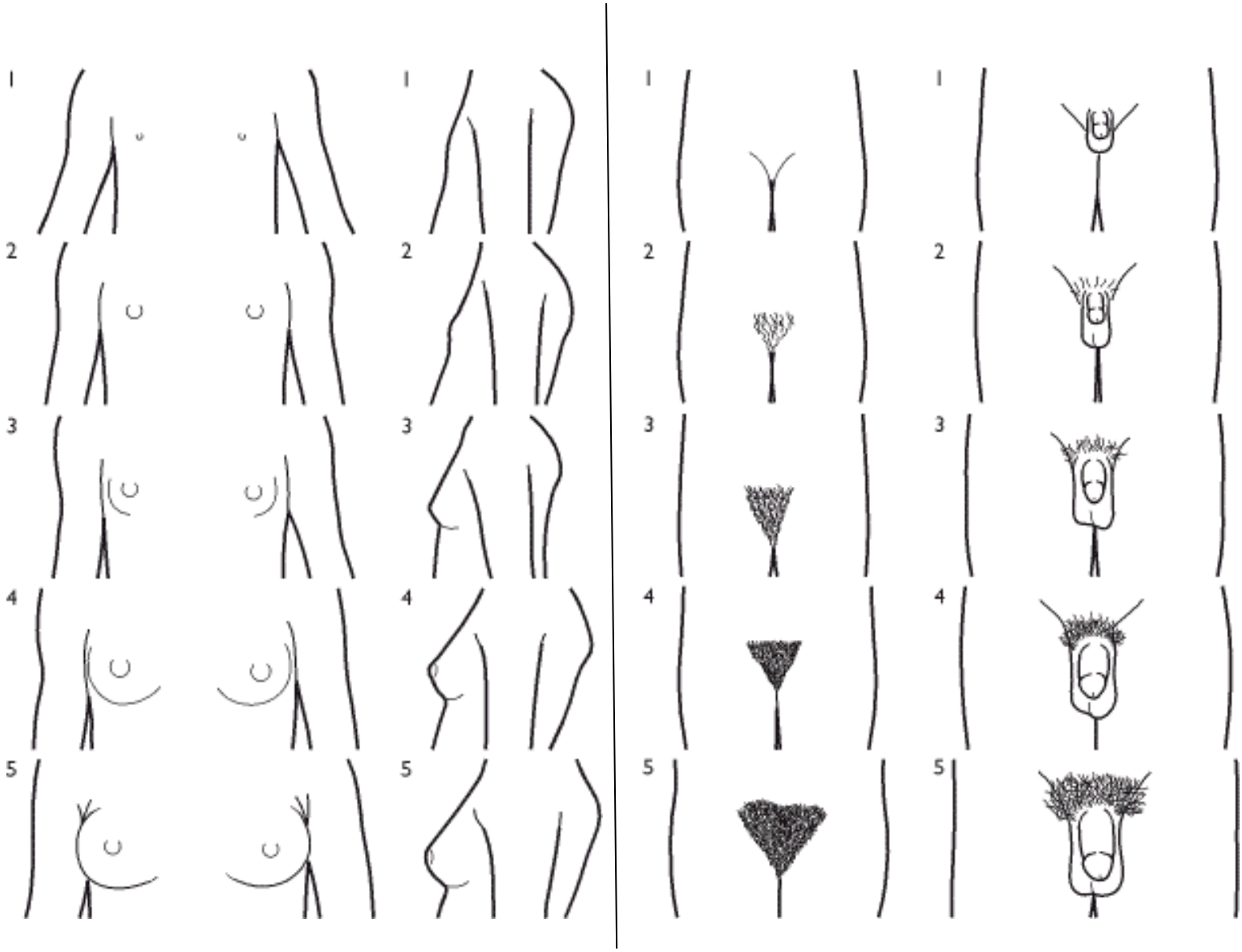
5. APPENDIX

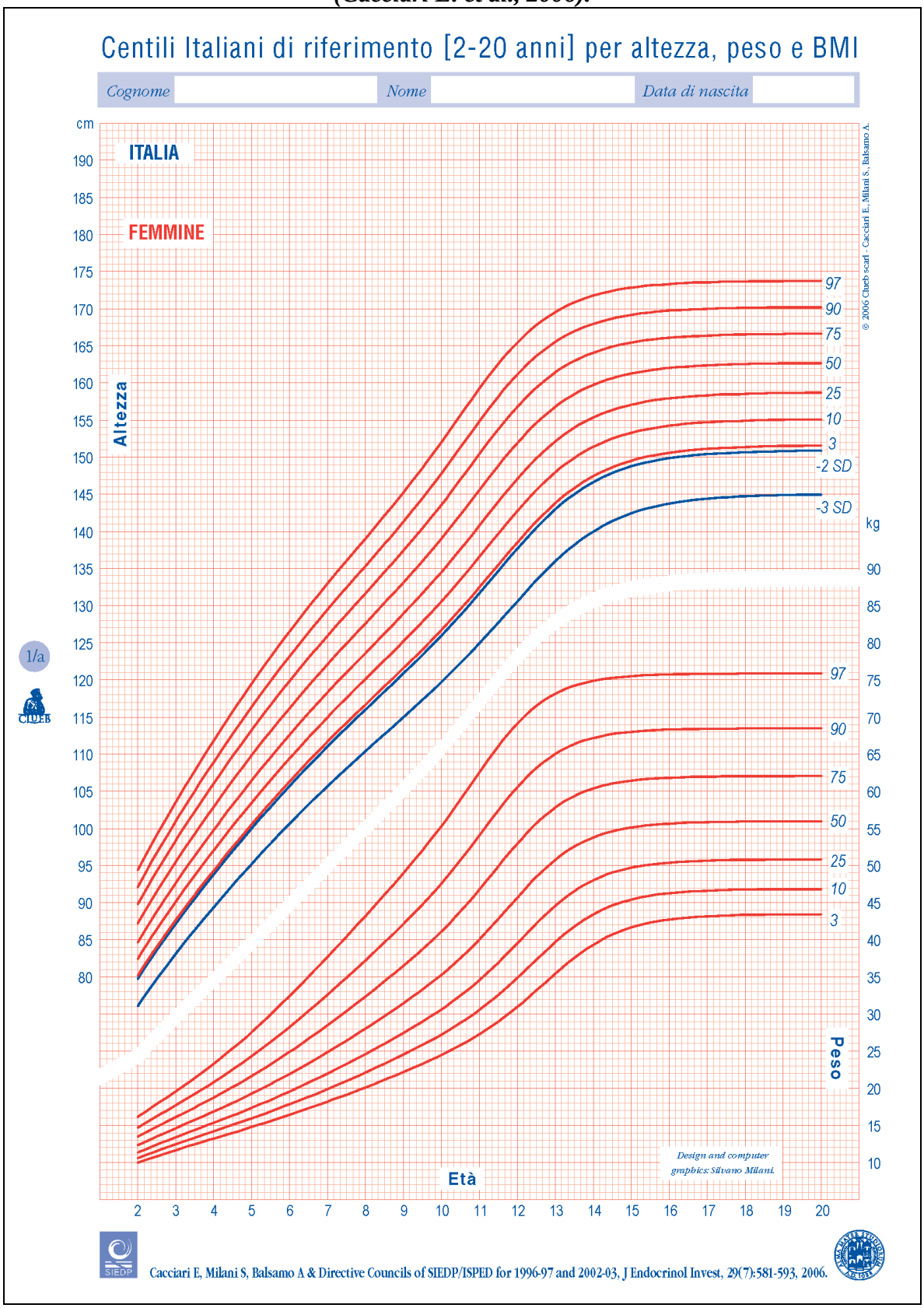
Appendix 1.

Tanner Stages in females (left panel) and in males (right panel)



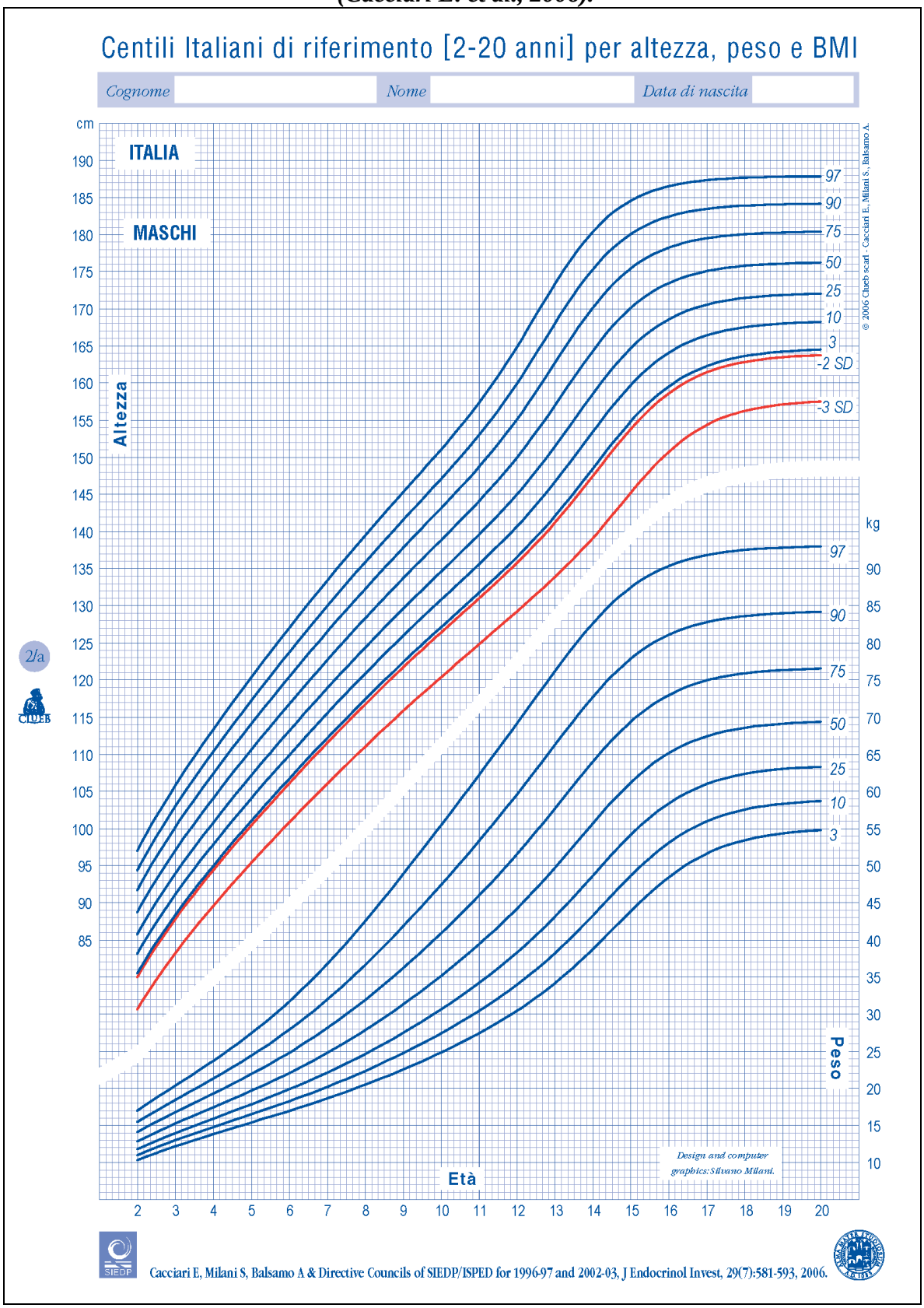
Appendix 2a.

Italian percentiles (2-20 years) in females: height and weight; (Cacciari E. et al., 2006).

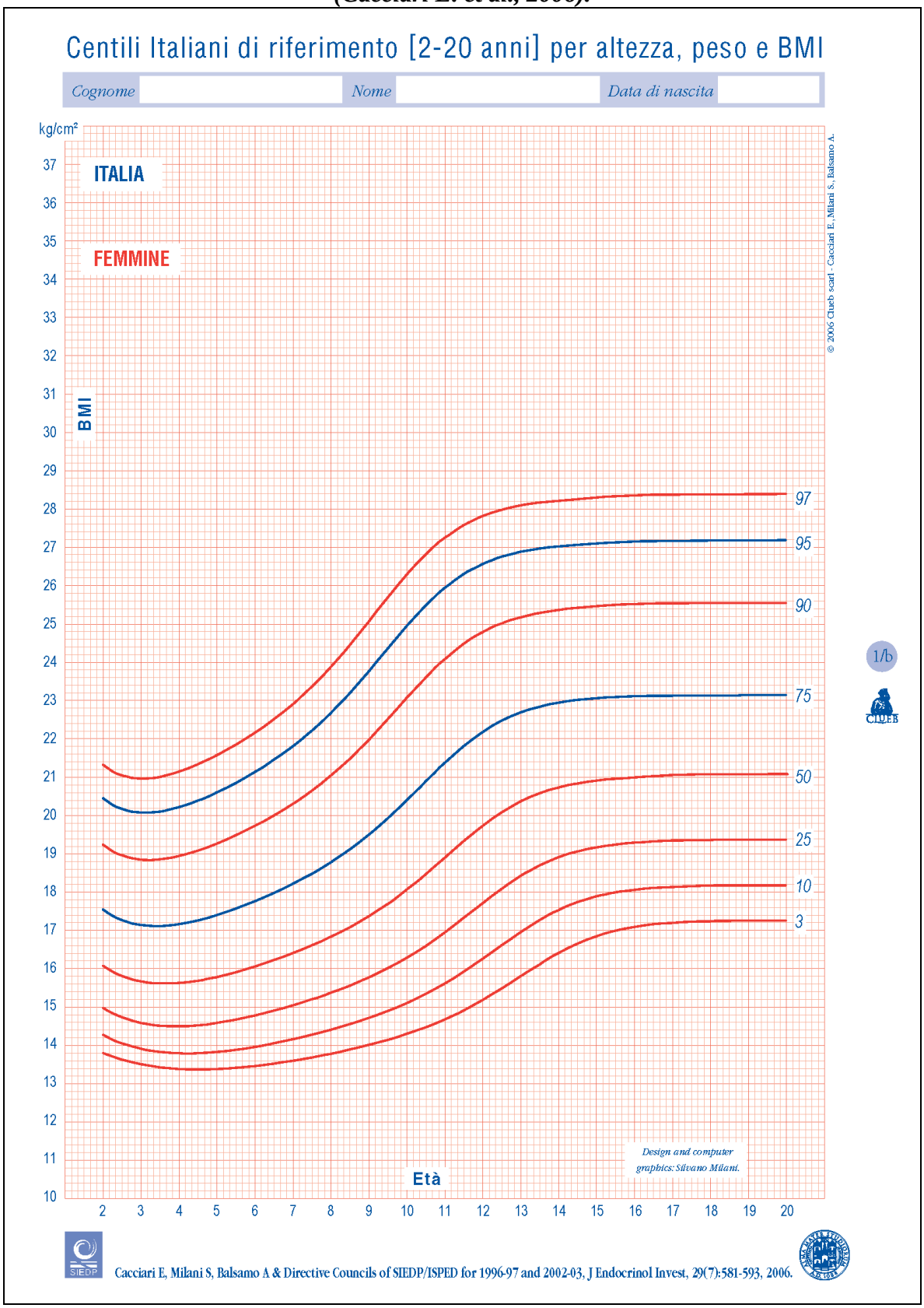


Appendi 2b.

Italian percentiles (2-20 years) in males: height and weight; (Cacciari E. et al., 2006).

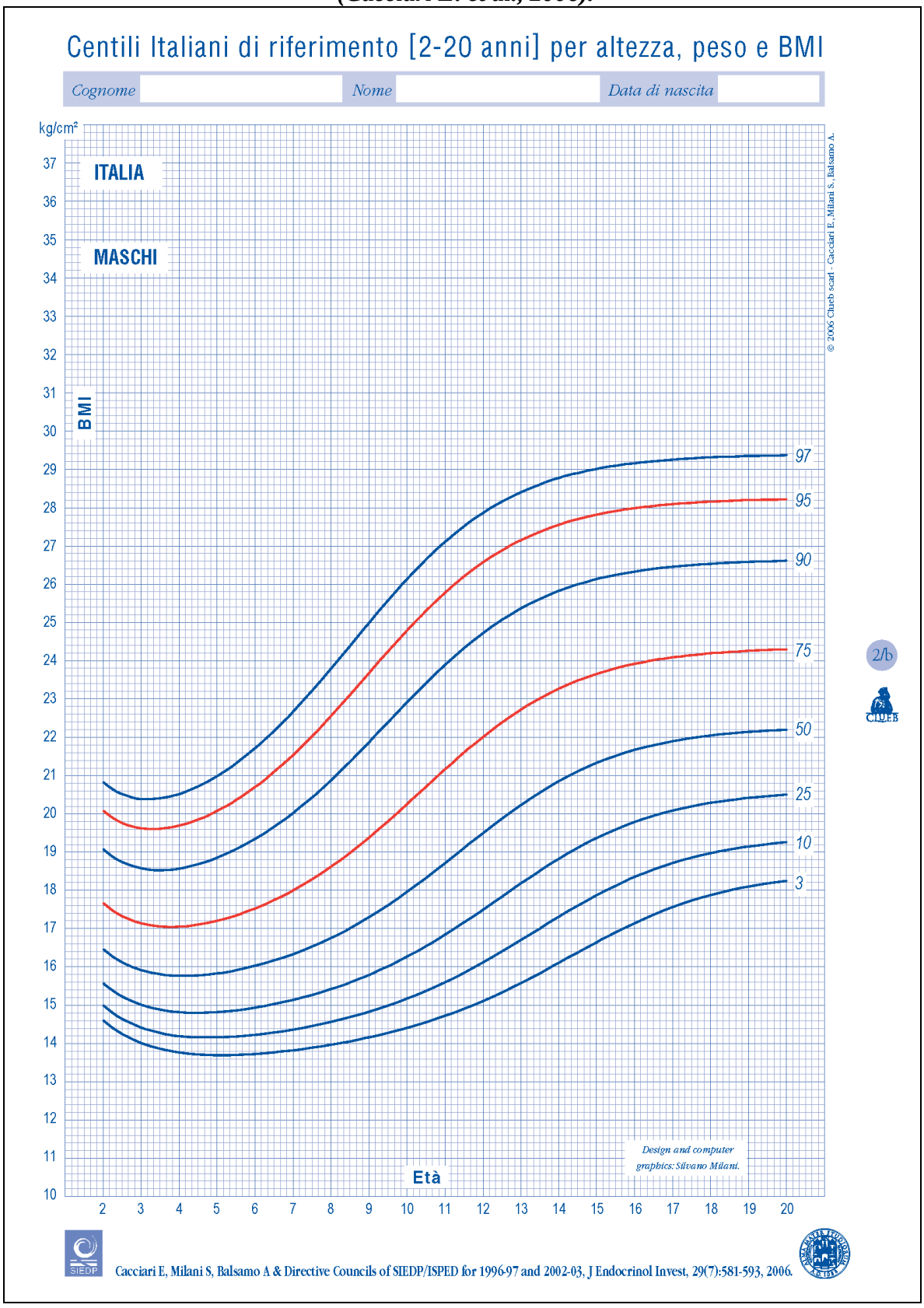


Appendix 2c.
Italian percentiles (2-20 years) in females: BMI;
(Cacciari E. et al., 2006).



Appendix2d.

Italian percentiles (2-20 years) in males: BMI;
(Cacciari E. et al., 2006).



Appendix 3.
Waist circunference at 90° percentile;
(McCarthy HD. et al., 2001).

MASCHI		**FEMMINE**	
Età (anni)	**Circonferenza vita (cm) al 90° percentile**	**Età (anni)**	**Circonferenza vita (cm) al 90° percentile**
5	**55,6**	**5**	**55,4**
6	**57,1**	**6**	**57,0**
7	**58,8**	**7**	**58,7**
8	**60,9**	**8**	**60,4**
9	**63,2**	**9**	**62,0**
10	**65,6**	**10**	**63,6**
11	**67,9**	**11**	**65,4**
12	**70,4**	**12**	**67,3**
13	**73,1**	**13**	**69,1**
14	**76,1**	**14**	**70,6**
15	**79,0**	**15**	**71,7**
16	**81,8**	**16**	**72,6**

Appendix 4a.

Blood pressure percentiles in females;
(National High Blood Pressure Education Program Working Group on High Blood Pressure in Children and Adolescents, 2004).

BP Levels for Girls by Age and Height Percentile

Age, y	BP Percentile	SBP, mm Hg							DBP, mm Hg						
		Percentile of Height							Percentile of Height						
		5th	10th	25th	50th	75th	90th	95th	5th	10th	25th	50th	75th	90th	95th
1	50th	83	84	85	86	88	89	90	38	39	39	40	41	41	42
	90th	97	97	98	100	101	102	103	52	53	53	54	55	55	56
	95th	100	101	102	104	105	106	107	56	57	57	58	59	59	60
	99th	108	108	109	111	112	113	114	64	64	65	65	66	67	67
2	50th	85	85	87	88	89	91	91	43	44	44	45	46	46	47
	90th	98	99	100	101	103	104	105	57	58	58	59	60	61	61
	95th	102	103	104	105	107	108	109	61	62	62	63	64	65	65
	99th	109	110	111	112	114	115	116	69	69	70	70	71	72	72
3	50th	86	87	88	89	91	92	93	47	48	48	49	50	50	51
	90th	100	100	102	103	104	106	106	61	62	62	63	64	64	65
	95th	104	104	105	107	108	109	110	65	66	66	67	68	68	69
	99th	111	111	113	114	115	116	117	73	73	74	74	75	76	76
4	50th	88	88	90	91	92	94	94	50	50	51	52	52	53	54
	90th	101	102	103	104	106	107	108	64	64	65	66	67	67	68
	95th	105	106	107	108	110	111	112	68	68	69	70	71	71	72
	99th	112	113	114	115	117	118	119	76	76	76	77	78	79	79
5	50th	89	90	91	93	94	95	96	52	53	53	54	55	55	56
	90th	103	103	105	106	107	109	109	66	67	67	68	69	69	70
	95th	107	107	108	110	111	112	113	70	71	71	72	73	73	74
	99th	114	114	116	117	118	120	120	78	78	79	79	80	81	81
6	50th	91	92	93	94	96	97	98	54	54	55	56	56	57	58
	90th	104	105	106	108	109	110	111	68	68	69	70	70	71	72
	95th	108	109	110	111	113	114	115	72	72	73	74	74	75	76
	99th	115	116	117	119	120	121	122	80	80	80	81	82	83	83
7	50th	93	93	95	96	97	99	99	55	56	56	57	58	58	59
	90th	106	107	108	109	111	112	113	69	70	70	71	72	72	73
	95th	110	111	112	113	115	116	116	73	74	74	75	76	76	77
	99th	117	118	119	120	122	123	124	81	81	82	82	83	84	84
8	50th	95	95	96	98	99	100	101	57	57	57	58	59	60	60
	90th	108	109	110	111	113	114	114	71	71	71	72	73	74	74
	95th	112	112	114	115	116	118	118	75	75	75	76	77	78	78
	99th	119	120	121	122	123	125	125	82	82	83	83	84	85	86
9	50th	96	97	98	100	101	102	103	58	58	58	59	60	61	61
	90th	110	110	112	113	114	116	116	72	72	72	73	74	75	75
	95th	114	114	115	117	118	119	120	76	76	76	77	78	79	79
	99th	121	121	123	124	125	127	127	83	83	84	84	85	86	87
10	50th	98	99	100	102	103	104	105	59	59	59	60	61	62	62
	90th	112	112	114	115	116	118	118	73	73	73	74	75	76	76
	95th	116	116	117	119	120	121	122	77	77	77	78	79	80	80
	99th	123	123	125	126	127	129	129	84	84	85	86	86	87	88
11	50th	100	101	102	103	105	106	107	60	60	60	61	62	63	63
	90th	114	114	116	117	118	119	120	74	74	74	75	76	77	77
	95th	118	118	119	121	122	123	124	78	78	78	79	80	81	81
	99th	125	125	126	128	129	130	131	85	85	86	87	87	88	89
12	50th	102	103	104	105	107	108	109	61	61	61	62	63	64	64
	90th	116	116	117	119	120	121	122	75	75	75	76	77	78	78
	95th	119	120	121	123	124	125	126	79	79	79	80	81	82	82
	99th	127	127	128	130	131	132	133	86	86	87	88	88	89	90
13	50th	104	105	106	107	109	110	110	62	62	62	63	64	65	65
	90th	117	118	119	121	122	123	124	76	76	76	77	78	79	79
	95th	121	122	123	124	126	127	128	80	80	80	81	82	83	83
	99th	128	129	130	132	133	134	135	87	87	88	89	89	90	91
14	50th	106	106	107	109	110	111	112	63	63	63	64	65	66	66
	90th	119	120	121	122	124	125	125	77	77	77	78	79	80	80
	95th	123	123	125	126	127	129	129	81	81	81	82	83	84	84
	99th	130	131	132	133	135	136	136	88	88	89	90	90	91	92
15	50th	107	108	109	110	111	113	113	64	64	64	65	66	67	67
	90th	120	121	122	123	125	126	127	78	78	78	79	80	81	81
	95th	124	125	126	127	129	130	131	82	82	82	83	84	85	85
	99th	131	132	133	134	136	137	138	89	89	90	91	91	92	93
16	50th	108	108	110	111	112	114	114	64	64	65	66	66	67	68
	90th	121	122	123	124	126	127	128	78	78	79	80	81	81	82
	95th	125	126	127	128	130	131	132	82	82	83	84	85	85	86
	99th	132	133	134	135	137	138	139	90	90	90	91	92	93	93
17	50th	108	109	110	111	113	114	115	64	65	65	66	67	67	68
	90th	122	122	123	125	126	127	128	78	79	79	80	81	81	82
	95th	125	126	127	129	130	131	132	82	83	83	84	85	85	86
	99th	133	133	134	136	137	138	139	90	90	91	91	92	93	93

Appendix 4b.

Blood pressure percentiles in males

(National High Blood Pressure Education Program Working Group on High Blood Pressure in Children and Adolescents, 2004).

BP Levels for Boys by Age and Height Percentile

Age, y	BP Percentile	SBP, mm Hg							DBP, mm Hg						
		Percentile of Height							Percentile of Height						
		5th	10th	25th	50th	75th	90th	95th	5th	10th	25th	50th	75th	90th	95th
1	50th	80	81	83	85	87	88	89	34	35	36	37	38	39	39
	90th	94	95	97	99	100	102	103	49	50	51	52	53	53	54
	95th	98	99	101	103	104	106	106	54	54	55	56	57	58	58
	99th	105	106	108	110	112	113	114	61	62	63	64	65	66	66
2	50th	84	85	87	88	90	92	92	39	40	41	42	43	44	44
	90th	97	99	100	102	104	105	106	54	55	56	57	58	58	59
	95th	101	102	104	106	108	109	110	59	59	60	61	62	63	63
	99th	109	110	111	113	115	117	117	66	67	68	69	70	71	71
3	50th	86	87	89	91	93	94	95	44	44	45	46	47	48	48
	90th	100	101	103	105	107	108	109	59	59	60	61	62	63	63
	95th	104	105	107	109	110	112	113	63	63	64	65	66	67	67
	99th	111	112	114	116	118	119	120	71	71	72	73	74	75	75
4	50th	88	89	91	93	95	96	97	47	48	49	50	51	51	52
	90th	102	103	105	107	109	110	111	62	63	64	65	66	66	67
	95th	106	107	109	111	112	114	115	66	67	68	69	70	71	71
	99th	113	114	116	118	120	121	122	74	75	76	77	78	78	79
5	50th	90	91	93	95	96	98	98	50	51	52	53	54	55	55
	90th	104	105	106	108	110	111	112	65	66	67	68	69	69	70
	95th	108	109	110	112	114	115	116	69	70	71	72	73	74	74
	99th	115	116	118	120	121	123	123	77	78	79	80	81	81	82
6	50th	91	92	94	96	98	99	100	53	53	54	55	56	57	57
	90th	105	106	108	110	111	113	113	68	68	69	70	71	72	72
	95th	109	110	112	114	115	117	117	72	72	73	74	75	76	76
	99th	116	117	119	121	123	124	125	80	80	81	82	83	84	84
7	50th	92	94	95	97	99	100	101	55	55	56	57	58	59	59
	90th	106	107	109	111	113	114	115	70	70	71	72	73	74	74
	95th	110	111	113	115	117	118	119	74	74	75	76	77	78	78
	99th	117	118	120	122	124	125	126	82	82	83	84	85	86	86
8	50th	94	95	97	99	100	102	102	56	57	58	59	60	60	61
	90th	107	109	110	112	114	115	116	71	72	72	73	74	75	76
	95th	111	112	114	116	118	119	120	75	76	77	78	79	79	80
	99th	119	120	122	123	125	127	127	83	84	85	86	87	87	88
9	50th	95	96	98	100	102	103	104	57	58	59	60	61	61	62
	90th	109	110	112	114	115	117	118	72	73	74	75	76	76	77
	95th	113	114	116	118	119	121	121	76	77	78	79	80	81	81
	99th	120	121	123	125	127	128	129	84	85	86	87	88	88	89
10	50th	97	98	100	102	103	105	106	58	59	60	61	61	62	63
	90th	111	112	114	115	117	119	119	73	73	74	75	76	77	78
	95th	115	116	117	119	121	122	123	77	78	79	80	81	81	82
	99th	122	123	125	127	128	130	130	85	86	86	88	88	89	90
11	50th	99	100	102	104	105	107	107	59	59	60	61	62	63	63
	90th	113	114	115	117	119	120	121	74	74	75	76	77	78	78
	95th	117	118	119	121	123	124	125	78	78	79	80	81	82	82
	99th	124	125	127	129	130	132	132	86	86	87	88	89	90	90
12	50th	101	102	104	106	108	109	110	59	60	61	62	63	63	64
	90th	115	116	118	120	121	123	123	74	75	75	76	77	78	79
	95th	119	120	122	123	125	127	127	78	79	80	81	82	82	83
	99th	126	127	129	131	133	134	135	86	87	88	89	90	90	91
13	50th	104	105	106	108	110	111	112	60	60	61	62	63	64	64
	90th	117	118	120	122	124	125	126	75	75	76	77	78	79	79
	95th	121	122	124	126	128	129	130	79	79	80	81	82	83	83
	99th	128	130	131	133	135	136	137	87	87	88	89	90	91	91
14	50th	106	107	109	111	113	114	115	60	61	62	63	64	65	65
	90th	120	121	123	125	126	128	128	75	76	77	78	79	79	80
	95th	124	125	127	128	130	132	132	80	80	81	82	83	84	84
	99th	131	132	134	136	138	139	140	87	88	89	90	91	92	92
15	50th	109	110	112	113	115	117	117	61	62	63	64	65	66	66
	90th	122	124	125	127	129	130	131	76	77	78	79	80	80	81
	95th	126	127	129	131	133	134	135	81	81	82	83	84	85	85
	99th	134	135	136	138	140	142	142	88	89	90	91	92	93	93
16	50th	111	112	114	116	118	119	120	63	63	64	65	66	67	67
	90th	125	126	128	130	131	133	134	78	78	79	80	81	82	82
	95th	129	130	132	134	135	137	137	82	83	83	84	85	86	87
	99th	136	137	139	141	143	144	145	90	90	91	92	93	94	94
17	50th	114	115	116	118	120	121	122	65	66	66	67	68	69	70
	90th	127	128	130	132	134	135	136	80	80	81	82	83	84	84
	95th	131	132	134	136	138	139	140	84	85	86	87	87	88	89
	99th	139	140	141	143	145	146	147	92	93	93	94	95	96	97

Appendix 5.
Standard Neonatali Italiani: peso (Kg);
(SEP: Montecatini, 1996).

Standard neonatali: PESO (Kg)								
età gestazionale (settimane)	***soggetti***	**3°**	**10°**	**25°**	**50°**	**75°**	**90°**	**97°**
								femmine
26	**21**	**0,572**	**0,590**	**0,662**	**0,776**	**0,889**	**0,962**	**0,979**
27	**24**	**0,566**	**0,629**	**0,734**	**0,875**	**1,015**	**1,120**	**1,184**
28	**24**	**0,605**	**0,709**	**0,842**	**1,1007**	**1,171**	**1,304**	**1,409**
29	**27**	**0,683**	**0,823**	**0,981**	**1,166**	**1,352**	**1,510**	**1,650**
30	**62**	**0,795**	**0,967**	**1,146**	**1,349**	**1,552**	**1,732**	**1,903**
31	**67**	**0,936**	**1,133**	**1,331**	**1,550**	**1,768**	**1,966**	**2,164**
32	**93**	**1,099**	**1,318**	**1,532**	**1,763**	**1,995**	**2,208**	**2,428**
33	**125**	**1,278**	**1,516**	**1,742**	**1,985**	**2,227**	**2,453**	**2,691**
34	**270**	**1,469**	**1,721**	**1,957**	**2,209**	**2,461**	**2,698**	**2,949**
35	**441**	**1,665**	**1,927**	**2,172**	**2,432**	**2,692**	**2,936**	**3,198**
36	**917**	**1,861**	**2,130**	**2,381**	**2,647**	**2,914**	**3,165**	**3,434**
37	**2208**	**2,051**	**2,323**	**2,579**	**2,851**	**3,123**	**3,379**	**3,651**
38	**5819**	**2,229**	**2,502**	**2,761**	**3,038**	**3,315**	**3,574**	**3,847**
39	**12007**	**2,389**	**2,661**	**2,921**	**3,203**	**3,484**	**3,745**	**4,016**
40	**16421**	**2,527**	**2,794**	**3,055**	**3,341**	**3,627**	**3,888**	**4,155**
41	**7400**	**2,636**	**2,896**	**3,157**	**3,447**	**3,737**	**3,999**	**4,259**
42	**1863**	**2,710**	**2,961**	**3,222**	**3,517**	**3,812**	**4,073**	**4,324**
43	**252**	**2,744**	**2,984**	**3,245**	**3,545**	**3,845**	**4,105**	**4,346**
								maschi
26	**20**	**0,639**	**0,657**	**0,730**	**0,843**	**0,957**	**1,029**	**1,047**
27	**26**	**0,639**	**0,702**	**0,807**	**0,948**	**1,088**	**1,193**	**1,257**
28	**29**	**0,683**	**0,787**	**0,921**	**1,085**	**1,249**	**1,383**	**1,487**
29	**44**	**0,767**	**0,907**	**1,065**	**1,250**	**1,435**	**1,593**	**1,734**
30	**51**	**0,884**	**1,056**	**1,235**	**1,438**	**1,641**	**1.821**	**1,992**
31	**77**	**1,030**	**1,228**	**1,426**	**1,644**	**1,863**	**2,060**	**2,258**
32	**99**	**1,199**	**1,418**	**1,632**	**1,863**	**2,095**	**2,308**	**2,328**
33	**154**	**1,384**	**1,621**	**1,847**	**2,090**	**2,333**	**2,559**	**2,796**
34	**297**	**1,580**	**1,831**	**2,068**	**2,320**	**2,572**	**2,808**	**3,060**
35	**492**	**1,781**	**2,043**	**2,288**	**2,548**	**2,808**	**3,053**	**3,315**
36	**1167**	**1,982**	**2,251**	**2,502**	**2,769**	**3,035**	**3,286**	**3,555**
37	**2551**	**2,178**	**2,450**	**2,706**	**2,978**	**3,250**	**3,506**	**3,778**
38	**6494**	**2,361**	**2,634**	**2,893**	**3,170**	**3,447**	**3,706**	**3,979**
39	**12657**	**2,527**	**2,798**	**3,059**	**3,341**	**3,622**	**3,883**	**4,154**
40	**16267**	**2,670**	**2,937**	**3,198**	**3,484**	**3,770**	**4,032**	**4,298**
41	**7297**	**2,784**	**3,044**	**3,306**	**3,596**	**3,886**	**4,148**	**4,408**
42	**2034**	**2,864**	**3,115**	**3,376**	**3,671**	**3,966**	**4,227**	**4,478**
43	**227**	**2,903**	**3,144**	**3,404**	**3,704**	**4,004**	**4,265**	**4,505**

Appendix 6.
National Cholesterol Education Programme Adult Treatment Panel III (NCEP ATP III) criteria of Cruz and Goran 2001

	NCEP ATP III (2004) **Cruz e Goran**
Central Obesity	**Waist circunference: ≥ 90° percentile** **by age and sex.**
Hypertension:	**Blood pressure** **systolic or diastolic: ≥ 90° percentile by age and sex.**
Dislipidemia:	**Triglicerides: ≥ 90° percentile** **By age and sex.** **HDL Cholesterol (High Density Lipoprotein):** **≤ 10° percentile by age and sex**
Disglycemia:	**Fasting glucose: ≥ 100 mg/dL** **or** **after OGTT (Oral Glucose Tolerance Test):** **≥ 140 mg/dL and < 200 mg/dL.**

Appendix 7

The BMI standard deviation score (BMISDS), sex and age dependent; (Cacciari E. et al., 2006)

- Whole Italy. Height, weight and body mass index (BMI) growth norms, expressed as LMS. The SD score corresponding to the value (y) of the auxometric trait is computed according to formula 3 (see text).

	Height (cm)						Weight (kg)						BMI (kg/m²)					
Age	Girls			Boys			Girls			Boys			Girls			Boys		
(yrs)	L	M	S	L	M	S	L	M	S	L	M	S	L	M	S	L	M	S
2.0	0.768	87.2	0.043	0.964	88.8	0.050	-0.982	12.3	0.127	-0.974	12.8	0.131	-2.743	16.1	0.106	-3.721	16.5	0.084
2.5	0.708	91.4	0.044	0.947	93.1	0.049	-0.982	13.4	0.131	-0.978	14.0	0.133	-2.900	15.8	0.104	-3.622	16.1	0.086
3.0	0.656	95.4	0.044	0.926	97.0	0.048	-0.985	14.5	0.136	-0.987	15.2	0.135	-2.934	15.7	0.105	-3.507	15.9	0.089
3.5	0.612	99.2	0.045	0.903	100.7	0.047	-0.987	15.7	0.141	-0.998	16.3	0.138	-2.886	15.6	0.107	-3.392	15.8	0.091
4.0	0.579	102.8	0.045	0.880	104.1	0.047	-0.984	16.8	0.148	-1.010	17.4	0.141	-2.791	15.6	0.110	-3.284	15.8	0.094
4.5	0.556	106.4	0.046	0.856	107.4	0.047	-0.972	18.0	0.154	-1.020	18.5	0.145	-2.672	15.7	0.113	-3.184	15.8	0.097
5.0	0.541	109.7	0.046	0.834	110.7	0.047	-0.950	19.2	0.162	-1.026	19.6	0.150	-2.548	15.8	0.116	-3.089	15.8	0.101
5.5	0.534	113.0	0.046	0.813	113.8	0.046	-0.920	20.5	0.169	-1.025	20.8	0.155	-2.426	15.9	0.119	-2.996	15.9	0.104
6.0	0.531	116.1	0.046	0.795	116.8	0.046	-0.881	21.9	0.176	-1.015	22.0	0.161	-2.312	16.1	0.122	-2.900	16.0	0.108
6.5	0.530	119.2	0.046	0.779	119.8	0.046	-0.836	23.3	0.183	-0.995	23.3	0.168	-2.210	16.2	0.125	-2.796	16.2	0.112
7.0	0.528	122.0	0.046	0.766	122.8	0.046	-0.789	24.8	0.189	-0.965	24.7	0.175	-2.118	16.4	0.128	-2.682	16.3	0.117
7.5	0.520	124.8	0.046	0.753	125.6	0.046	-0.740	26.4	0.195	-0.927	26.2	0.182	-2.036	16.6	0.132	-2.554	16.5	0.121
8.0	0.503	127.6	0.047	0.739	128.4	0.046	-0.691	28.0	0.200	-0.881	27.8	0.189	-1.957	16.9	0.136	-2.412	16.8	0.127
8.5	0.474	130.3	0.047	0.719	131.1	0.046	-0.644	29.6	0.204	-0.831	29.5	0.195	-1.873	17.1	0.140	-2.257	17.0	0.132
9.0	0.438	133.1	0.047	0.686	133.8	0.046	-0.598	31.4	0.208	-0.778	31.3	0.201	-1.775	17.4	0.144	-2.091	17.3	0.137
9.5	0.406	135.9	0.048	0.630	136.4	0.046	-0.553	33.2	0.211	-0.724	33.2	0.206	-1.653	17.7	0.149	-1.916	17.6	0.143
10.0	0.399	139.0	0.048	0.539	139.0	0.046	-0.503	35.2	0.215	-0.668	35.1	0.210	-1.504	18.1	0.154	-1.736	18.0	0.148
10.5	0.443	142.2	0.049	0.401	141.6	0.046	-0.442	37.5	0.218	-0.612	37.2	0.213	-1.333	18.5	0.158	-1.557	18.3	0.152
11.0	0.551	145.5	0.049	0.220	144.3	0.047	-0.361	40.0	0.220	-0.556	39.4	0.215	-1.151	18.9	0.160	-1.382	18.7	0.155
11.5	0.717	148.8	0.048	0.026	147.1	0.048	-0.260	42.7	0.219	-0.499	41.7	0.217	-0.983	19.4	0.161	-1.217	19.1	0.157
12.0	0.909	151.9	0.047	-0.116	150.2	0.050	-0.153	45.6	0.214	-0.439	44.2	0.217	-0.854	19.8	0.159	-1.069	19.5	0.159
12.5	1.085	154.6	0.045	-0.126	153.5	0.052	-0.070	48.4	0.205	-0.379	46.9	0.216	-0.789	20.1	0.156	-0.943	19.9	0.159
13.0	1.211	156.8	0.044	0.050	157.2	0.053	-0.041	50.7	0.193	-0.317	49.7	0.213	-0.797	20.4	0.152	-0.844	20.2	0.157
13.5	1.274	158.4	0.042	0.401	161.0	0.053	-0.080	52.5	0.181	-0.260	52.7	0.208	-0.874	20.6	0.147	-0.776	20.6	0.155
14.0	1.283	159.7	0.040	0.855	164.6	0.052	-0.172	53.7	0.171	-0.214	55.8	0.202	-0.997	20.8	0.142	-0.740	20.9	0.153
14.5	1.257	160.6	0.039	1.305	167.8	0.049	-0.285	54.6	0.163	-0.189	58.7	0.193	-1.137	20.9	0.137	-0.738	21.1	0.149
15.0	1.215	161.2	0.038	1.649	170.4	0.047	-0.391	55.1	0.157	-0.197	61.3	0.184	-1.270	21.0	0.134	-0.765	21.3	0.146
15.5	1.170	161.7	0.038	1.829	172.3	0.044	-0.475	55.4	0.154	-0.240	63.5	0.174	-1.380	21.0	0.131	-0.818	21.5	0.142
16.0	1.130	162.0	0.037	1.847	173.7	0.041	-0.536	55.6	0.151	-0.314	65.2	0.165	-1.463	21.0	0.129	-0.890	21.7	0.139
16.5	1.098	162.2	0.037	1.754	174.7	0.039	-0.578	55.7	0.150	-0.406	66.6	0.158	-1.520	21.1	0.128	-0.974	21.8	0.136
17.0	1.072	162.3	0.037	1.612	175.3	0.038	-0.605	55.8	0.149	-0.501	67.5	0.152	-1.557	21.1	0.127	-1.065	21.9	0.133
17.5	1.053	162.4	0.037	1.466	175.8	0.037	-0.623	55.8	0.148	-0.588	68.2	0.148	-1.581	21.1	0.127	-1.156	22.0	0.130
18.0	1.039	162.5	0.036	1.341	176.0	0.036	-0.635	55.9	0.148	-0.660	68.7	0.145	-1.594	21.1	0.126	-1.242	22.0	0.128
19.0	1.021	162.6	0.036	1.172	176.4	0.036	-0.647	55.9	0.147	-0.758	69.2	0.141	-1.604	21.1	0.126	-1.391	22.1	0.124
20.0	1.012	162.6	0.036	1.084	176.5	0.035	-0.653	55.9	0.147	-0.811	69.5	0.139	-1.605	21.1	0.126	-1.501	22.2	0.122

SIEDP-2006 Italian growth charts.