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Evaluation of kisspeptin levels in prepubertal obese and overweight children: sexual dimorphism and modulation of antioxidant levels

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Abstract. – OBJECTIVE: Kisspeptin, neuropeptide involved in puberty beginning and regulation of pituitary-gonadal axis, has been shown to stimulate antioxidant defenses in murine models. Its levels are greater in females than males and also in obese prepubertal girls. Therefore, our aim was to evaluate sex-related differences in prepubertal obese patients and the relationships of Kisspeptin with metabolic/hormonal parameters.

PATIENTS AND METHODS: We studied Kisspeptin concentrations in 54 children (22 males and 32 females, Tanner stage 1), 5-12 ys, classified according to Cole's criteria into 17 overweight and 37 obese; 25 normal-weight children, aged 6-12 years, were studied as controls. We evaluated metabolic (glucose and insulin levels after oral glucose load, total- LDL- HDL-cholesterol, triglycerides, uric acid) and hormonal (fT3, fT4, TSH, IGF-1, leptin) parameters. Moreover, total antioxidant capacity (TAC) was evaluated by spectrophotometric method, using the system H202-metmyoglobin-ABTS. Kisspeptin levels were measured by RIA.

RESULTS: We did not find significant differences between obese and normal-weight children, but obese males presented significantly lower levels than females. Kisspeptin did not correlate with BMI, HOMA-IR, Insulin peak levels and TAC; a significant correlation was found between Kisspeptin and fT3 (r^2=0.25; p=0.003) in the obese group; leptin levels, significantly greater in obese vs. overweight and control children, significantly correlated with TAC (r^2=0.39; p=0.03).

CONCLUSIONS: These data suggest that both hormones could modulate antioxidants, Kisspeptin indirectly via influence on thyroid hormones, and Leptin by a direct effect. This mechanism seems to be sex-related, not attributable to peripheral steroid levels. Further studies can clarify the complex interrelationship between central and peripheral Kisspeptin secretion and oxidative stress in children obesity.

Key Words:
Obesity, Insulin-resistance, Antioxidants, Thyroid.

Introduction

The name of Kisspeptin (Kiss) includes a family of peptides, derived from the primary product of translation of the Kiss1 gene; it has 154 aminoacids and is rapidly processed to a shorter and unstable form with 54 aminoacids, further cut into peptides with 14, 13 or 10 aminoacids[1-3]. All these forms interact with a G protein-coupled receptor.

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The name of metastatin, initially thought for a hypothetical involvement in neoplastic spreading, is not more used, since the well-recognized functions concern physiology of reproduction and GnRH neurons stimulation. It has been extensively studied in pubertal disorders[4-6].

However, together with leptin produced by adipose tissue, it seems to cover a metabolic role, connecting nutritional status with puberty and reproduction, which is still poorly understood. Other than in hypothalamus, Kiss is expressed in other tissues, such as adipose tissue, pancreas, adrenal glands, gonads of both sexes[7].

A sex dimorphism has been demonstrated in humans, with higher levels in females than in males. It has been shown that Kiss levels increase from puberty to adult age, while this pattern is not present in boys[8]. Moreover, prepubertal female obese children have been shown to have greater levels than non-obese children, but no data are reported on obese prepubertal males. Whether or not the dimorphism is present also before puberty, and therefore can be independent from sexual hormones milieu, is not addressed in the cited paper.

Finally, a role of Kiss as antioxidant has been proposed[9]. Oxidative stress is present in obese children and their antioxidant defenses are not entirely developed, making them particularly vulnerable with a precocious beginning of metabolic and cardiovascular complications[10,11].

To gain insight this topic and to further explore the possible metabolic role of Kisspeptin, we have performed an observational case-control study, comparing Kiss levels in obese and overweight children, both males and females, and normal weight controls and investigated its correlations with leptin, index of insulin resistance and total antioxidant capacity.

Patients and Methods

Population Enrollment

A total of 79 prepubertal (Tanner's stage I) children (33 males and 46 females) of Caucasian origin, from 5 to 12 years were enrolled in the study. Informed consent was obtained from children's parents and the study protocol was approved by our Institutional Review Board.

Since BMI values in childhood, in contrast to adulthood, are dependent on age and gender, the Standard Deviation (SD) of BMI was used for data analysis, considering as overweight children with BMI greater than 1.6 SD (corresponding to 85°centile) and obese ones with a BMI greater than 1.8 SD (corresponding to 95° centile).

According to Cole et al[12] weight references (2000), three groups were defined: obese (OB) (n = 37, 17 males and 20 females), overweight (OW) (n = 17, 5 males and 12 females) and normal weight (controls) (n = 25, 11 males and 14 females) children.

Based on phenotypic, anamnestic and hormonal data, the following conditions were excluded:

– monogenic obesity;
– syndromic obesity;
– obesity due to endocrine diseases (Cushing syndrome, hypothyroidism, hypopituitarism);
– obesity due to diencephalic or encephalic lesions or malformations;
– iatrogenic obesity related to drugs taken during the previous 6 months (antiepileptics, antidepressant, corticosteroids);
– acute or chronic inflammatory diseases.

Anthropometric Parameters

For each child the following parameters were measured: standing height (Harpenden stadiometer), naked weight (precision scales), waist circumference (WC; flexible meter). Italian growth charts have been used as reference standards for height and weight. Waist circumferences have been compared to the values set out in McCarthy et al[13] in 2001. Body Mass Index (BMI) was calculated by the formula: Weight (kg)/Height² (meter).

Biological Sample Collection

All children underwent venous sampling at 08.00 am, after an overnight fasting, collecting blood samples by three pyrogen-free tubes, one containing lithium-heparin as anticoagulant, one containing EDTA and one without anticoagulant to obtain serum. All samples were centrifuged immediately after basal sampling at a temperature of 4°C at 2700 rpm for 11 minutes. Plasma or serum aliquots were then separated and stored at a temperature of -80°C.

We determined plasmatic glucose, C-reactive protein (CRP), total cholesterol, HDL-cholesterol, triglycerides, uric acid and total antioxidant capacity (TAC); serum insulin, insulin-like growth factor-1 (IGF-1), free triiodothyronine (fT3), free thyroxine (fT4) and thyroid stimulating hormone (TSH); from EDTA samples of we assayed kisspeptin; finally, from lithium-hep-

arin tubes of subgroup of 50 children (24 ob/ow and 26 controls) we also determined leptin levels.

Moreover, in the population of obese and overweight children, an oral glucose tolerance test (OGTT) was performed (oral glucose dose of 1.75 g / Kg of ideal weight, max 75 gr), with blood samples taken every 30 min until 120 min to evaluate glucose and insulin response.

Homeostasis model of insulin resistance (HOMA-IR), an insulin resistance index, was calculated according to the formula: [fasting insulin (U/ml)] * [fasting glucose (mmol/l)]/405. In the absence of a unanimous consensus about the definition of insulin resistance in pediatric age, we chose a cut-off of HOMA greater than 2.67 in males and 2.22 in females in a population of 268 obese children and adolescents[14].

Assays

Plasma concentrations of glucose, total cholesterol, HDL-cholesterol, triglycerides, uric acid were measured by using enzymatic assays and CRP by an immunoturbidimetric assay on Olympus AU2700 chemistry analyzer (Olympus America Inc., Center Valley, PA, USA). The intra-and inter-assay coefficients of variation (CV) for total cholesterol, triglycerides, and uric acid were < 1.5%, and < 2.5%, respectively. The intra-and inter-assay CV for HDL-cholesterol and CRP were < 2.5%, and < 3.0%, respectively. LDL cholesterol was calculated by Friedewald's equation: LDL = total cholesterol - (HDL + triglycerides/5).

Serum concentrations of insulin, IGF-1, TSH, fT3, and fT4 were measured by using immunochemiluminometric assays on a Roche Modular E170 analyzer (Roche Diagnostics, Indianapolis, IN, USA). The intra- and inter-assay CV for insulin, IGF-1, TSH, fT3, and fT4 were, respectively, < 5.0% and < 7.0%.

Plasma TAC was evaluated using the method developed by Rice-Evans and Miller[14] with some modifications[11]. The method is based on the formation of the radical cation 2,2I-azinobis(3-ethylbenzothiazoline-6-sulphonate) ($ABTS^{+}$) formed by interaction between ABTS (150 mM) and ferrylmyoglobin (radical species), generated by activation of metmyoglobin (2.5 mM) with H_2O_2 (75mM); the radical cation is spectroscopically revealed. This reaction is inhibited by the antioxidant content of the sample and the presence of chain-breaking antioxidants induces a lag time (the "lag phase", LAG in seconds).

The Leptin levels were measured using the DRG Diagnostics kit (DRG Instruments, GmbH, Marburg, Germany) by enzyme-linked immunoassay (ELISA) method. The detection limit of leptin by this assay was 1.0 ng/ml. The intra and inter-assay CV were < 5.9% and <8.6%, respectively.

For the assay of Kisspeptin, the plasma was first acidified and then the peptides were extracted using a C-18 SEP-COLUMN (Phenomenex, Inc., Torrance, CA, USA) containing 200 mg of C18. Samples were eluted, evaporated dry and then stored at a temperature of -80°C until the assay. The concentration of Kisspeptin (in pg/ml) was measured using the KISS1 (68-121) Amide-Metastin (1-54) -NH2 kit (RK-048-59, Phoenix Pharmaceuticals, Inc., Burlingame, CA, USA) based on the RIA (Radio Immuno Assay) method.

Statistical Analysis

Continuous variables were expressed as Mean±SEM. Shapiro test was performed preliminarily to evaluate the distribution of data in the population studied. Correlation analysis was performed using Spearman coefficient. The comparison between groups was performed using the Mann-Whitney test. Statistical analysis was performed using the XLSTAT software (2015.1 version, Addinsoft, NY, USA).

Results

Hormonal and Metabolic Parameters

Table I summarizes mean values and standard error (SEM) of the anthropometric, metabolic and hormonal parameters in the three groups (obese, overweight children *vs.* normal subjects). Basal blood glucose was significantly higher in obese and overweight patients compared to normal subjects, while there was no difference between obese and overweight. As for basal insulinemia, there was a significant difference between obese and normal weight and obese and overweight, but not between overweight and controls, maybe due to a not adequate compensatory insulinemic response in overweight children. The most interesting data are HOMA index values, progressively and significantly higher by going from the normal to the overweight and the obese (Figure 1).

Figure 2 right panel shows the percentage of IR subjects, according to the definition of HOMA above reported; left panel shows the mean gly-

Table I. Mean ± SEM of anthropometric, hormonal and metabolic parameters in the three groups.

	Obese subjects	Overweight subjects	Normal subjects
Weight (Kg)	49.19 ± 8.19	40.83 ± 2.35	21.63 ± 2.7
Height (m)	1.35 ± 0.22	1.35 ± 0.02	1.16 ± 0.06
Age (yrs)	8.49 ± 1.39	8.73 ± 0.56	6.98 ± 0.52
BMI	26.37 ± 4.39	21.68 ± 0.48	15.75 ± 0.88
IGF-1 (ng/ml)	187.27 ± 39.92	219.46 ± 23.67	148.09 ± 18.34
TSH (μUI/L)	2.5 ± 0.41	2.37 ± 0.37	1.93 ± 0.21
FT3 (pg/ml)	3.89 ± 0.64	3.92 ± 0.19	4.08 ± 0.23
FT4 (pg/ml)	11.52 ± 1.92	11.5 ± 0.57	10.87 ± 0.27
Total Cholesterol (mg/dl)	161.70 ± 26.58	160.92 ± 3.99	147.86 ± 25.25
LDL (mg/dl)	92.63 ± 15.43	93.92 ± 4.33	79.33 ± 9.23
HDL (mg/dl)	53.59 ± 8.81	58.38 ± 2.63	55.78 ± 3.13
Triglycerides (mg/dl)	78.29 ± 12.87	59.21 ± 5.59	56.92 ± 10.06
Glucose (mg/dl)	84.45 ± 13.88*	83.66 ± 1.59*	70.66 ± 1.95
Insulin (μUI/mL)	11.46 ± 1.88*°	6.27 ± 0.84	4.35 ± 1.42
Hb1A1c (%)	35.76 ± 6.13	35.33 ± 0.74	
HOMA-IR	2.40 ± 0.39*°	1.31 ± 0.18*	0.33 ± 0.15
Uric Acid (mg/dl)	4.48 ± 0.78	4.1 ± 0.22	3.84 ± 0.27

*$p < 0.05$ *vs*. controls. °$p < 0.05$ *vs*. overweight.

caemia and insulinemia values after oral glucose load in overweight and obese children. Although the mean glycaemia values fall into normal values, insulinemia shows an abnormal high peak at 30 min (76.25 ± 9.39 μUI / ml), with stable levels at the end of two hours (68.99 ± 8.56 μUI / ml) in both groups. There were no cases of altered fasting blood glucose (IFG) or reduced glucose tolerance (IGT) except for two patients with borderline blood glucose levels after two hours.

Total Antioxidant Capacity

Regarding the comparison of overweight and obese children with the control group, no differences were observed in LAG values (Figure 3 left panel), nor between males and females.

LAG values were also studied in relation to metabolic parameters (HOMA Index, glycemia and fasting insulinemia, uricemia) and hormone values (IGF-1, TSH, fT3, fT4). No significant correlation between total antioxidant capacity and hormone parameters emerged; however, while there was no statistically significant correlation (r^2=0.10) between LAG and HOMA Index, the uric acid value, which is an indirect sign of insulin resistance, appears to be correlated with the TAC value with a directed proportional relationship (r^2 =0.43) (Figure 3, right panel).

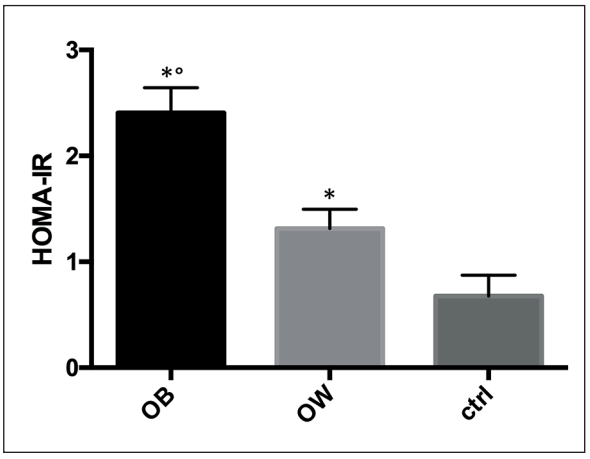

Figure 1. Mean±SEM HOMA-IR in Obese (OB), Overweight (OW) and control (ctrl) children. *p<0.05 *vs*. controls; °p<0.05 *vs*. OW.

Leptin

Leptin was evaluated taking regard of the differences in sex and age. As expected, leptin was significantly higher in obese subjects than overweight and in these compared to normal (Figure 4).

Leptin values were higher in females, but not significantly, in the group of controls (4.8 ± 1.8 *vs.* 1.4 ± 0.5 ng/ml); an opposite pattern was present in the obese group (23.0 ± 6.9 in males *vs.* 14.3 ± 5.1 ng/ml in females), with a similar trend in overweight (12.4 ± 3.2 in males *vs.* 10.7 ± 3.6 ng/ml in females). As expected, leptin values were higher in children older than 8 years (21.9 ±3.1 *vs.* 14.9 ±2.9 in obese; 14.8 ±1.8 *vs.* 8.9 ±3.6 in overweight; 4.6 ±2.2 *vs.* 2.3 ±1.1 ng/ml in controls). A correlation between leptin and LAG was observed (r^2=0.39; p=0.03).

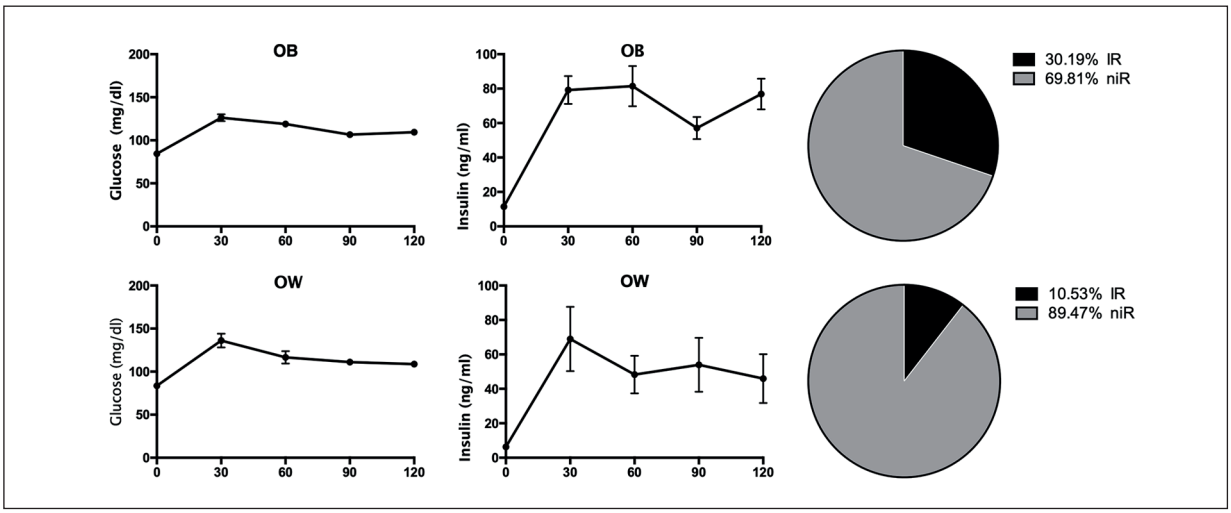

Figure 2. Left panel: Mean±SEM glucose and insulin levels after oral glucose load in obese (OB) and overweight (OW) children; right panel: percentage of insulin-resistant children in OB and OW groups.

Kisspeptin

Finally, we studied plasma values of Kisspeptin, evaluating the differences of this protein related to sex and age. We did not find statistically significant differences in its plasma concentrations between normal, overweight and obese patients (17.4±0.8, 18.4±1.7 and 16.4±1.4 pg/ml, respectively). A significant difference related to sex was observed (with lower values in males), but this difference was observed only in obese subjects (Figure 5).

In order to evaluate the changes in plasma concentrations of Kisspeptin during development, we divided children into two groups depending on their age (a group was under 8 years old and a group was under that age, since this value coincided with the average and median of our sample of obese children).

Analyzing data regarding the two populations, the age-related difference, descripted in literature, was not found in our population. Kisspeptin values of children under the age of 8 were not different from those of older children (15.9 ±2.6 *vs.* 15.4 ±2.2 in obese, 16.8 ±3.1 *vs.* 19.8 ±1.4 in overweight, 19.0 ±1.1 *vs.* 15.8 ±1.3 pg/ml in control group).

We then studied the existing correlations between Kisspeptin and nutritional status of the obese children. No statistically significant correlation was found with BMI expressed in absolute value or in terms of SDS. Regarding correla-

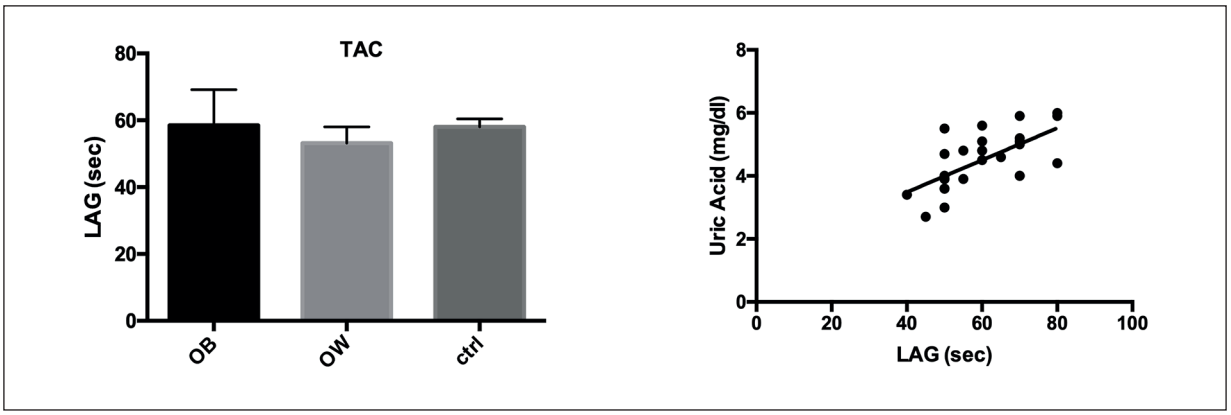

Figure 3. Left panel: Mean±SEM TAC, expressed as Lag (sec), in three groups in Obese (OB), Overweight (OW) and control (ctrl) children; right panel: linear correlation between TAC, expressed as Lag (sec), and uric acid in obese children (r^2=0.43; p= 0.0001).

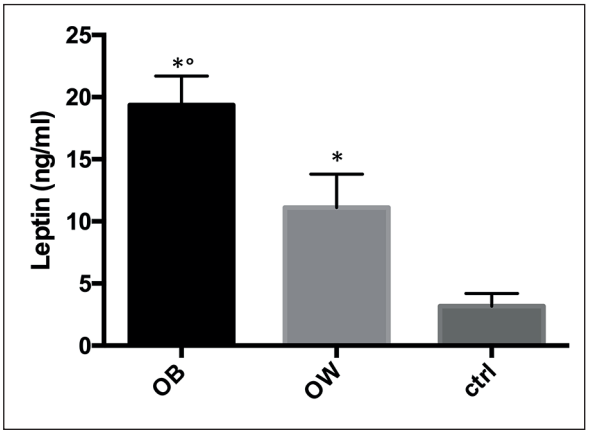

Figure 4. Mean±SEM Leptin in Obese (OB), Overweight (OW) and control (ctrl) children. *p<0.05 *vs.* controls; °p<0.05 *vs.* OW.

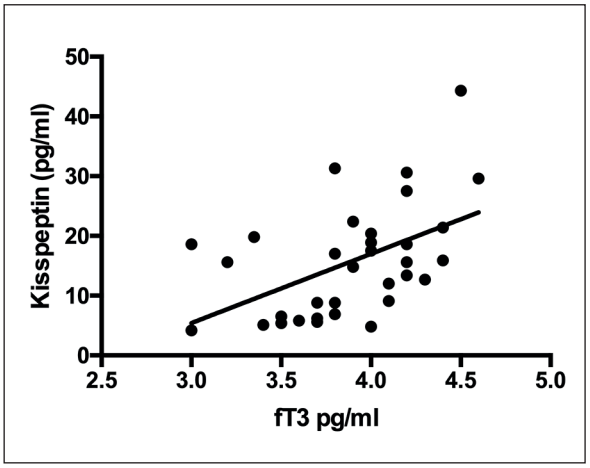

Figure 6. Linear correlation between Kisspeptin and fT3 in obese children (r^2 0.25; p=0.003).

tions with metabolic parameters, Kisspeptin did not seem to correlate with the HOMA index and LAG values. As regards hormone parameters, no correlation was found with the levels of IGF-1, leptin or TSH; however, a significant correlation was present between Kisspeptin and fT3 in the group of obese subjects (Figure 6).

Discussion

The main novel result of the study is the sex-related difference in Kiss levels among prepuberal obese children. This datum had been previously demonstrated in the population of adult obese[8]. Moreover, a sexual dimorphism has been more recently described in different pubertal stages of Chinese children and adolescents, in a longitudinal observational study including both normal and overweight/obese subjects[15]: in particular, girls reached higher kisspeptin levels in Tanner 2 stage, while in boys the levels of this hormone increased in stage T4 and T5. A discrepant result was reported in another study, describing age-dependent elevations in plasma Kiss in children in comparison with adults, with a peak at about 9-12 ys; in this study, no sex-related difference was noted[16].

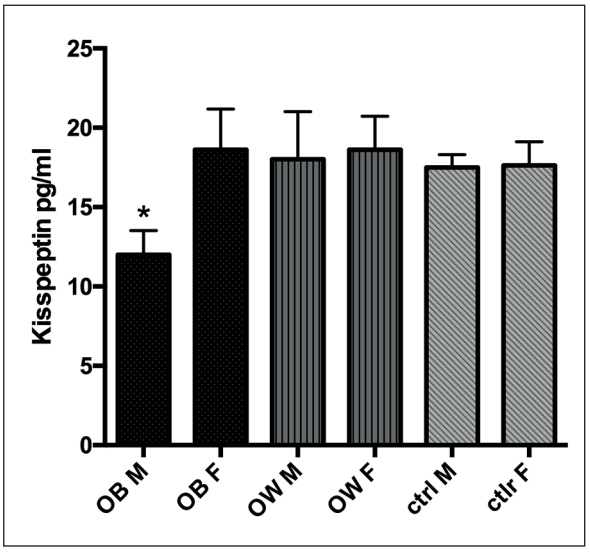

Figure 5. Mean±SEM Kisspeptin in Obese (OB), Overweight (OW) and control (ctrl) children divided in subgroups according to sex. *p<0.05 *vs.* female obese children.

Anyway, both in adult and adolescents, a key role could be exerted by sex steroid levels. In fact, different experimental evidences support the hypothesis that estrogens exert, via estrogen receptor alpha, important roles in the regulation of hypothalamic Kiss expression during pubertal development[6]. Estrogens have been shown to differentially regulate Kiss1 expression in a nucleus-specific way (inhibitory in the arcuate nucleus and stimulatory in antero-periventricular nucleus)[17].

However, we studied a cohort of prepubertal subjects; therefore, the results suggest that the difference is not related to the milieu of circulating steroids, but, on the contrary, could be related to a differential hypothalamic control also present before puberty. Such difference could develop in very early stages of life: a number of studies indicate a sex dimorphism in the hypothalamic Kiss1-receptor in different animal species, including mice[18,19], rats[20] and sheep[21]. The topic of

sexual brain maturation in relation of circulating steroids during development is well discussed by Bianco[22]: manipulation of steroid environment in mouse embryos induced abnormalities in the sex-dimorphic expression of kisspeptin in the hypothalamus[20,23].

Finally, we want to underline that the sexual difference was present only in our obese group and not in normal weight controls. This result could be explained by the higher levels of leptin present in overweight and obese children, due to the stimulating effect of leptin on kisspeptin: variations of circulating leptin or the nutritional status influence kiss expression[22,24-26], suggesting that kiss has a key role in the link between nutritional signals and reproduction. We could further suggest that in our population, increased leptin triggers an amplification of sex-related Kiss pattern.

The second novel result is that Leptin showed a direct correlation with LAG, while other characteristics of leptin secretion were expected as already discussed (higher levels in OB *vs.* OW *vs.* controls). A bidirectional relation between antioxidant and leptin could be present since increased food can induce augmented production of radical oxygen species and oxidative stress[27].

Finally, the third novel finding is the positive significant correlation between Kiss and fT3. Although we did not find correlation between Kiss and TAC, a significant positive correlation with fT3 was observed only in obese patients. fT3 is in turn related to antioxidant regulation as previously shown[28,29]. Both hyper- and hypothyroidism can be associated with oxidative stress, but many studies underline beneficial effects of thyroid hormones on antioxidants, via mechanisms still poorly known; in particular T3 can have positive effects on redox cellular status, *via* UCP-3[30,31], sirtuins and AMPK-activation[32]; a complex interplay is present among thyroid hormones, oxidative stress and inflammation and, as above stated, some data link Kiss and inflammation. Since obesity is a condition with low-grade inflammation and, therefore, oxidative stress, as documented also in our patients by increase compensatory TAC, it can be speculated that Kisspeptin, via thyroid hormones, can induce an increase in antioxidant capacity. Data concerning Kisspeptin and pituitary-thyroid axis are still inconclusive; despite most studies did not show a direct effect of Kisspeptin on TSH release, both *in vitro* and *in vivo* experiments[33], these models are not superimposable to our study. In fact, hypothalamic and peripheral Kisspeptin can have differential effects. Moreover, other studies seem to suggest a link between Kisspeptin and metabolic status, at least parallel to pituitary-thyroid axis, in models such as anorexia nervosa[34] or after pharmacological treatments[35]. The correlation between Kisspeptin and fT3 could not demonstrate a causal relationship; moreover, both hormones can be increased by a common mechanism, such as obesity and/or pubertal advancement. Whatever the reason, however, the sexual dimorphism, seems to indicate that this hormonal response could be more marked in prepubertal females than in males, thus explaining the well-known earlier onset of puberty in obese females.

While the role of Kiss as a sensor of metabolic status, therefore influencing reproductive system and in particular the timing of puberty is largely accepted, our data suggest that it can also cover an active role contributing to modulation of antioxidants. A limit of our study is that we evaluated only TAC, using a method which measures non proteic non enzymatic chain braking substances, which, however, have a key role in antioxidant defense against oxidative stress, as demonstrated in previous papers[36], also in agreement with other data in literature. In fact, the effects of Kisspeptin on liver antioxidant have been studied in young male rats; moreover, a possible indirect action, mediated by GnRH neurons and steroid secretion, has been investigated by comparison with other treatment (GnRH agonist goserelin and Kiss plus goserelin). Kisspeptin-10 was able to induce an increase in liver SOD and CAT and a decrease in MDA levels[9]; however, the first action seemed to be indirect (no effect when Kiss was administered with goserelin), while the last one was attributed to reduction in lipid peroxidation by direct mechanism (effects with Kiss alone or Kiss plus goserelin).

Taken together our data suggest a possible metabolic role of both Kiss and Leptin and a modulation of antioxidant systems, even if not entirely over-imposable for the two peptides.

Conclusions

To sum up, we demonstrated a sex related difference in Kiss levels in prepubertal obese patients, with higher levels in females. Therefore, this dimorphism, previously observed in adult obese people, is not related to circulating steroid levels. We also showed a correlation of

leptin with LAG and of Kisspeptin with fT3, only in obese subjects, suggesting that increased oxidative stress and/or low-grade inflammation can modulate both hormones, for compensatory mechanism, with a direct effect on antioxidant levels by leptin and an indirect one by Kiss, mediated by thyroid hormones. However, we cannot establish direct causal relationships between these data, but they suggest that a link can be sustained by metabolic status and hormones traditionally considered as regulators of pituitary-gonadal axis.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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