Impact of child obesity on adipose tissue physiology: assessment of adipocytokines and inflammatory cytokines as biomarkers of obesity

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Abstract

Obesity could be interpreted as a low grade inflammatory state. The role of cytokines for innate and acquired immune response and adipocytokines in pathogenesis of obesity is not completely understood. The aim of the study was to evaluate anthropometric parameters, adipocytokines and inflammatory cytokine levels as biomarkers of childhood obesity. This investigation was designed as a longitudinal observational study. Forty-seven obese children (19 males and 28 females) were enrolled by Pediatric Clinic of the Foundation IRCCS Policlinico San Matteo, Pavia, Italy. For each patient a blood sample, used for other biochemical evaluations, was collected. Cytokines and adipocytokines plasmatic levels were determined using an ELISA method. Plasma leptin levels are in correlation with age (r=0.5; P<0.001) and BMI-z score (r=0.36; P<0.001), particularly in girls; plasma resistin levels are in inverse correlation with age, particularly in boys (r=-0.67; P<0.001) and in correlation with BMI-z score (r=0.52; P=0.002). Plasma leptin and resistin levels show a good correlation with anthropometric parameters of childhood obesity (sex and BMI z score). This study suggests that leptin and resistin, can be considered as biomarkers of childhood obesity.

Introduction

The innate immune system seems to participate in the regulation of energy balance and insulin resistance in response to changes in the nutritional environment.1 A very interesting feature of the inflammatory response that emerges in the presence of obesity is that it appears to be triggered and to reside predominantly in adipose tissue.2 The deal of white adipose tissue varied in function of age and sex and the plasma levels of adipocytokines are correlated to the grade of obesity. It is important to look for a positively correlation between plasma cytokines and adipocytokines levels and clinical parameters of obesity, with the aim of identify those patients that could present in adult age a major risk to develop one or more of the diseases related to obesity.

It has also been widely recognised that obesity is a state of low-grade inflammation, with adipose tissue generating substantial quantities of pro-inflammatory molecules; the pathophysiological mechanisms of which remained poorly understood, underlining the relationship between adipose tissue and the immune system. The inflammatory processes are mediated by several factors secreted by adipocytes collectively called adipocytokines (adiponectin, leptin, ghrelin, visfatin and resistin) some of which seem to play an important role in obesity-associated insulin resistance and cardiovascular complications.3,4

The chronic inflammatory response associated with obesity is characterized not only by an abnormal production of adipokines, but also with an increase in the plasma levels of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and other biological markers of inflammation. The increase in pro-inflammatory cytokine levels, such as TNF-α and IL-6, is partly due to the infiltration of macrophages in white adipose tissue (WAT).5,6 A number of metabolic changes are caused by childhood obesity, including insulin resistance, diabetes and dyslipidemia. Lifestyle modification with changes in dietary habits and physical activity is the primary intervention suggested to the patients. Anthropometric parameters may not identify all positive changes associated with lifestyle modifications, whereas circulating adipokines may represent an alternative as biomarkers.

In this study 47 simply obese pediatric patients were enrolled by the Department of Pediatrics of the Pediatric Clinic of IRCCS Foundation Policlinico S. Matteo.

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and 28 girls, presenting during 2005 to the Outpatients’ Department of Nutritional Disorders of Pediatric Clinic of IRCCS Foundation Policlinico San Matteo, Pavia, Italy were included in the study **Multidisciplinary study of child obesity.**

**Clinical evaluation**

For each patient the study included: complete case-history, clinical evaluation, formulation of a suitable diet after adequate examination, evaluation of plasmatic and serological immunological parameters. Children with genetic and endocrine causes of obesity were excluded. Clinical evaluation included case-history and examination to detect secondary causes of obesity. Moreover an accurate anamnesis among the other members of the family were conducted to identify possible familial predisposition to obesity, cardiovascular diseases, hypertension, hypercholesterolemia, endocrine and metabolic disorders. Clinical features such as dysmorphism, striae, acanthosis nigricans, gibbus, knee valgus condition, flat feet were detected. Body height was measured using a standard beam balance to the nearest 0.1 kg while wearing light indoor clothing. Body weight was measured using a stadiometer to the nearest 1 mm. BMI was calculated by standard formula (body weight in kilograms divided by height in meters squared).* It’s important to remember that for children and young people (those aged <18 years), BMI is not a static measurement, but varies from birth to adulthood, and is different between boys and girls. For this reason we calculated also BMI z-score, based on the Center for Disease Control (CDC) growth charts, that seems to be optimal for assessing adiposity on a single occasion (in accordance to international literature, which used a definition of BMI ≥85th centile of reference data for overweight and BMI ≥95th centile of reference data for obesity).8,11

**Plasma samples**

For each patient a blood sample in heparinized syringe was collected. Plasma sample was obtained after centrifugation of whole blood for 10 min. at 1400 rpm. Plasma samples were frozen at -20°C until use.

**Plasmatic adipocytokines and cytokines production**

Adipocytokines production in plasma samples was evaluated with an ELISA assay according to manufacturer instructions. Briefly: microtiter plates were coated with purified monoclonal antibody anti-human IL-6, IL-8, TNF-α (respectively 1 µg/mL for IL-6 and 2 µg/mL for IL-8, IL-12, TNF-α obtained from Endogen-Tema, USA) and TGF-β (2 µg/mL obtained from R&D System, USA). After stabi-lization with 2% BSA (Bovine Serum Albumin, Sigma) in PBS (Euroclone, Italy) for 1h, samples were added. Monoclonal biotin labeled antibody anti-human IL-6, IL-8, IL-12, TNF-α (respectively 0.25 µg/mL, 0.05 µg/mL, 0.25 µg/mL and 0.25 µg/mL Endogen) and detection antibody for TGF-β (300 ng/mL R&D System, USA) were used. Reproducibility and specificity of the assay were verified previously.

**Statistical analysis**

The Shapiro-Wilk’s W test was used to evaluate data distribution. Descriptive results are presented as means and SD or median and interquartile range (25° and 75° percentile). Inferential univariate analysis using either parametric or nonparametric tests were used, as appropriate for the data distribution. Correlations between parameters were evaluated with the Pearson correlation coefficient. P<0.05 was deemed statistically significant and all tests were two-sided. Data analysis was performed with STATA statistical package (Stata Corporation, College Station, TX, USA).

**Results**

Characteristics of the 47 children are summarized in Table 1.

**Table 1. Anthropometric parameters, expressed as mean and standard deviation, in both genders.**

| Total | Boys | Girls |
|-------|------|-------|
| Age, years | 9.95 (SD:3.47) | 10.12 (SD:3.44) | 9.74 (SD:3.54) |
| Weight, kg | 52.80 (SD:19.46) | 55.83 (SD:18.81) | 50.75 (SD:19.96) |
| Height, cm | 138.52 (SD:19.99) | 141.5 (SD:17.75) | 136.50 (SD:21.46) |
| BMI z-score | 2.18 (SD:0.67) | 2.33 (SD:0.84) | 2.07 (SD:0.52) |

Plasmatic adipocytokines and cytokines production

Adipocytokines production in plasma samples was evaluated with an ELISA assay using a competition ELISA kits specific for each adipocytokine: adiponectin (B-Bridge International, Otsuka Pharmaceutical Co., Ltd., Japan), leptin (Diagnostics Biochem, Canada), ghrelin (Phoenix Pharmaceuticals; Inc., USA), visfatin (Phoenix Pharmaceuticals), resistin (Biovendor).

Cytokines production in plasma samples was evaluated with an ELISA assay according to manufacturer instructions. Briefly: microtiter plates were coated with purified monoclonal antibody anti-human IL-6, IL-8, TNF-α (respectively 1 µg/mL for IL-6 and 2 µg/mL for IL-8, IL-12, TNF-α obtained from Endogen-Tema, USA) and TGF-β (2 µg/mL obtained from R&D System, USA). After stabilization with 2% BSA (Bovine Serum Albumin, Sigma) in PBS (Euroclone, Italy) for 1h, samples were added. Monoclonal biotin labeled antibody anti-human IL-6, IL-8, IL-12, TNF-α (respectively 0.25 µg/mL, 0.05 µg/mL, 0.25 µg/mL and 0.25 µg/mL Endogen) and detection antibody for TGF-β (300 ng/mL R&D System, USA) were used. Reproducibility and specificity of the assay were verified previously.

Moreover we considered also pubertal develop-ment, by physical examination according to Tanner staging. In particular, we examined the development of testis and pubic hair for boys and the breast enlargement and pubic hair for girls. We considered in pre-pubertal stage (I Tanner stage) 23 patients (49% of all enrolled patients), 15 girls (65% of pre-pubertal children) and 8 boys (35% of pre-pubertal children). We considered in pubertal stage (II-IV Tanner stage) 24 patients (51% of all enrolled patients), 13 girls (54% of pubertal children) and 11 boys (46% of pubertal children). BMI was significant higher in all pubertal subjects compared to pre-pubertal children (28.14±4.53 vs 24.87±3.41, P<0.05, data not shown). We analyzed correlation between plas-ma adipocytokines levels and clinical variables, such as age and BMI z score. Plasmatic leptin levels were in correlation with age and BMI z score, particularly in girls (respectively r =0.5; P<0.001 and r = 0.36; P<0.001) (Figure 1). Plasma resistin levels were in inverse correlation with age, particularly in boys (r =0.67; P<0.001) and in correlation with BMI z score (r=0.52; P=0.002) (Figure 2).

Plasma adiponectin, leptin, ghrelin levels were higher in girls than in boys, even if not significantly as summarized in Table 2.

There is no difference in plasma leptin, adiponectin, ghrelin, resistin and visfatin levels between pre-pubertal and pubertal children (data not shown).

We also evaluated plasma inflammatory cytokines levels (IL-6, IL-8, TNF-α, TGF-β), but we didn’t observe a significant correlation between their plasma levels and BMI z score, even if expressed with gender specification (Table 2).

We observed an increment in pro-inflam-matory cytokines (IL-6, IL-8, TNF-α) and TGF-β production in pubertal children compared to pre-pubertal children, even if this correlation is not statistically significant (data not shown).
Discussion

The etiology of obesity represents a complex interaction of genetics, diet, metabolism and physical activity levels. Adipocytokines and adipose tissue directly contributes to the pathogenesis of obesity related disorders. Pediatricians should focus preventive efforts on childhood obesity, with its associated pathologic conditions in childhood and likelihood of persistence into adulthood. Obesity runs in family: in our study, about 57% of overweight and obese children had a family history of obesity. We have evaluated plasmatic levels of adipocytokines and cytokines, measured in basal conditions, in function of clinical parameters of obesity, such as sex and body weight.

Leptin has emerged over the past decade as a key hormone involved in the regulation not only of food intake and energy expenditure, but also of neuroendocrine and immune function as well as in the modulation of glucose and fat metabolism. Leptin levels correlate with adiposity: plasmatic leptin levels, that plays a major role in the control of body fat store, within so called brain-gut axis, are so elevated in obese patients to induce leptin-resistance. Leptin levels are closely related to the fat mass and decrease with weight reduction. In our study we observed a linear correlation of plasmatic leptin levels with age and BMI-z score, particularly in girls. Higher leptin levels in obese girls are attributed to a major production of subcutaneous adipose tissue, inhibited by androgen hormones and stimulated by estrogen. Leptin expression may be increased by the actions of several inflammatory cytokines, such as TNF-α and IL-6, as well as by conditions of acute inflammation. In this study no linear correlation was observed between leptin levels and IL-6 or TNF-α (data not shown), probably because all patients are obese.

There is considerable controversy about the role of resistin in humans. Resistin is a protein hormone produced both by adipocytes and immune cells including those invading adipose tissue. Several studies found higher circulating resistin levels and resistin mRNA expression in adipose tissue of obese patients. Circulating levels of resistin should increase in obese humans and resistin seems to act on adipocytes themselves leading to insulin resistance. In our study plasmatic resistin levels were in inverse correlation with age, particularly in boys, probably related to pubertal hormonal production, and in correlation with BMI-z score, as expected.

Adiponectin levels in plasma uniformly decrease in obese patients, conferring a substantially increased risk for obesity comorbidi-

![Figure 1. Correlation between plasma leptin levels and age (months) or BMI z score. In all patients and particularly in girls leptin levels are in correlation with age (respectively, r=0.30 and r=0.5) and BMI z score (respectively, r=0.31 and 0.35).](image1)

| Adipocytokines | Leptin, ng/mL | 48.45 (SD 41.49) |
|---------------|--------------|-----------------|
|               | Boys         | 44.29 (SD 23.62) |
|               | Girls        | 52.42 (SD 51.46) |
| Adiponectin, ng/mL | 17.14 (SD 7.16) | 15.62 (SD 7.19) |
|               | Boys         | 18.41 (SD 7.07) |
|               | Girls        | 16.52 (SD 7.19) |
| Viscatin, ng/mL | 9.08 (SD 3.08) | 9.33 (SD 2.66) |
|               | Boys         | 8.92 (SD 3.32) |
|               | Girls        | 8.92 (SD 3.32) |
| Resistin, ng/mL | 3.01 (SD 1.17) | 3.05 (SD 1.50) |
|               | Boys         | 2.97 (SD 0.85) |
|               | Girls        | 2.67 (SD 0.74) |
| Grelin, ng/mL | 2.20 (SD 0.99) | 1.92 (SD 1.00) |
|               | Boys         | 2.47 (SD 0.74) |
|               | Girls        | 2.47 (SD 0.74) |

**Inflammatory cytokines**

| IL6, pg/mL | 1.12 (IQR: 0.13-4.34) |
|------------|-----------------------|
| Boys       | 2.425 (IQR: 0.62-16.34) |
| Girls      | 0.93 (IQR: 0.05-2.13) |
| TNFα, pg/mL | 0.68 (IQR: 0.13-3.77) |
| Boys       | 0.56 (IQR: 0.09-1.78) |
| Girls      | 0.91 (IQR: 0.67-1.34) |
| IL8, pg/mL | 17.16 (IQR: 13.43-21.69) |
| Boys       | 18.66 (IQR: 13.43-22.76) |
| Girls      | 16.64 (IQR: 13.25-20.83) |

**Table 2. Plasmatic adipocytokines and inflammatory cytokines levels.**

Figure 1.

Figure 2. Correlation between plasma resistin levels and age (months) or BMI z score. In all patients plasma resistin levels are in correlation with age (r=0.42) and BMI z score (r=0.52); in boys resistin levels are in stronger correlation with age (r = -0.67) and BMI z score (r=0.8).
Circulating adiponectin may represent a good biomarker to evaluate the efficacy of lifestyle intervention in overweight/obese children. Adiponectin levels are lower in obese children whereas markers of inflammation and pro-inflammatory cytokines are higher: hypoadiponecemia may contribute to the low-grade systemic chronic inflammatory state associated with childhood obesity. We didn’t observe a significant correlation between plasma adiponectin levels and BMI z score. Plasmatic adiponectin levels were higher in girls than in boys, as previously shown by other authors. Plasma concentration of total adiponectin were also inversely related to age, as Sabin et al. demonstrated, even if not significantly (data not shown).

Visfatin appears to be preferentially produced by visceral adipose tissue, has immunimimetic action and was also identified in inflammatory cells. Visfatin plasma concentrations seem to be positively correlated with the visceral visfatin-mRNA expression and percentage of body fat, but negatively with subcutaneous visfatin mRNA expression, as Berndt et al. suggested. This effect probably becomes evident only in more advanced age: children abdominal fat mass is indeed lower than in adulthood and it’s not able to influence visceral visfatin-mRNA expression. As expected in pediatric patients, we didn’t observe any significant correlation between visfatin plasmatic concentration and BMI z-score.

Ghrelin is implicated in the coordination of energy balance and weight regulation and its regulation seems to be an important factor in the pathogenesis of obesity. Mechanism of appetite and energy metabolism are mediated through hormones, leptin and ghrelin, neuropeptide Y as well as genetic factors. In our study we didn’t find a significant correlation between plasma ghrelin levels and age or gender, as previously shown by Zou et al.

Obesity and correlated metabolic pathologies are associated with a chronic inflammatory response, characterized by abnormal cytokine production. Macrophage infiltration of adipose tissue is characteristic of human obesity. These cells appear to be activated both from a morphologic (giant cells) and functional (cytokine production) standpoint. Circulating levels of TNF-α (cytokine production) are associated with future weight gain. Inflammation to clinical and biochemical parameters linking adipose tissue, inflammation and obesity. Inflammation-sensitive plasma proteins and inflamatory condition? Exp Biol Med 2010;:19:932-7.

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