Inflammatory Markers in Obese Adolescents with Type 2 Diabetes and Their Relationship to Hepatokines and Adipokines

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Objectives To analyze inflammatory markers, adipokines, and hepatokines in obese adolescents with and without type 2 diabetes mellitus (T2DM).

Study design We studied high sensitivity C-reactive protein (hsCRP), tumor necrosis factor (TNF)-α, interleukin-1β, and interferon-γ, the hepatokines (fetuin-A and fibroblast growth factor [FGF]-21), and the adipokines (adiponectin and leptin) in a cross-sectional study of 74 predominately Caucasian adolescents with T2DM aged 12-18 years and in 74 body mass index (BMI)-, age–, and sex-matched controls.

Results Adolescents with T2DM had significantly higher concentrations of hsCRP, TNF-α, and interleukin-1β compared with obese controls without T2DM. Interferon-γ was not detectable in obese adolescents with or without T2DM. In multiple linear regression analysis, hsCRP was significantly associated with FGF-21 and BMI, but not with hemoglobin A1c, adiponectin, leptin, fetuin-A, sex, or age. TNF-α was significantly related negatively to leptin, positively to BMI, but not to hemoglobin A1c, adiponectin, fetuin-A, FGF-21 sex, or age in multiple linear regression analysis.

Conclusions Increased inflammatory markers are associated with T2DM in adolescents. Because hsCRP was related to FGF-21 and TNF-α was associated with leptin, these findings suggest a link between increased levels of these adipokines and hepatokines and chronic inflammation. Future longitudinal studies in humans are necessary to confirm these hypotheses. (J Pediatr 2016;173:131-5.)

Type 2 diabetes mellitus (T2DM), a chronic metabolic disease, results from insulin resistance in association with dysregulated insulin secretion and loss of β-cell mass. It is now widely accepted that chronic inflammation plays an important role in the development of T2DM even if the mechanisms are not fully understood. In animal studies, inflammatory stress promoted insulin resistance. In line with these animal studies, epidemiologic studies in humans demonstrated an increase of inflammatory markers such as C-reactive protein (CRP) and tumor necrosis factor (TNF)-α in obese patients with T2DM.

Apart from a direct link between chronic inflammation and insulin resistance, the involvement of hepatokines and adipokines in the pathogenesis in T2DM has also been discussed. Adipose and hepatic tissues act as inflammatory immune tissue leading to chronic inflammatory states in obese humans. For example, adipocytes secrete adipokines such as adiponectin and leptin, which influence insulin resistance. Interestingly, these adipokines have been proposed to link obesity and chronic inflammation. Among the hepatokines, fibroblast growth factor (FGF)-21 and fetuin-A have received increasing attention because they affect glucose metabolism and may be related to chronic inflammation.

However, the role of adipokines and hepatokines and their relationship to chronic inflammation in the natural history of early onset T2DM has not been studied. Therefore, we compared markers of inflammation such as CRP, TNF-α, interleukin (IL)-1β, and interferon (IFN)-γ as well as leptin, adiponectin, FGF-21, and fetuin in a cross-sectional study, comparison adolescents with T2DM and age–, sex–, and body mass index (BMI)-matched adolescents without

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<td>BMI</td>
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<td>CRP</td>
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<td>TNF</td>
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T2DM to identify associations for the development of T2DM. We studied high sensitivity CRP (hsCRP), TNF-α, IL-1β, and IFN-γ as they have been reported to be increased in the prediabetes state, and IL-1β and IFN-γ have been proposed to lead to β-cell failure.

Methods

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

A total of 74 obese adolescents aged 12-18 years with T2DM and 74 age-, sex- and BMI-matched obese adolescents without diabetes were investigated in this cross-sectional study. Adolescents with T2DM were recruited from the Diabetes Patienten Verlaufs dokumentation dataset (for details of this cohort see Reinehr et al11,12). T2DM was diagnosed according to the criteria of the American Diabetes Association identical to the guidelines of the German Diabetes Society. In all adolescents with T2DM, autoantibodies against β-cells were negative and maturity onset of diabetes in youth type 2 and 3 was excluded by genetic analyses.11,12 The obese adolescents without T2DM were recruited from the obesity cohort at the outpatient obesity clinic of the Vesti sche Children’s Hospital, University of Witten/Herdecke as described previously.13 Diabetes mellitus was excluded in these adolescents by normal hemoglobin (Hb)A1c levels and oral glucose tolerance tests.

None of the adolescents in this study suffered from an acute infection, endocrine disorders, or syndromal obesity. Details of excluding these diseases were published in details elsewhere.11

Height was measured to the nearest centimeter using a rigid stadiometer. Weight was measured in underwear to the nearest 0.1 kg using a calibrated balance scale. BMI was calculated as weight in kilograms (kg) divided by the square of height in meters (m²). The degree of overweight was quantified using Cole LMS method, which normalizes the BMI skewed distribution and expresses BMI as a SDS.14 Reference data for German children were used.14

Systolic and diastolic blood pressure were measured 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.

After clotting, blood samples were centrifuged for 10 minutes at 8000 rpm. Serum was stored at −81°C for later determination of markers of inflammation, adiponectin, leptin, fetuin-A, and FGF-21. These samples were analyzed in 1 central laboratory. All samples were thawed only once.

TNF-α was measured by a specific ultrasensitive commercially available enzyme-linked immunosorbent assay kit (Invitrogen, Camarillo, California). The minimum detectable concentrations of TNF-α is <0.09 pg/mL. The intra- and interassay coefficients of variations were <10%.

hsCRP was determined using a commercially available immunonephelometric method on a Dimension Vista analyzer (CardioPhase hsCRP Flex, Siemens, Erlangen, Germany). The minimum detectable concentration of hsCRP is 0.16 mg/L. The intra- and interassay coefficients of variation were 5.2% (mean = 0.37 mg/L, n = 20) and 5.7% (mean = 0.38 mg/L, n = 20), respectively.

Human IL-1β was measured using a commercially available enzyme-linked immunosorbent assay kit (Thermo Scientific, Rockford, Illinois). The detection limit is 1 pg/mL. The intra- and interassay coefficients of variation were <10%.

IFN-γ was determined by a commercially available enzyme-linked immunosorbent assay kit (Invitrogen, Camarillo, California). The detection limit is 0.03 IU/mL. The intraassay coefficients of variation were <5%, the interassay coefficients of variation were <10%.

The methods and findings concerning HbA1c, low density lipoprotein- and high density lipoprotein-cholesterol, fetuin-A, FGF-21, leptin, and adiponectin have been reported previously.11,12

Statistical Analyses

Statistical analyses were performed using the Winstat software package (R. Fitch Software, Bad Krozingen, Germany) and SAS v 9.4 (SAS Institute, Cary, North Carolina). All variables except hsCRP, IL-1β, IFN-γ, TNF-α, and FGF-21 were normally distributed as tested by the Kolmogorov-Smirnov test. To compare variables between adolescents with and without T2DM, χ² test, Mann Whitney U test, and Student t test for unpaired observations were used as appropriate. We calculated a multiple logistic regression with T2DM as dependent variable, and the independent variables inflammatory markers adjusted to age, sex, and BMI. Furthermore, multiple linear regression analyses with inflammatory markers as the dependent variable and the independent variables inflammatory markers adjusted to age, sex, BMI, leptin, adiponectin, HbA1c, fetuin-A, and FGF-21 were calculated to identify associations among inflammatory markers, hepatokines, and adipocytokines. Sex was used as categorical variable in these models. All variables not normally distributed were log-transformed in these models. A P value of <.05 was considered as significant. Data were presented as mean and SD or median and IQR if variables were not normally distributed.

Results

Adolescents with T2DM were treated by lifestyle intervention only (n = 24), metformin (n = 23), insulin (n = 9), or the combination of both insulin and metformin (n = 18; Table). Adolescents without T2DM had 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 had significantly higher hsCRP and TNF-α concentrations compared with the age-, sex-, and
BMI-matched obese adolescents without T2DM (Figures 1 and 2). Furthermore, the IL-1β concentrations were significantly (P < .001) higher in adolescents with T2DM compared with adolescents without T2DM, who all showed IL-1β concentrations below the detection limit of 1 pg/mL. However, IL-1β above the sensitivity of 1 pg/mL could be measured in 13.9% of the adolescents with T2DM. The IL-1β levels of these adolescents were in mean ± SD 42 ± 16 pg/mL. IFN-γ levels above the sensitivity of 0.03 IU/mL could not be detected in any adolescent with or without T2DM.

In a multiple logistic regression adjusted to age, sex, and BMI, hsCRP (OR estimate 1.21; 95% CI 1.04-1.42) and TNF-α (OR 3.04; 95% CI 2.02-4.59) were associated with T2DM. Because IL-1β and IFN-γ were detectable only in a minority of the participants, these variables were not included in this model.

We found no sex-related differences regarding inflammatory markers: HsCRP levels did not differ (P = .384) between the 86 boys (median 2.8, IQR 0.9-6.7 mg/L) and 62 girls (median 2.4, IQR 0.9-4.8 mg/L). The TNF-α concentrations did not differ (P = .205) between the 86 boys (median 6.5, IQR 5.4-8.5 pg/mL) and the 62 girls (median 6.1, IQR 4.5-8.0 pg/mL). Because of the low number of detectable IL-1β and IFN-γ levels in our study, we did not perform any sex-based analyses for these variables.

In a multiple linear regression analysis (r² = 0.28), log-transformed hsCRP was significantly associated with log-transformed FGF-21 (β-coefficient 0.32, 95% CI ±0.15, P < .001) and BMI (β-coefficient 0.015, 95% CI ±0.014, P = .039), but not with HbA1c, adiponectin, leptin, fetuin-A, sex, or age. Log-transformed TNF-α was significantly related to leptin (β-coefficient −0.005, 95% CI ±0.002, P < .001) and BMI (β-coefficient 0.02, 95% CI ±0.01, P < .001), but not to HbA1c, adiponectin, fetuin-A, log-transformed FGF-21 sex, or age in multiple linear regression analysis (r² = 0.30).

**Discussion**

Our finding of increased IL-1β concentrations in adolescents with T2DM is new, but the findings concerning TNF-α and hsCRP are consistent with previous studies. Elevated...
concentrations of hsCRP and TNF-α were reported in obese adults with T2DM compared with lean adults or obese adults without T2DM. Most previous studies in adolescents, with smaller sample sizes than in our study, found increased hsCRP and TNF-α levels in obese adolescents with T2DM compared with obese adolescents without T2DM.

Our findings support the hypothesis that systemic inflammation could be one causative link between obesity and diabetes. TNF-α and IL-1β can impair insulin signaling or directly induce β-cell apoptosis, and are increased in animal models of T2DM. IL-1β is expressed in and secreted from adipose tissue. Another cytokine leading to β-cell apoptosis and dysfunction is IFN-γ. Even though we measured increased hsCRP, TNF-α and IL-1β concentrations in our adolescents with T2DM supporting the above mentioned animal experiments, we did not find elevated IFN-γ levels in obese adolescents with T2DM. However, systemic IFN-γ concentrations may not represent local IFN-γ concentrations in the pancreas. Furthermore, we were not able to detect IFN-γ concentrations because all measurements were below the sensitivity of our test kit.

Inflammatory cytokines influence insulin signaling not only directly but possibly also via adipokines and hepatokines. Furthermore, adipokines also regulate inflammation. For example, leptin is an adipokine with immune modulating properties. In our study, leptin correlated negatively with TNF-α in a multiple linear regression analysis accounting for multiple confounders. In contrast, leptin correlated positively with inflammatory markers in a recent study in adults. However, these correlations were obesity-dependent, and our study was matched for BMI. There is consensus that adiponectin generally exerts insulin sensitizing, anti-inflammatory, and antiapoptotic actions on a number of different cell types. Interestingly, TNF-α downregulates adiponectin expression and secretion in fat cells in vitro. In contrast to these cell experiments, we did not find a correlation between any inflammatory markers including TNF-α and adiponectin. This finding is in agreement with a study in adults reporting no correlation between adiponectin and inflammatory markers.

FGF-21 and fetuin-A are hepatokines thought to be involved in the pathogenesis of T2DM. Although T2DM is regarded as a FGF-21 resistant state, fetuin-A interferes with insulin action at peripheral tissues through its interaction with insulin receptor. Consistent with the hypothesis of a FGF-21 resistant state in T2DM, we measured increased FGF-21 levels in obese adolescents with T2DM compared with matched obese adolescents without T2DM. FGF-21 resistance can be mediated through altered expression of both the FGF receptor and the adapter molecule β-klotho. Interestingly, TNF-α represses β-klotho expression and impairs FGF-21 action in adipose cells. In contrast to this animal model, we found no significant relationship between FGF-21 and TNF-α in multiple linear regression analyses. However, another inflammatory marker, hsCRP, was significantly associated with FGF-21 in our study. Fetuin-A was shown to induce cytokine expression and low-grade inflammation in animal models. In contrast, we found no correlation between fetuin-A and any inflammation marker in our study.

The findings of increased fetuin-A and FGF-21 levels as associations for T2DM in adolescents have been reported and discussed previously by our research group, as well as decreased leptin levels in obese adolescents with T2DM pointing against leptin resistance in T2DM and supporting results from animal studies suggesting that leptin improves insulin resistance.

The strengths of this study include the large cohort of mostly Caucasian adolescents with proven T2DM by genetic exclusion of type 2 and 3 maturity onset of diabetes in youth diabetes and exclusion of β-cell specific autoantibodies, the short diabetes duration, and the comparison with an age-, sex-, and BMI-matched control group. However, our study also has some potential limitations. First, BMI percentiles were used to classify being overweight. Although BMI is a good measure for being overweight, one needs to be aware of its limitation as an indirect measure of fat mass. Second, we have no data concerning pubertal stage. It is well known that adiponectin and leptin levels depend on pubertal stage. However, the mean age of our cohort was >15 years when the great majority of girls and most boys are at a late pubertal or postpubertal stage. Third, we had only indirect measures of insulin resistance such as high-density lipoprotein-cholesterol and lipids, but no fasting insulin levels and corresponding glucose levels. Fourth, we cannot rule out an effect of antidiabetic treatment on inflammatory markers, adipokines, or hepatokines. Fifth, as racial/ethnic differences in insulin secretory capacity in periaadolescent children, insulin sensitivity, and inflammatory markers including adiponectin, CRP, and TNF-α have been reported; our findings cannot be generalized to other ethnicities. Sixth, there are several other adipokines and cytokines (eg, IL-18 and IL-6) related to insulin resistance, which were not analyzed in this study. Seventh, although changes of inflammatory markers in T2DM are speculated to be caused by hyperglycemia, glycemic control (HbA1c) showed no significant correlation with any inflammatory marker in multiple linear regression analyses. Finally, the cross-sectional design has an inherent limitation of potential selection bias and limits our ability to definitively identify which factors and pathways account for T2DM. For example, the observed significant relationship between TNF-α and leptin or between hsCRP and FGF-21 does not allow a conclusion regarding whether inflammation leads to changes of adipokines and hepatokines or vice versa. Future longitudinal studies in humans are necessary to confirm the relative timing of these relationships.

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