Retinol-Binding Protein 4 and Lipocalin-2 in Childhood and Adolescent Obesity: When Children Are Not Just “Small Adults”

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BACKGROUND: Although there is much evidence regarding the physiologic and pathogenic roles of the newly described adipokines retinol-binding protein 4 (RBP4) and lipocalin-2 as potential promoters of insulin resistance in obese adults, relatively little information exists regarding their roles in obese children.

METHODS: We investigated the circulating concentrations of RBP4 and lipocalin-2 in 80 obese girls (ages 9–15 years) and their relationships with high-sensitivity C-reactive protein (hs-CRP) and the adipokines leptin and adiponectin. We divided participants by their body mass index standard deviation scores (BMI SDSs) into 4 groups of 20 girls each: overweight [mean BMI SDS (SD), 1.8 (0.4)], obese [2.2 (0.4)], morbidly obese [3.6 (0.4)], and lean controls [0.11 (0.4)]. We measured plasma-soluble RBP4, the RBP4-binding protein transthyretin, lipocalin-2, hs-CRP, leptin, and adiponectin and calculated the homeostatic assessment model (HOMA) index from fasting glucose and insulin concentrations.

RESULTS: Unexpectedly, plasma RBP4 and lipocalin-2 concentrations were correlated negatively with BMI SDS values ($P = 0.005$, and $P = 0.03$, respectively). These results were different from those of adults and were not correlated with the HOMA index. In contrast, hs-CRP and leptin concentrations were positively correlated with BMI SDS values ($P < 0.0001$, and $P < 0.00001$, respectively), as expected, whereas the adiponectin concentration was negatively correlated ($P = 0.008$).

CONCLUSIONS: Although the correlations of leptin, adiponectin, and hs-CRP concentrations with BMI in children are similar to those of adults, the correlations of RBP4 and lipocalin-2 with BMI in children are the inverse of those observed in adults. Thus, although systemic inflammation and mild insulin resistance are present in childhood obesity, RBP4 and lipocalin-2 concentrations are not increased in children as they are in obese adults with long-standing severe insulin resistance and type 2 diabetes.

Adipose tissue is no longer considered merely an energy-storing depot but is now thought to be a metabolically active endocrine organ that secretes many information-carrying molecules, some of which, such as leptin and adiponectin, confer insulin-sensitizing action, whereas others, such as resistin and tumor necrosis factor α, promote insulin resistance (1, 2). Recent animal and human studies have suggested that the soluble form of retinol-binding protein 4 (RBP4),4 initially thought to be only a retinol (vitamin A) transporter, is a major circulating adipokine implicated in systemic insulin resistance (3). RBP4 is a small 21-kDa protein that circulates as an 80-kDa protein complex with transthyretin that is not easily filtered through the kidneys (3).

Increases in the serum concentration of RBP4 have been observed in obese adults with insulin-resistant type 2 diabetes, whereas reductions in the circulating concentrations of this adipokine have been associated with improved insulin action (3, 4). Furthermore, increased RBP4 concentrations have been observed in lean individuals with insulin resistance (4, 5), and regulatory single-nucleotide polymorphisms of the RBP4 gene are associated with insulin resistance in adults (6).
gene have recently been described in Mongolian patients with type 2 diabetes mellitus (6). Another study, however, did not confirm the presence of increased serum RBP4 concentrations in obese postmenopausal women compared with women of normal weight (7). Although it is not yet clear whether RBP4 is the cause and/or the consequence of systemic insulin resistance or is merely a biomarker of the systemic insulin-resistant state (3–7), this protein appears to be an important adipokine implicated in the interplay between obesity and insulin resistance. Two recent studies of adolescent obesity found increased RBP4 concentrations (8, 9), although RBP4 concentration showed no clear correlation with the homeostatic assessment model (HOMA) index in the obese youngsters (8).

Lipocalin-2 belongs to the same protein family as RBP4 and is a known inflammatory biomarker that is positively correlated with body mass index (BMI) and other variables of the metabolic syndrome (10, 11). Lipocalin-2 was found in a recent study to promote insulin resistance, a result resembling findings for RBP4 (12).

We investigated the circulating concentrations of RBP4 and lipocalin-2 in overweight, obese, and morbidly obese female children and adolescents and compared the results with those of lean, healthy age-matched controls. We studied the correlations of concentrations of these adipokines with BMI, markers of inflammation such as high-sensitivity C-reactive protein (hs-CRP), and the concentrations of the well-studied adipokines leptin and adiponectin. We elected to include only girls in this study to control for sexual dimorphism, because the RBP4 concentration, like leptin and adiponectin concentrations, shows sex-related differences (8, 13).

**Participants and Methods**

The children and adolescents included in this study were recruited from the outpatient Obesity Clinic of the Division of Endocrinology, Diabetes and Metabolism of the First Department of Pediatrics of the University of Athens, “Aghia Sophia” Children’s Hospital, Athens, Greece. No participant was receiving any medication, and all individuals were in a good general condition. The categories of overweight and obesity were defined on the basis of BMI (weight in kilograms divided by the square of the height in meters) according to WHO criteria (14) and modified for childhood and adolescent obesity as suggested by Cole et al. (15). We categorized the girls, ages 9–15 years, on the basis of their BMI standard deviation score (SDS) values (z score) into 4 groups of 20 girls each: overweight [mean BMI SDS (SD), 1.8 (0.4)], mildly obese [2.2 (0.4)], and morbidly obese [3.6 (0.4)], and healthy, lean age-matched controls [−0.11 (0.4)] (Table 1). We used the recent Hellenic BMI charts for BMI SDS calculations (16). The Ethics Committee of the “Aghia Sophia” Children’s Hospital approved the study protocol, and girls were included in the study only after informed consent had been obtained from their parents. Table 1 summarizes the participants’ family histories and clinical profiles. All girls underwent a full physical examination, including measurements of weight, height, BMI, arterial blood pressure, and pubertal status.

After the participants had fasted overnight, we withdrew venous blood and measured their fasting glucose and insulin concentrations. The HOMA index was used as a marker of insulin resistance and was calculated as: HOMA Index = [(Fasting Insulin Concentration) × (Fasting Glucose Concentration)]/161, where the insulin concentration is expressed in picomoles per
liter and the glucose concentration is expressed in millimoles per liter (17). We also obtained a lipid profile, which included measurements of total cholesterol, triglycerides, and HDL and LDL cholesterol, for all participants. Finally, we measured RBP4, transthyretin, lipocalin-2, hs-CRP, leptin, and adiponectin concentrations in all individuals.

Serum concentrations of glucose, total cholesterol, triglycerides, and HDL and LDL cholesterol were measured with the Siemens Advia 1650 Clinical Chemistry System (Siemens Healthcare Diagnostics), and serum insulin concentrations were measured via chemiluminescence detection with the automated Siemens ACS180 System Analyzer (Siemens Healthcare Diagnostics).

To measure RBP4 concentrations in serum, we used a sandwich ELISA assay (Immunodiagnostik) identical in protocol and reagent composition with that from ALPCO Diagnostics [as tested by Graham et al. (18)]. Serum samples were diluted so that absorbance measurements fell in the middle of the linearity range for this assay. According to the manufacturer, intraassay and interassay CVs for RBP4 measurements are 5.0% and 9.7%, respectively.

Serum lipocalin-2 concentrations were measured with a solid-phase ELISA (R&D Systems). Intraassay and interassay CVs were 3.1%–4.1% and 5.6%–7.9%, respectively, according to the manufacturer.

Transthyretin and hs-CRP were measured on the BN ProSpec nephelometer (Dade Behring, Siemens Healthcare Diagnostics) with fully automated latex particle–enhanced immunonephelometric assays. The intraassay and interassay CVs were <6% and <7%, respectively. Serum leptin and adiponectin concentrations were measured with sensitive ELISAs from R&D Systems and B-Bridge International, respectively. Intraassay CVs were 3.0%–3.3% for leptin and 3.3%–5.8% for adiponectin; interassay CVs were 3.5%–5.4% for leptin and 3.2%–7.3% for adiponectin.

### Statistical Analysis

Differences between groups were evaluated with the Mann–Whitney U-test. The Spearman rank correlation test was used to examine the relationships between various variables. All P values are the results of 2-sided tests. Statistical analyses were performed with the STATGRAPHICS PLUS version 5.1 for Windows (Statpoint).

### Results

Table 2 summarizes the results for fasting glucose and insulin concentrations, the HOMA index, lipid variables, and RBP4, transthyretin, lipocalin-2, hs-CRP, leptin, and adiponectin concentrations for the 4 BMI groups.

<table>
<thead>
<tr>
<th>Table 2. Fasting serum glucose and insulin concentrations, HOMA index values, lipid profiles, and circulating concentrations of RBP4, transthyretin, lipocalin-2, hs-CRP, leptin, and adiponectin in the 4 BMI groups.a</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fasting glucose, mmol/L</strong></td>
</tr>
<tr>
<td>Controls</td>
</tr>
<tr>
<td>---------------------------------------------------------------</td>
</tr>
<tr>
<td>4.66 (0.11)</td>
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<tr>
<td>---------------------------------------------------------------</td>
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<tr>
<td>23.3 (4.6)</td>
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* Data are presented as the mean (SD). Statistical results are for comparisons with the control group. *P < 0.05; **P < 0.01; ***P < 0.001.

b HDL-C, HDL cholesterol; LDL-C, LDL cholesterol.
lesterol concentrations in the 4 groups were similar, triglyceride concentrations were significantly higher, and HDL cholesterol concentrations were significantly lower in the obese and morbidly obese groups, compared with the healthy controls (Table 2).

The lean and overweight groups were not significantly different with respect to RBP4 concentration (P > 0.21), nor were the lean and obese groups (P > 0.97). RBP4 concentrations in the morbidly obese group, however, were significantly decreased compared with the obese, overweight, and control groups (P < 0.004, P < 0.0001, and P < 0.002, respectively; Table 2).

Transthyretin concentrations in the obese and morbidly obese groups were not significantly different from those in the lean control group (P > 0.06, and P > 0.72, respectively; Table 2).

Both hs-CRP and leptin concentrations were significantly increased in the overweight/obese groups compared with the controls (P < 0.01), whereas adiponectin concentrations in the obese and morbidly obese groups were significantly decreased (P < 0.003, and P < 0.006, respectively).

An evaluation of the correlation of BMI SDS with all of the measured variables revealed that both RBP4 and lipocalin-2 concentrations were negatively correlated with the BMI SDS [r = −0.350 (P < 0.0005), and r = −0.239 (P < 0.03), respectively]. More specifically, a consideration of the impact of menarche on the RBP4 concentration revealed the RBP4 concentration not to be significantly correlated with BMI SDS in girls before menarche (r = −0.30; P > 0.08), whereas RBP4 concentration and BMI SDS showed a statistically significant negative correlation after menarche (r = −0.35; P < 0.03). hs-CRP and leptin concentrations were positively correlated with BMI-SDS values [r = 0.552 (P < 0.0001), and r = 0.847 (P < 0.00001), respectively], whereas adiponectin concentrations were negatively correlated (r = −0.296; P < 0.008) (Fig. 1).

Discussion

Both RBP4 and lipocalin-2 concentrations were significantly negatively correlated with the BMI SDS in children and adolescents. Additionally, hs-CRP and leptin concentrations were positively correlated with the BMI SDS, whereas the adiponectin concentration was negatively correlated. Similar studies of RBP4 and lipocalin-2 in obese adults, however, have yielded opposite results; that is, RBP4 and lipocalin-2 concentrations were positively correlated with BMI (3–12). On the other hand, the correlations of the hs-CRP, leptin, and adiponectin concentrations with BMI in this study were similar to the results found in previous studies of both adult and pediatric populations (2, 19–23).

RBP4 has recently been proposed to be the circulating adipokine that confers systemic insulin resistance to skeletal muscle and liver in obese adults (3–5, 24). In mice and in some human studies (3, 4), RBP4 was found to be negatively correlated with GLUT4 production in adipose tissue (3, 4, 24–26), although other studies of humans by Janke et al. (7) found a robust positive correlation between RBP4 and GLUT4 concentrations that was completely independent of confounding variables. This finding has subsequently been confirmed at the mRNA level in human adipocytes (27) and challenges the assumption of the unequivocal accuracy of extrapolating findings from murine models to human pathophysiology (28).

Quantitative western blotting standardized to the full-length RBP4 protein is generally accepted as the gold standard method for measuring RBP4 (18), although the various commercially available kits for RBP4 may perform differently. In the present study, we measured the serum RBP4 concentration with an Immunodiagnostik sandwich ELISA that is identical in protocol and reagent composition with the ALPCO Diagnostics ELISA validated by Graham et al. (4, 18). Serum concentrations of RBP4 are lower in females than in males (8, 13), and postmenopausal obese women do not demonstrate the same increases in RBP4 concentration observed in premenopausal obese women (7). To circumvent the known sex-related differences in RBP4 concentration and to study the impact of sex steroids and menarche on RBP4 concentration, we included only girls in this study and further subdivided our cohort into pre- and postmenarche populations.

We found no significant correlation between RBP4 concentration and the BMI SDS in premenarche girls (r = −0.30; P > 0.08), whereas the correlation was significant after menarche (r = −0.35; P < 0.03), confirming that menarche may influence the correlation between RBP4 concentration and BMI.

Treatment of adult mice with fenretinide, a drug that disrupts the interaction of RBP4 with transthyretin and causes increased renal RBP4 excretion, was found to lead to enhanced insulin sensitivity. Although whether fenretinide also has a beneficial effect in humans is not yet clear, the RBP4–transthyretin interaction has been proposed as a new target for the development of drugs for combating insulin resistance (3, 5, 29). In our study, we hypothesized that reduced transthyretin concentrations might have led to increased renal clearance of RBP4 and thereby produce the lower RBP4 concentrations in our morbidly obese group (29, 30); however, we observed no significant reductions in transthyretin concentration in our obese and morbidly obese participants compared with the lean control individuals (P > 0.06, and P > 0.072, respectively).
RBP4 concentration is positively correlated with BMI and insulin resistance in obese adults, and increases in the RBP4 concentration precede the development of frank diabetes (4, 13). Moreover, RBP4 concentrations are increased in healthy nonobese individuals who are genetically susceptible to diabetes because of having at least one first-degree relative with type 2 diabetes (4). One may therefore argue that if the girls in our lean group had a higher incidence of first-degree relatives with type 2 diabetes, they would be genetically more susceptible to developing diabetes and thus would have higher RBP4 concentrations; however, the incidence of first-degree relatives with type 2 diabetes was lower in our lean, overweight, and obese groups and higher in the morbidly obese group, which had the lowest RBP4 concentrations.

In this study, all the children in all BMI categories had nonpathologic fasting glucose concentrations, a
finding that excluded any severe glucose intolerance at this period of their lives. In new-onset type 1 diabetes, serum RBP4 concentrations are reduced and return to typical concentrations after insulin treatment (4, 31); however, the enhanced osmotic diuresis in such patients because of hyperglycemia and the resulting polyuria might lead to renal loss of RBP4, a phenomenon that is reversed with insulin treatment. This explanation cannot account for the reduced RBP4 values found in our morbidly obese participants, who had neither hyperglycemia nor enhanced diuresis.

In our cohort, insulin resistance, as expressed in the HOMA index, was significantly increased in the obese and morbidly obese individuals compared with the lean control individuals (P < 0.04, and P < 0.02, respectively). This relationship is similar to that found in adult populations (4, 13). Irrespective of BMI category, however, we found no significant correlation between RBP4 concentration and the HOMA index, the fasting insulin concentration, or the fasting glucose concentration, suggesting that RBP4 may not be a good marker of insulin resistance at young ages. Some earlier studies reported a positive correlation between RBP4 concentration and the HOMA index in adults (4, 32), whereas others have failed to find such a correlation, challenging the role of an increased RBP4 concentration in insulin resistance (7, 33). Lee et al. (8) found the RBP4 concentration to be positively correlated with the HOMA index in nonobese adolescents, but not in obese adolescents. This finding is in accord with our conclusion that RBP4 concentration may not be a good predictor of insulin resistance in young age groups. Another study of the RBP4 concentration in obese adolescents included a small number participants of both sexes (4 males and 3 females), but because RBP4 concentrations show sex-related differences, drawing accurate conclusions from the results of such a small cohort is difficult (9). We conclude that robust data on RBP4 concentration in adolescent populations are sparse (8, 9) and that no clear-cut correlations with insulin resistance have been shown.

We found no correlation in our cohort between the concentration of circulating RBP4 and the leptin or adiponectin concentration in any group, although such correlations have been described for adults (33). We found a negative correlation between lipocalin-2 concentration and BMI similar to that found between RBP4 concentration and BMI. Our data and analyses show that lipocalin-2 and RBP4 are markedly similar on all counts. Studies of these 2 adipokines in different populations and health states are definitely needed to obtain a better functional profile of these highly related proteins.

In conclusion, whereas hs-CRP, leptin, and adiponectin demonstrated relationships with BMI in children that were similar to those observed in adults, the concentrations of RBP4 and lipocalin-2 in these young individuals appeared to have relationships with BMI that were the inverse of those found in adults, suggesting the existence of substantial physiological differences between children and adults. It is also possible that the discrepancies between the findings for the children in our study and those for adults in previously published studies may also indicate that an increased RBP4 concentration is simply a consequence of advancing age, a long-standing period of insulin resistance, or even type 2 diabetes. Although subtle insulin resistance, as indicated by the HOMA index, and systemic inflammation, as indicated by an increased hs-CRP concentration, are already present in childhood obesity, they may not have been present for a sufficiently long time to lead to an increase in the RBP4 or lipocalin-2 concentration.

Therefore, if increases in RBP4 and lipocalin-2 concentrations are causally related to severe insulin resistance, given that temporality (i.e., a cause preceding an effect) may be a prerequisite for causality as suggested by van Dam et al. (11), then prospective studies that assess these biomarkers are critical. Of utmost importance, therefore, will be to study young obese and morbidly obese individuals longitudinally to determine the critical time after RBP4 and lipocalin-2 concentrations have increased that these factors become coupled to severe insulin resistance and, ultimately, to glucose intolerance and type 2 diabetes mellitus.

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**References**

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