Fibroblast Growth Factor 21 and Fetuin-A in Obese Adolescents With and Without Type 2 Diabetes

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Context: Hepatokines such as fetuin-A or fibroblast growth factor 21 (FGF21) are reasonable candidates affecting the pathophysiology of type 2 diabetes mellitus (T2DM). However, studies in humans at the onset of disease are scarce.

Objective: The objective of the study was to compare FGF21 and fetuin-A levels between adolescents with and without T2DM.

Design: This was a cross-sectional comparison of adolescents with and without T2DM.

Setting: The study was conducted at diabetes and obesity treatment centers.

Patients: Seventy-four predominantly Caucasian adolescents with T2DM aged 12–18 years and 74 body mass index (BMI)-, age-, and gender-matched controls participated in the study.

Intervention: There were no interventions.

Main Outcome Measures: FGF21 and fetuin-A and their correlation to age, BMI, glycated hemoglobin, blood pressure, lipids, adiponectin, and leptin were measured.

Results: Adolescents with T2DM showed significant higher FGF21 serum concentrations compared with obese controls without T2DM [median 277 pg/mL (interquartile range [IQR] 161–586) vs 200 pg/mL (IQR 116–323), respectively, P = .009] and higher fetuin-A serum concentrations (median 0.30 g/L (IQR 0.27–0.33) vs 0.28 g/L (IQR 0.25–0.30), respectively, P = .005). In a multiple linear regression analysis, fetuin-A was positively associated with glycated hemoglobin [β-coefficient 0.005 (95% confidence interval ± 0.004), P = .013], negatively with adiponectin (β-coefficient –0.004 (95% confidence interval ± 0.002, P = .006) but not with BMI, age, gender, ethnicity, or leptin. FGF21 was not associated with any parameter in multiple linear regression analysis.

Conclusions: Increased FGF21 serum levels in obese adolescents with T2DM compared with obese adolescents without T2DM suggest a FGF21-resistant state in T2DM because FGF21 improves insulin sensitivity. The increase of fetuin-A levels in obese adolescents with T2DM supports the hypothesis that fetuin-A is involved in the pathogenesis of T2DM because this hepatokine leads to insulin resistance. (J Clin Endocrinol Metab 100: 3004–3010, 2015)
Type 2 diabetes mellitus (T2DM), a chronic debilitating disease, results when insulin resistance develops in association with dysregulated insulin secretion and loss of β-cell mass (1). Numerous adipose tissue-derived hormones have been shown to predict T2DM and to be involved in the pathogenesis of T2DM. For example, we have recently reported that leptin levels but not adiponectin levels differ between obese adolescents with and without T2DM (2). Moreover, recent data revealed increasing evidence that liver-derived hormones also might affect glucose metabolism. Among these hepatokines, fibroblast growth factor-21 (FGF21) and fetuin-A have received increasing attention (3).

FGF21 is mainly produced by the liver but also by other tissues including white adipose tissue, skeletal muscle, and pancreatic β-cells (4). FGF21 signaling requires the FGF receptor and the adapter molecule β-klotho (5), which targets FGF21 primarily to the liver itself but also to pancreas and adipose tissue. FGF21 induces glucose uptake and decreases glucose concentrations in obese mice (6) and diabetic monkeys (7). Furthermore, FGF21 potentially regulates insulin secretion in humans (8). Therefore, FGF21 is suggested to be a reasonable candidate directly affecting the pathophysiology of T2DM (3). Because human studies observed increased circulating FGF21 levels in insulin resistance, impaired glucose tolerance, and incident diabetes (9–11), T2DM has been suggested as a state of FGF21 resistance (12). However, because obesity per se is associated with increased FGF21 levels both in adults (10, 13, 14) and children (15), it is unclear whether the increase of FGF21 measured in T2DM is caused by obesity and/or is involved in the pathogenesis of T2DM. Most studies of FGF21 in T2DM have used lean controls (9–11). Therefore, the question remains whether FGF21 is related to obesity, T2DM, or both.

Another protein secreted by the liver, fetuin-A, was recently proposed as a link between obesity, nonalcoholic fatty liver disease (NAFLD), and diabetes (16). Fetuin-A is exclusively expressed in the liver in humans except for the tongue and placenta (3). Animal studies have shown that fetuin-A inhibits insulin receptor tyrosine kinase activity in muscles and in the liver (17, 18). Furthermore, fetuin-A induces cytokine expression and low-grade inflammation (19). The human fetuin-A gene resides on chromosome 3q27, which has been mapped as a T2DM locus (20). Higher fetuin-A concentrations were associated with manifest T2DM in humans (21, 22) and in women with gestational diabetes mellitus (23). Furthermore, fetuin-A has been reported to predict T2DM in adults (16, 24).

Because studies concerning hepatokines such as fetuin-A or FGF21 are missing so far in adolescents with T2DM, we compared fetuin-A and FGF21 concentrations between adolescents with T2DM and age-, gender-, and body mass index (BMI)-matched adolescents without T2DM. Studies in adolescents seem preferable because analyses in this age range address the onset of T2DM. Furthermore, studies in adolescents have the advantage that there is no potential interaction with other diseases and medications. We hypothesized that fetuin-A and FGF21 concentrations are higher in adolescents with T2DM as compared with matched obese controls, suggesting a FGF21-resistant state and increased fetuin-A concentrations as causes of insulin resistance in the pathogenesis of T2DM.

Subjects and Methods

Subjects

Written informed consent was obtained from all children and their parents. The study was approved by the local ethics committee of the University of Witten/Herdecke (Datteln, Germany) and the University of Ulm (Ulm, Germany) for the pediatric Bundesministerium für Bildung und Forschung diabetes biobank.

We examined 74 obese adolescents with T2DM and 74 age-, gender-, and BMI-matched obese adolescents without diabetes. Adolescents with T2DM were recruited from the DPV data set (for details of this cohort see references 2 and 25). Briefly, T2DM was diagnosed based on the criteria of the American Diabetes Association identical to German guidelines (26). In none of the adolescents with T2DM were autoantibodies detectable, and maturity onset of diabetes in youth was excluded by genetic analyses (25). The obese adolescents without T2DM were recruited from the obesity cohort at the outpatient obesity clinic of the Vestische Children’s Hospital, University of Witten/Herdecke (for details see reference 2). Diabetes mellitus was excluded in these adolescents by normal glycated hemoglobin (HbA1c) levels and oral glucose tolerance tests.

None of the adolescents in the current study suffered from endocrine disorders or syndromal obesity. Details of excluding these diseases were published in details elsewhere (2, 25).

Anthropometric measurements

Height was measured to the nearest centimeter using a rigid stadiometer. Weight was measured unclothed in underwear to the nearest 0.1 kg using a calibrated balance scale. BMI was calculated as weight in kilograms divided by the square of height in meters. The degree of overweight was quantified using Cole’s method, which normalized the BMI skewed distribution and expressed BMI as a SD score (BMI-SDS) (27). Reference data for German children were used (28).

Blood pressure was measured using a validated protocol (29). Systolic and diastolic blood pressure (BP) were measured at the right arm twice after a 10-minute rest in the supine position by using a calibrated sphygmomanometer and averaged. The cuff size was based on the length and circumference of the upper arm and was as large as possible without having the elbow skin crease obstructing the stethoscope (29).
**Biochemical measurements**

After clotting, blood samples were centrifuged for 10 minutes at 8000 rpm. Serum was stored at −81°C for later determination of adiponectin, leptin, fetuin-A, and FGF21. All samples were thawed only once. Fetuin-A, FGF21, leptin, and adiponectin concentrations were analyzed in one central laboratory. Serum FGF21 levels were measured with a highly specific commercially available ELISA kit (BioVendor). No cross-reactivity with human FGF19 and human FGF23 has been observed. The sensitivity of the assay was 7 pg/mL, the intraassay coefficient of variation (CV) and the interassay CV were below 5%. Serum fetuin-A concentrations were measured by an ELISA kit (BioVendor Laboratory Medicine). The antibodies are highly specific for the human fetuin-A protein. The assay uses a two-sided sandwich technique with two selected polyclonal antibodies that bind to different epitopes of human fetuin-A. The intra- and interassay CVs were less than 10%. Serum adiponectin was determined by an ELISA (human adiponectin kit, Mediagnost; the intraassay CV was < 5%; the interassay CV was < 7%, sensitivity was 0.6 ng/mL). Serum leptin was measured by an ELISA (human leptin kit; Mediagnost; the intraassay CV was < 5%; the interassay CV was < 7%, sensitivity was 0.2 ng/mL).

HbA1c, low-density lipoprotein (LDL)- and high-density lipoprotein (HDL)-cholesterol, aspartattransaminases (AST), and alanintransaminases (ALT) were determined by commercially available test kits in the participating centers. Intra- and interassay CVs were less than 5% in all these methods. HbA1c was measured by an ELISA kit (BioVendor). No cross-reactivity with human FGF19 and human FGF23 has been observed. The sensitivity of the assay was 7 mg/dL, the intraassay coefficient of variation (CV) and the interassay CV were below 5%. Serum fetuin-A concentrations were measured by an ELISA kit (BioVendor Laboratory Medicine). The antibodies are highly specific for the human fetuin-A protein. The assay uses a two-sided sandwich technique with two selected polyclonal antibodies that bind to different epitopes of human fetuin-A. The intra- and interassay CVs were less than 10%. Serum adiponectin was determined by an ELISA (human adiponectin kit, Mediagnost; the intraassay CV was < 5%; the interassay CV was < 7%, sensitivity was 0.6 ng/mL). Serum leptin was measured by an ELISA (human leptin kit; Mediagnost; the intraassay CV was < 5%; the interassay CV was < 7%, sensitivity was 0.2 ng/mL).

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**Results**

The patient characteristics are demonstrated in Table 1. Adolescents without T2DM showed mean fasting glucose levels of 89 ± 7 mg/dL and 2-hour glucose levels in the oral glucose tolerance test of 118 ± 28 mg/dL.

Adolescents with T2DM were treated by lifestyle intervention only (n = 24), metformin (n = 23), insulin (n = 9), or the combination of insulin and metformin (n = 18).

Patients with T2DM demonstrated significantly lower HDL-cholesterol and leptin levels and higher systolic BP and transaminases as compared with the age-, gender-, and BMI-matched obese adolescents without T2DM (see Table 1).

Adolescents with T2DM showed significantly higher FGF21 and fetuin-A levels as compared with the age-, gender-, and BMI-matched obese adolescents without T2DM (see Figures 1 and 2).

**Table 1.** Anthropometrics, Cardiovascular Risk Factors, and Leptin, Adiponectin, Transaminases, and HbA1c Levels in 74 Obese Adolescents With T2DM and 74 Obese Age-, Gender-, and BMI-Matched Adolescents Without T2DM

<table>
<thead>
<tr>
<th>Variable</th>
<th>Obese Adolescents With T2DM</th>
<th>Obese Adolescents Without T2DM</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>15.5 ± 0.2</td>
<td>15.2 ± 0.2</td>
<td>.179</td>
</tr>
<tr>
<td>Gender</td>
<td>58% male</td>
<td>58% male</td>
<td>.999</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>32.0 ± 7.1</td>
<td>32.2 ± 6.4</td>
<td>.437</td>
</tr>
<tr>
<td>BMI-SDS</td>
<td>2.40 ± 0.90</td>
<td>2.54 ± 0.62</td>
<td>.293</td>
</tr>
<tr>
<td>Ethnicity</td>
<td>95% Caucasian</td>
<td>97% Caucasian</td>
<td>.406</td>
</tr>
<tr>
<td>Systolic BP, mm Hg</td>
<td>129 ± 12</td>
<td>123 ± 21</td>
<td>.020</td>
</tr>
<tr>
<td>Diastolic BP, mm Hg</td>
<td>73 ± 9</td>
<td>73 ± 12</td>
<td>.815</td>
</tr>
<tr>
<td>HDL cholesterol, mg/dL</td>
<td>42 ± 11</td>
<td>48 ± 11</td>
<td>.004</td>
</tr>
<tr>
<td>LDL cholesterol, mg/dL</td>
<td>113 ± 33</td>
<td>108 ± 34</td>
<td>.450</td>
</tr>
<tr>
<td>HbA1c, %</td>
<td>7.2 ± 2.0</td>
<td>5.5 ± 0.4</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>HbA1c, mmol/mL</td>
<td>54.2 ± 24.3</td>
<td>36.4 ± 4.8</td>
<td>.408</td>
</tr>
<tr>
<td>AST, U/L</td>
<td>28 (IQR 21–42)</td>
<td>25 (IQR 21–30)</td>
<td>.048</td>
</tr>
<tr>
<td>ALT, U/L</td>
<td>34 (IQR 19–60)</td>
<td>25 (IQR 19–35)</td>
<td>.056</td>
</tr>
<tr>
<td>Leptin, ng/mL</td>
<td>18 ± 12</td>
<td>37 ± 23</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Adiponectin, µg/mL</td>
<td>5.0 ± 2.5</td>
<td>4.9 ± 2.5</td>
<td>.833</td>
</tr>
<tr>
<td>Age at diabetes manifestation, y</td>
<td>12.8 ± 2.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duration of diabetes, y</td>
<td>2.8 ± 2.3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data represent percentage, mean and SD, or median and IQR.
We found no sex-related differences regarding FGF21 or fetuin-A: the FGF21 levels did not differ (P = 0.642) between the 86 boys [median 212 pg/mL (IQR 141–530)] and 62 girls [median 228 pg/mL (IQR 136–436)]. In addition, the fetuin-A concentrations did not differ (P = 0.189) between the 86 boys (mean ± SD 0.29 ± 0.04 g/L) and the 62 girls (mean 0.28 ± 0.04 g/L). Separating the adolescents to with and without suffering from T2DM revealed the same findings (data not shown).

The associations between fetuin-A and FGF21, respectively, on the one hand and weight status, cardiovascular risk factors, leptin, adiponectin, and HbA1c on the other hand are demonstrated in Table 2. FGF21 and fetuin-A were significantly related to HbA1c. Fetuin-A but not FGF21 was negatively associated with adiponectin, and positively with ALT.

In a multiple linear regression analysis, log-transformed FGF21 was not significantly associated with BMI, age, gender, ethnicity, leptin, adiponectin, or HbA1c. Fetuin-A was positively associated with HbA1c [β-coefficient 0.005 (95% confidence interval [CI] ± 0.004), P = .013] and negatively with adiponectin [β-coefficient −0.004 (95%CI ± 0.002), P = .006] but not with BMI, age, gender, ethnicity, or leptin in multiple linear regression analysis.

Fetuin-A levels did not differ (P = 0.747) between the adolescents with T2DM treated with metformin [median 216 pg/mL (IQR 162–1077)], insulin [median 260 pg/mL (IQR 105–1735)], the combination of metformin and insulin [median 307 pg/mL (IQR 197–563)], or solely lifestyle intervention [median 263 pg/mL (IQR 145–457)].

Calculating multiple linear regression analyses in the subgroup of adolescents with T2DM including drug treatment as independent variable demonstrated that FGF21 (P = .126) and fetuin-A (P = .663) were not significantly related to medication. FGF21 was not significantly related to any variable in this model. Fetuin-A was negatively associated with adiponectin [β-coefficient −0.006 (95% CI ± 0.004), P = .006], whereas a significant relationship to HbA1c was not detectable (P = .284).

Discussion

To the best of our knowledge, this is the first study at the onset of T2DM comparing fetuin-A and FGF21 levels be-
tween obese adolescents with and without T2DM. According to our hypotheses, FGF21 and fetuin-A levels were significantly higher in obese adolescents with T2DM compared with BMI-, age-, and gender-matched adolescents without T2DM. These relationships were also not related to the adipokytokine leptin, suggesting an independent role of hepatokines in the early pathogenesis of T2DM in obese adolescents.

The observed increased FGF21 levels in obese adolescents with T2DM point toward a FGF21-resistant state in the early stage of T2DM (12) because FGF21 has many glucose-lowering properties: FGF21 induces glucose uptake and decreases glucose concentrations in obese animals and increases insulin-independent glucose uptake in vitro in adipocytes, thereby improving insulin resistance (6, 10, 30). Furthermore, FGF21 potentially regulates insulin secretion in humans (8). Consistent with the hypothesis of a FGF21-resistant state, one recent study reported that acute continuous infusion of FGF21 to control mice leads to reduced hepatic glucose output and increased insulin sensitivity while having no effect in obese mice (31).

Interestingly, despite high endogenous levels in obese mice, exogenous FGF21 administered at pharmacological doses appears to improve metabolic parameters and induces weight loss (32). A metabolic state in which high endogenous levels of a physiological regulator appear to be ineffective but in which high pharmacological doses induce the expected results suggests a state of hormone resistance (12). In this line, a pharmacological variant of FGF21 showed a significant trend toward glucose lowering in a randomized, placebo-controlled, double-blind, proof-of-concept trial in patients with T2DM (33).

FGF21 resistance can be mediated through the altered expression of both the FGF receptor and the adapter molecule β-klotho (5). Islets of diabetic and healthy mice treated with high glucose ex vivo displayed reduced β-klotho expression, resistance to FGF21, and decreased peroxisome proliferator-activated receptor-γ expression (30). These data indicate that hyperglycemia in T2DM may lead to FGF21 resistance in pancreatic islets, probably through the reduction of peroxisome proliferator-activated receptor-γ expression, which provides a novel mechanism for glucose-mediated islet dysfunction (30). Accordingly, FGF21 was significantly related to HbA1c in a univariate analysis in our study. However, in a multiple linear regression model accounting for multiple confounders, this significant relationship got lost. Furthermore, serum FGF21 levels were reported to be decreased in type 1 diabetes mellitus (T1DM) (11) pointing against a role of hyperglycemia in the pathogenesis of increased FGF21 levels. However, a comparison between adolescents with T1DM and adolescents with T2DM matched to age, gender, and degree of overweight would be ideal to analyze whether hyperglycemia is a main predictor of FGF21 levels.

The increase of FGF21 in T2DM is not only attributed to obesity as previous studies suggest (10, 13–15, 34), but also in our study obese adolescents with T2DM showed significantly higher FGF21 levels than age-, gender-, and BMI-matched obese adolescents without T2DM. Because a decrease of FGF21 concentrations was reported in obese children and adults with weight loss (15, 34), these findings point toward a reversibility of increased FGF21 levels in the early diabetes stage when weight loss can be achieved. This scenario is comparable with hyperinsulinemia in T2DM.

In our study, fetuin-A serum levels were significantly higher in obese adolescents with T2DM compared with serum concentrations in age-, gender-, and BMI-matched adolescents without T2DM. This finding is in line with adult studies demonstrating that higher fetuin-A concentrations were associated with T2DM and insulin resistance in middle-aged and elderly Chinese adults (21), that serum levels of fetuin A are increased in women with gestational diabetes mellitus (23), and that serum fetuin-A concentrations predict T2DM (16, 22, 24). Because prior studies in animals suggest that fetuin-A interferes with insulin action at peripheral tissues through its interaction with insulin receptor (17, 18) and fetuin-A knockout mice have enhanced glucose clearance and insulin sensitivity (18), these findings suggest a pathogenetic role of increased fetuin-A in the development of T2DM. Furthermore, fetuin-A was demonstrated to induce low-grade inflammation, which is also associated with insulin resistance (19). Additionally, Hennige et al (19) demonstrated that fetuin-A represses adiponectin production in animals and humans. In our study, fetuin-A was also significantly and negatively associated with adiponectin. In contrast, a study in adults reported that adiponectin and fetuin-A independently of each other associate with the diabetes risk and that they are involved in the development of T2DM via different mechanisms (35). Both in vivo and in vitro experiments confirm that fetuin-A is not regulated by leptin (36). Accordingly, fetuin-A was not significantly related to leptin in our study.

One might speculate that hyperglycemia per se is associated with increased fetuin-A levels because fetuin-A was significantly related to HbA1c. Indeed, fetuin-A levels were also increased in children with T1DM and associated with the duration of disease (37). However, in our study fetuin-A was not associated with HbA1c if only adolescents with T2DM were analyzed, pointing against a relevant relationship between fetuin-A and hyperglycemia. A comparison between adolescents with T1DM and adoles-
cents with T2DM matched to age, gender, and degree of overweight would be ideal to analyze whether insulin resistance or quality of diabetes control is the main predictor of fetuin-A levels.

Interestingly, there is a strong relationship between NAFLD and T2DM, which has been suggested to be mediated by hepatokines (3, 16, 35). Accordingly, we found higher transaminases serum levels in obese adolescents with T2DM compared with obese adolescents without T2DM. Although a relationship between FGF21 and NAFLD has been reported in adults (14), there was no significant relationship between FGF21 and transaminases as markers of NAFLD in our study in concordance with a previous study in children (15). In contrast, fetuin-A correlated significantly to ALT according to a previous study in obese children (38). Higher fetuin-A levels have been also reported in adults with NAFLD (3, 16). Stefan and Haring (3) and Stefan et al (16) found that fetuin-A expression was significantly elevated in mice with fatty liver.

The strengths of our study are the large cohort of mostly Caucasian adolescents with proven T2DM by genetic exclusion of maturity onset of diabetes in youth diabetes and exclusion of autoimmune bodies, the short diabetes duration, and an age-, gender-, and BMI-matched control group. However, our study has a few potential limitations. First, BMI percentiles were used to classify overweight. Although BMI is a good measure for overweight, one needs to be aware of its limitation as an indirect measure of fat mass. Second, we have no data concerning pubertal stage, whereas it is well known that, for example, adiponectin and leptin levels depend on pubertal stage and especially entry into puberty is associated with a change of these adipokines. However, fetuin-A and FGF21 are independent of pubertal stage (15, 38). Furthermore, the mean age of our cohort was older than 15 years when the great majority of girls and most boys are at a late or postpubertal stage. Third, we have only indirect parameters of insulin resistance such as HDL-cholesterol and lipids but no fasting insulin levels and corresponding glucose levels. Fourth, the cross-sectional data limits our ability to definitively identify which factors and pathways account for T2DM, and the case-control design has an inherent limitation of potential selection bias. Fifth, we cannot rule out an effect of antidiabetic treatment on adipokines or hepatokines. For example, metformin has been reported to decrease fetuin-A levels (39). However, we did not find significant relationships between medications and FGF21 or fetuin-A levels in our study. Sixth, we have not performed liver biopsy for diagnosis of NAFLD, whereas it is known that transaminases are only a poor marker of the severity of NAFLD. Liver biopsies in children are difficult to perform and also for ethical reasons because no specific therapy follows up histological diagnosis of NAFLD apart from recommending reduction of overweight, which is generally advised to all obese children. Finally, recent studies have suggested that FGF21 concentrations are affected by the glomerular filtration rate and therefore may be related to renal function (40). Although none of the participants enrolled in our study had impaired renal function, glomerular filtration rate spanned a wide range.

In summary, this is the first study analyzing hepatokines in a relative large cohort of obese adolescents with T2DM and age-, gender-, and BMI-matched controls. The higher FGF21 levels in obese adolescents with T2DM compared with adolescents without T2DM points toward a FGF21-resistant state in T2DM because FGF21 improves glucose metabolism in animal models. On the other hand, the higher fetuin-A levels in obese adolescents with T2DM compared with adolescents without T2DM suggest that this hepatokine is involved in the pathogenesis of T2DM because fetuin-A increases insulin resistance. Future longitudinal studies in humans are necessary to prove these hypotheses.

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This study is registered at clinicaltrials.gov with the identifier of NCT00435734.

Authors contributions: T.R., J.W., and R.W.H. developed the study design. All authors discussed the findings. Thomas Reinehr wrote the first draft of the paper.

The hypothesis development, analysis, interpretation, and conclusions contained in this study are those of the author’s alone and not any of the supporters.

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