Impact of Metabolic Control and Serum Lipids on the Concentration of Advanced Glycation End Products in the Serum of Children and Adolescents With Type 1 Diabetes, as Determined by Fluorescence Spectroscopy and \( N_\varepsilon-(\text{carboxymethyl}) \) Lysine ELISA

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OBJECTIVE — Advanced glycation end products (AGEs) are a complex and heterogeneous group of proteins that are formed by nonenzymatic glycation in a series of reactions. It is hypothesized that they may play a role in the pathogenesis of diabetes-related complications; at present, however, their exact biological role is scarcely understood. Clinical studies so far have shown that serum levels of AGEs are correlated with clinical stages of diabetes complications such as retinopathy and nephropathy. This study was performed in children and adolescents with type 1 diabetes to examine the putative role of serum AGEs in respect to metabolic control and diabetes complications in relation to a number of clinical and laboratory parameters.

RESEARCH DESIGN AND METHODS — We studied 99 children and adolescents up to the age of 20 years with type 1 diabetes and 60 control subjects. Serum levels of AGEs were measured with two different methods [fluorescence spectroscopy and \( N_\varepsilon-(\text{carboxymethyl}) \)lysine (CML) enzyme-linked immunosorbent assay] and correlated with clinical data, such as age, diabetes duration, BMI, and long-term metabolic control determined by HbA1c, and laboratory parameters, such as serum lipids.

RESULTS — Serum levels of fluorescent AGEs, but not of CML-AGEs, in children and adolescents with type 1 diabetes were significantly higher compared with control subjects. There was an age-dependent increase of fluorescent AGEs in children and adolescents with diabetes that was not seen in healthy children and adolescents. Levels of fluorescent AGEs in patients with diabetes between 13 and 16 years of age correlated positively with HbA1c levels. No significant association between levels of AGEs and diabetes duration was found. Children and adolescents with diabetes and high serum triglycerides had significantly higher serum levels of fluorescent AGEs. Children and adolescents with diabetes between the age of 13 and 16 years with high levels of LDL had significantly higher levels of fluorescent AGEs.

CONCLUSIONS — In this study we demonstrated a clear age-dependent increase of fluorescent AGEs but not of CML-AGEs in children and adolescents with diabetes type 1. Moreover, we showed a strong association between serum AGEs and serum triglycerides and cholesterol. The observed effect may be caused by a loss of optimal regulation of lipid metabolism. It could suggest a link between triglycerides and formation of AGEs. This new and interesting finding and its impact on metabolic control and the development of diabetes complications should be examined in the future.

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Type 1 diabetes is one of the most common chronic disorders in children and adolescents. Many genetic or metabolic factors seem to play a role in the pathogenesis of diabetes complications, such as retinopathy, neuropathy, nephropathy, and macrovascular disease (1,2). Good glycemic control reduces the rate of development and progression of diabetes complications in patients with type 1 diabetes, as has been demonstrated by many studies (for example, the Diabetes Control and Complication Trial [3]). Glycemic control is usually determined by measurement of glycated protein HbA1c and, at present, is the most commonly measured predictor of microvascular and neuropathic complications (3). Because HbA1c does not seem to be directly involved in the development of vascular complications, it would be beneficial to find additional predictors that distinguish between those patients who have a greater risk of developing complications from those who do not. In view of the increasing incidence of childhood diabetes, this would be especially important for subjects with type 1 diabetes whose illness started in childhood (4,5). Recently, research has shown that advanced
AGEs in diabetic children

glycation end products (AGEs) may be implicated in the pathogenesis of diabetes-related macrovascular and microvascular complications, including neuropathy, nephropathy, and retinopathy (6,7).

AGEs are generated by the nonenzymatic reaction of a sugar ketone or aldehyde group with the free amino groups of proteins or amino acids. In the first step, a labile Schiff base is formed, which subsequently rearranges into the stable Amadori-product. Finally, AGEs are formed as a mixture of protein-bound nitrogen and oxygen containing heterocyclic compounds through a complex cascade of dehydration, condensation, fragmentation, oxidation, and cyclization reactions. AGEs are a complex and heterogeneous group of proteins, and only the structures of some AGEs have been identified. Best characterized are pentosidine and Ne-(carboxymethyl)lysine (CML). So far no universally accepted method to detect AGEs or an internationally recognized standard unit of measurement exists (6,7). Common methods of measurement used at present are fluorescence spectroscopy or, more advanced, fast-protein liquid chromatography or high-performance liquid chromatography, competitive enzyme-linked immunosorbent assays (ELISAs), and immunohistochemistry (6,8–14). AGEs demonstrate a yellow-brown color and have a diffuse absorption spectrum without discrete peaks but have a characteristic fluorescence with an excitation maximum at 370 and emission at ~445 nm (11,15). Recently, a competitive ELISA using a monoclonal antibody recognizing CML became commercially available (13,14).

The aim of this study was to determine the levels of serum AGEs by fluorescence spectroscopy and CML-ELISA in young subjects with and without diabetes and to determine whether there was an association with disease-specific parameters. The priori hypothesis stated that serum levels of AGEs determined by fluorescence spectroscopy and CML-ELISA are higher in children and adolescents with type 1 diabetes than in healthy subjects. Furthermore, we asked whether disease-specific parameters such as diabetes duration and long-term metabolic control determined by HbA1c correlated with the presence of AGEs. Clinical parameters such as age and BMI and different laboratory parameters such as cholesterol and triglycerides were also examined and correlated with levels of AGEs.

RESEARCH DESIGN AND METHODS

Subjects
The 99 subjects with diabetes in this retrospective study regularly attend the Diabetes Outpatient Clinic of the University Children’s Hospital (University of Leipzig, Germany). To be included in the study, patients had to be <20 years of age and have diabetes type 1 as defined by the American Diabetes Association 1998. Parameters such as age, sex, weight, height, BMI, duration of diabetes, and daily insulin doses were recorded. Levels of HbA1c were examined 3 months before, 3 months after, and at the time of examination. Renal function status was determined by measuring serum creatinine once a year and albumin excretion rate in timed overnight urine collections every 3 months. Serum creatinine in all subjects with diabetes was found to be within the normal range. No persistent microalbuminuria (as defined as an albumin excretion rate >20 μg/min in a minimum of two of three consecutive urine specimens) was observed. Nonfasting venous blood was collected for determination of levels of triglycerides, cholesterol, LDL, and HDL. In 15.2% (n = 15) of all children and adolescents with diabetes, the nonfasting triglyceride levels were elevated (normal range, <1.7 mmol/l). The 15 children with elevated nonfasting levels were subsequently reexamined, and their fasting triglyceride levels were found to be within the normal range. Elevated (nonfasting) cholesterol levels (normal range, <5.2 mmol/l) were found in 16.2% (n = 16) of subjects; when reexamined in a fasting state, they were found to be normal. Remaining serum was stored at −20°C until further analysis. Sixty healthy control subjects without diabetes and between the age of 9 and 16 years were recruited along with regular public health service examinations in school classes from a study on anthropometric measurements (“Leipzig school children’s project”). Their HbA1c and random glucose levels were measured and found to be normal. Investigators performing the laboratory analyses were blinded to the study hypothesis. The study protocol was approved by the Ethical Committee of the Medical Faculty of the University of Leipzig. Written informed consent was obtained from parents and subjects depending on their age.

Determination of HbA1c
HbA1c levels were determined by a commercial kit using a turbidimetric inhibition immunoassay (Tina Quant aHbA1c; Roche Diagnostics, Penzberg, Germany). The normal range was 4.8%–6.0%, with an interassay coefficient of variation of 8.8% for values within the normal range and of 4.1% for values above the normal range.

Determination of serum cholesterol and triglycerides
Levels of serum cholesterol, HDL, LDL, and triglycerides were measured using the commercially available enzymatic in vitro tests by Roche (Cholesterol CHOD-PAP, HDL-C Plus, LDL-C Plus, Triglycerides GPO-PAP; Hitachi, Roche Diagnostics).

Determination of AGEs by fluorescence spectroscopy (fluorescent AGEs)
Serum samples were diluted 100-fold in PBS and filtered (0.22 μm, Millex-GV; Millipore, Bedford, MA). Hemolytic sera were excluded from AGE measurements (n = 6). Fluorescence spectra (corrected for background) were recorded in triplicate on a Fluoromax spectrometer (Spex instruments) at room temperature. Emission and excitation slit widths were set to 1 nm. When excitation was performed at 370 nm, an emission maximum was found at ~445 nm, as previously described (11,15). To compare different sera, the excitation wavelength was set to 370 nm and the signal intensity was measured at the emission maximum of 445 nm and expressed in arbitrary units.

Measurement of AGEs as CML by a competitive ELISA (CML-AGEs)
The concentration of AGEs as CML was measured by the commercially available CML-ELISA from Roche Diagnostics. The polyclonal antibody used for the competitive ELISA was raised against CML hemoglobin. The CML-ELISA was performed following the protocol developed by Roche Diagnostics, as described previously (11,15). Briefly, 96-well microtiter plates were coated overnight with synthetic BSA-AGE (3 μg/ml) in 0.1 mol/l sodium carbonate buffer, pH 9.5. Wells were blocked for 1 h with PBS containing
6% BSA, 1% FCS, and 0.05% Tween 20. Serum samples, diluted 1:4 in assay buffer (PBS plus 0.5 mol/l KCl, 0.05% Tween 20, and 6% BSA), were added to anti-keyhole limpet hemocyanin rabbit anti-serum (diluted 1:400). After incubation for 2 h and three washing steps, alkaline phosphatase–linked anti-rabbit IgG (Boehringer Mannheim, diluted 1:2,000) in wash buffer (PBS with 0.05% Tween 20) was added, incubated for 1 h, and washed again. Wells were developed with p-nitrophenyl phosphate diluted in glycine buffer (pH 10.4); absorbance at 405 nm was read in a microplate reader and expressed as nanograms per milliliter.

Statistics

Data are presented as means ± SD. Statistical parameters were calculated using Microsoft Excel and SPSS program 9.0. For statistical analysis, groups of children and adolescents with diabetes were compared with their age-matched control groups. ANOVA was carried out for the control of the study groups (age-matched sub-jects with and without diabetes). Data were analyzed using correlation analysis. For comparison of different parameters, partial correlation and comparison of dependent correlation coefficients were performed. P values <0.05 were required for significance. For graphic presentation, SPSS 9.0 was used.

RESULTS

Study subjects

The children and adolescents with type 1 diabetes were 12.7 ± 3.9 years of age (range, 1.8–19.8 years) and had a mean duration of diabetes of 4.2 ± 3.1 years. Their mean HbA1c measured as mean of the HbA1c measured 3 months before, 3 months after, and at the time of examination, was 7.8% ± 1.6%. The healthy children and adolescents of the control group were 12.4 ± 2 years of age (range 9.8–15.8 years). Their random glucose level and HbA1c were normal. Clinical characteristics of the study subjects are shown in Table 1. Because of the differences in age range between the case and control subjects, case subjects were divided into two age-groups: 9–13 and 13–16 years of age. There was no significant difference in number, sex ratio, or BMI between the two groups. For statistical analysis, the two age-groups of children and adolescents with diabetes were compared with their age-matched control group.

Determination of AGES by fluorescence spectroscopy (fluorescent AGES) and competitive CML-ELISA (CML-AGES) in serum of children and adolescents with diabetes and control subjects and correlation to clinical and metabolic parameters

Serum AGES (fluorescent AGES measured by fluorescence spectroscopy and CML-AGES measured by CML-ELISA) were determined in subjects with and without diabetes. Levels of fluorescent AGES (excitation/emission wavelength of 370/445 nm) in the serum of children and adolescents with diabetes were higher compared with the control subjects (P ≤ 0.001, Δ = 43,114 [95% CI 32,052–54,176]). Furthermore, serum concentrations of fluorescent AGES were significantly higher in the older age-group of children and adolescents with diabetes compared with the control group (Fig. 1A). ANOVA showed that this age-dependent increase of fluorescent AGES in diabetic children and adolescents was not seen in healthy children and adolescents (P = 0.022, Δ = 14,957 for subjects with diabetes, Δ = −2,567 for subjects without diabetes; see Fig. 1A for 95% CIs). Relation of the parameters age and serum levels of fluorescent AGES in diabetic children and adolescents is depicted in Fig. 1B. No difference in the concentration of CML-AGEs between children and adolescents with and without diabetes was observed (P = 0.33, Δ = 91 [−47 to 229]; data not shown). Statistical analysis demonstrated no significant correlation between serum concentrations of fluorescent AGES and CML-AGEs (r = −0.18, P = 0.10). The serum level of AGES in children and adolescents with diabetes correlated positively with BMI (P = 0.001, r = 0.33), but when adjusted for age, the correlation became weakly significant in the older age-group (r = 0.28, P = 0.042).

Serum levels of fluorescent AGES were correlated with duration of diabetes and levels of HbA1c, triglycerides, cholesterol, HDL, and LDL to examine the relationship between AGES and metabolic control of diabetes. No significant age-adjusted correlation was found between serum fluorescent AGES and duration of diabetes in children with diabetes (r par-
A significant correlation was found when levels of fluorescent AGES were correlated with levels of triglycerides. Figure 1D shows the correlation between triglycerides and fluorescent AGES in children and adolescents with and without diabetes. Those children and adolescents with diabetes and high serum triglycerides had significantly higher serum levels of fluorescent AGES ($r = 0.39$, $P = 0.02$ [95% CI 0.07–0.62]) for the younger age-group, $r = 0.35$, $P = 0.01$ for the older age-group). After adjusting for HbA1c, the positive correlation between fluorescent AGES and triglycerides remained highly significant ($r = 0.39$, $P = 0.02$ for the younger age-group; $r = 0.61$, $P = 0.001$ for the older age-group). Accordingly, correlation analysis for fluorescent AGES and levels of LDL and HDL cholesterol was performed and showed that those subjects with diabetes in the older age-group who had higher LDL levels also demonstrated significantly higher levels of fluorescent AGES ($r = 0.47$, $P = 0.002$). When adjusted for levels of triglycerides, the same effect was observed ($r = 0.38$, $P = 0.014$). A similar effect was seen in the younger age-group, but this was not significant ($r = 0.31$, $P = 0.13$).

In contrast to the positive correlation of LDL, low levels of HDL were associated with high levels of fluorescent AGES in the older age-group of the children and adolescents with diabetes; however, there was borderline significance ($r = -0.26$, $P = 0.07$, figure not shown). Serum levels of CML did not correlate significantly with duration of diabetes ($r = -0.30$, $P = 0.79$, figure not shown). Levels of fluorescent AGES in the older age-group correlated significantly with mean HbA1c levels ($r = 0.32$, $P = 0.018$ [95% CI 0.06–0.55]) (Fig. 1C). There was no significant correlation between HbA1c and fluorescent AGES in the younger age-group ($r = -0.01$, $P = 0.94$ [−0.32–0.30]). Significance in the older age-group of subjects with diabetes still remained when adjusting the data for age ($r = 0.35$, $P = 0.01$). There was no significant correlation between the levels of fluorescent AGES and mean HbA1c levels ($r = 0.16$, $P = 0.15$ [−0.06–0.36]). Serum levels of fluorescent AGES or CML did not correlate with insulin doses in children and adolescents with diabetes ($r = 0.12$, $P = 0.28$ for fluorescent AGES and $r = -0.06$, $P = 0.58$ for CML; data not shown).

A signiﬁcant correlation was found when levels of fluorescent AGES were correlated with levels of triglycerides. Figure 1D shows the correlation between triglycerides and fluorescent AGES in children and adolescents with and without diabetes. Those children and adolescents with diabetes and high serum triglycerides had significantly higher serum levels of fluorescent AGES ($r = 0.39$, $P = 0.02$ [95% CI 0.07–0.62]) for the younger age-group, $r = 0.35$, $P = 0.01$ for the older age-group). After adjusting for HbA1c, the positive correlation between fluorescent AGES and triglycerides remained highly significant ($r = 0.39$, $P = 0.02$ for the younger age-group; $r = 0.61$, $P = 0.001$ for the older age-group). Accordingly, correlation analysis for fluorescent AGES and levels of LDL and HDL cholesterol was performed and showed that those subjects with diabetes in the older age-group who had higher LDL levels also demonstrated significantly higher levels of fluorescent AGES ($r = 0.47$, $P = 0.002$). When adjusted for levels of triglycerides, the same effect was observed ($r = 0.38$, $P = 0.014$). A similar effect was seen in the younger age-group, but this was not significant ($r = 0.31$, $P = 0.13$).

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Figure 1—A: Concentration of fluorescent AGES (determined by fluorescence spectroscopy) in serum of children and adolescents with type 1 diabetes and age-matched control subjects. Comparison of serum concentration of AGES determined by fluorescence spectroscopy in the two age-groups (dashed lines, 9–13 years; black lines, 13–16 years) of children and adolescents with type 1 diabetes (right) and the control subjects (left) shows significantly higher levels of fluorescent AGES in subjects with diabetes ($P \leq 0.001$, $\Delta = 43,411$ [95% CI 32,052–54,176]). Furthermore, an age-dependent increase of fluorescent AGES was seen in children and adolescents with diabetes ($P = 0.022$, $\Delta = -2,567$ for control subjects, $\Delta = 14,937$ for subjects with diabetes). B: Age-dependent increase of fluorescent AGES (determined by fluorescence spectroscopy) in serum of children and adolescents with type 1 diabetes. Serum concentrations of fluorescent AGES (determined by fluorescence spectroscopy) increased with age in children and adolescents with type 1 diabetes. C: Correlation of HbA1c and fluorescent AGES (determined by fluorescence spectroscopy) in serum of children and adolescents with type 1 diabetes. Also shown is the correlation of HbA1c and serum concentration of AGES, as determined by fluorescence spectroscopy (fluorescent AGES) in children and adolescents with diabetes $\leq 13$ years of age (△, dashed line) and $>13$ years of age (●, black line). Fluorescent AGES increased with HbA1c, in children and adolescents with diabetes $>13$ years of age ($r = 0.32$, $P = 0.018$). D: Correlation of triglycerides and fluorescent AGES (determined by fluorescence spectroscopy) in serum of children and adolescents with type 1 diabetes. Also shown is the correlation of triglycerides and serum concentration of AGES, as determined by fluorescence spectroscopy (fluorescent AGES), in children and adolescents with diabetes (△, black line) and control subjects (●, dashed line). Fluorescent AGES are higher in children and adolescents with diabetes than in healthy subjects ($P \leq 0.001$, $\Delta = 37,342$ [30,560–44,124]). Furthermore, fluorescent AGES increased with triglycerides in children and adolescents with and without diabetes ($P \leq 0.001$, $\Delta = 19,520$ [15,264–23,776]). AU, arbitrary units.
P = 0.07, figure not shown). No significant association between levels of CML and serum lipids was found (r = −0.06, P = 0.62 for triglycerides, r = 0.02, P = 0.89 for LDL, r = 0, P = 1.0 for HDL).

CONCLUSIONS — This study has demonstrated, as others have (16–20), that the serum concentrations of AGEs are elevated in children and adolescents with type 1 diabetes. AGEs are a heterogeneous group of proteins, and only the structures of some AGEs have been identified. Best characterized are pentosidine and CML. So far it is not exactly known which of the different AGE proteins is responsible for or associated with diabetes complications. AGE levels in serum or plasma can be measured by fluorescence spectroscopy, fast-protein liquid chromatography, or high-performance liquid chromatography (6). Fluorescence spectroscopy has been used as a valuable tool in the investigation of the Maillard reaction, e.g., in vivo assays nonenzymatic browning in lens proteins or in in vitro experiments examining the kinetics of the development of β-amyloid AGEs. However, use of this method to analyze AGEs levels in a complex body fluid such as serum is not used routinely. In a previous study, we have used this method to measure AGE levels in serum of Alzheimer’s patients and patients on maintenance hemodialysis (11). Recently, competitive ELISAs with antibodies raised against (carboxymethyl)lysine (CML) have become available that facilitate AGE determination in clinical practice (6).

The aim of this study was to compare AGE levels by fluorescence spectroscopy and CML-ELISA in children and adolescents with and without diabetes and to correlate them with different clinical and disease-specific clinical parameters. In the present study, serum levels of fluorescent AGEs, but not of CML-AGEs, in children and adolescents with diabetes were significantly higher compared with control subjects. The studies in children and adolescents by Berg and colleagues (16,17) and Chiarelli et al. (18) used mainly CML-ELISAs to measure serum concentrations of AGEs. In contrast to the results of Berg and colleagues and Chiarelli et al., we demonstrate that only fluorescent AGEs and not CML-AGEs are elevated in young subjects with diabetes. Our study suggests that CML-AGEs levels are inferior to HbA1c and fluorescent AGE measurements as a marker of the presence of type 1 diabetes. However, the CML value of our nondiabetic group is relatively high compared with other studies in children, adolescents, and adults (563 ± 198 ng/ml in our control group compared to 380 ± 100 ng/ml in adults in the study of Schwedler et al. [15] and 198.5 ± 42 ng/ml in children and 504 ± 195 ng/ml in adults in the study of Sekbekova et al. [20]). This difference may be caused by ingestion of food with high CML levels (e.g., roasted and baked foods including Coca-Cola). There is evidence indicating that food-derived AGEs contribute to the endogenous AGE pool (7,21). However, it is not clear what percentage of food-derived AGEs is absorbed and how rapid food-derived AGEs are eliminated (7,21). Koschinsky et al. (21) demonstrated that only approximately one-third of absorbed AGEs are excreted within 48 h in the urine of subjects with normal renal function. So far it is not clear how long children and adolescents have to consume a low-AGE diet to eliminate food-derived AGEs.

We demonstrate an age-dependent increase of fluorescent AGEs in children and adolescents with diabetes, which was not seen in healthy children and adolescents. Our data support the hypothesis that non–CML-AGEs, rather than CML-AGEs, may play a role in the evolution of accelerated micro- and macrovascular complications in diabetes (7,22). To our knowledge, this is the first study demonstrating an association between serum AGEs and serum triglycerides in children and adolescents with type 1 diabetes. In-
deed, those subjects with diabetes and high serum cholesterol and triglycerides also had significantly higher levels of serum fluorescent AGEs. There are several possible explanations for this observation. It could suggest a link between triglycerides and formation of AGEs. Recent investigations showed that some AGEs structures could also be formed from oxidized lipid precursors. These structures have been termed advanced lipoxidation end products (ALEs), and it has been suggested that ALEs and AGEs may lead to endothelial dysfunction, atherosclerosis, and diabetes complications (23). Unfortunately, fluorescence of serum proteins measured at an emission wavelength of 370 and an excitation of 445 nm is not specific for AGEs. We have shown previously that the majority of plasma fluorescence is bound to proteins, predominantly to albumin (11). However, protein-bound fluorescence is not only caused by AGEs but also by lipid peroxidation products such as malondialdehyde-protein adducts that have similar maximal excitation and emission wavelengths (390 nm/460 nm) as AGEs (24). Another explanation for the observed correlation between fluorescent AGEs and triglycerides could be the ingestion of different AGE-rich fatty food. For example, consumption of roasted and baked food may lead to high serum levels of triglycerides, AGEs, and ALEs. Thus, food consumed before taking the blood sample by the children and adolescents could be responsible for the elevated triglycerides and the high serum levels of AGEs. Alternatively, the observed effect may be probably caused by a loss of optimal regulation of lipid metabolism. For example, uptake of lipoproteins into cells has been shown to be inhibited by AGEs via competition of both molecules for the class B scavenger receptor (25). In a similar line of arguments, glycation of lipoproteins may inhibit their transporter functions, as it has been shown for glycated apoE particle, which loses its ability to be taken up by the heparan sulfates proteoglycan pathway (26). However, the cause-effect relationship for our observed correlation remains unsolved. Whether glycation of lipoproteins leads to impaired lipid clearance and to higher lipid plasma level or whether the higher level of oxidizable lipids leads to a higher level of fluorescent AGEs or ALEs is not clear. Several studies demonstrated that an increased chemical modification of proteins by carbohydrates and lipids plays an important but yet not clearly identified role in the pathogenesis of diabetes-related complications (27). So far, there is no consensus regarding the pathogenic link between diabetes and its complications, although hyperglycemia has been implicated as the major risk factor for development of diabetes complications. Among many factors, AGEs, as formed by nonenzymatic glycation, are thought to play an important role in this process (27). However, recent investigations showed that AGEs are also formed during lipid oxidation reactions, indicating that lipids could be one source of AGEs (23).

In summary, we show in this study that fluorescence spectroscopy, despite its potential unspecificity, is an interesting assay for the determination of AGEs in serum of children and adolescents with type 1 diabetes. Moreover, an association between triglycerides, cholesterol, and fluorescent AGEs was demonstrated for the first time. This new and interesting fact, the biochemical link between AGEs and serum lipids and the development of diabetes complications, should be examined in the future in more detail.

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