Monitoring of Early and Advanced Glycation in Relation to the Occurrence of Microvascular Complications in Children and Adolescents with Type 1 Diabetes Mellitus

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Summary

The authors aimed to evaluate if the monitoring of serum advanced glycation end-products (s-AGEs) could help to predict a development of diabetic complications. Clinical and biochemical parameters including fructosamine (FAM), glycated hemoglobin (HbA1c) and serum AGEs were investigated in children and adolescents with 1 type diabetes with (+DC) and without (−DC) complications. FAM levels (in mmol/l) were significantly elevated in +DC diabetic group compared to −DC one (3.043±0.459 vs. 2.614±0.430; $p<0.001$) or to controls (3.043±0.459 vs. 1.620±0.340; $p<0.001$) as well as in −DC compared to controls (2.614±0.430 vs. 1.620±0.340; $p<0.001$). HbA1c (in %) were significantly elevated in +DC diabetic group compared to −DC one (10.48±1.83 vs. 8.41±1.19; $p<<0.001$) or to controls (10.48±1.83 vs. 5.0±0.38, $p<<0.001$) and also in −DC compared to controls (8.41±1.19 vs. 5.0±0.38; $p<0.001$). Serum AGEs levels (in A. U.) were significantly higher in +DC group than in −DC (73.0±14.09 vs. 65.8±9.05; $p<0.05$) and in group +DC than in controls (73.0±14.09 vs. 60.17±13.78; $p<0.05$), whereas there was no difference between −DC and controls. FAM correlated with HbA1c in both diabetic groups (+DC: $r=0.374$; $p<0.05$; −DC: $r=0.719$; $p<0.001$), but not in controls. Serum AGEs were correlated with HbA1c ($r=0.478$; $p=0.003$) in +DC, but not in −DC or controls. Enhanced serum AGEs levels show that they could be not only an attendant phenomenon of microangiopathies, but also a predictor of their development.

Key words

Fructosamine • HbA1c • Serum AGEs • Glycation gap • Diabetic complications

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Introduction

Hyperglycemia and poor glycemic control are considered to be basal key factors in the development of diabetic complications. Acute and chronic hyperglycemia are known to enhance the forming of early (fructosamine (FAM), glycated hemoglobin (HbA1c), intermediate and advanced glycation products and is a primary factor that initiates and promotes diabetic complications (Little and Goldstein 1995, Hanssen 1997). Glycation has both physiological and pathophysiological significance (Rácz and Šipulová 1998, Jakuš and Rietbrock 2004).

FAM fraction reacts much more quickly than the HbA1c to a change in glucose situation and reflects a quality of diabetes control over the previous 2-3 weeks. FAM preceded an increase in albumin excretion rate and is associated with nephropathy and exerted a pathophysiological role in microvascular diabetic complications. The degree of glycation of hemoglobin provides information about the glucose level (quality of diabetes control) over previous 6-8 weeks (Michalková *et al.* 1981, Rácz *et al.* 1989, Gugliucci 2000, Cohen *et al.* 2002).

Hyperglycemia also accelerates the formation of advanced glycation end-products (AGEs). The AGEs are a heterogeneous group of fluorescent and non-fluorescent compounds including the following main subgroups: bis(lysyl)imidazolium cross-links, hydroimidazolones, 3-deoxyglucosone derivatives, and monolysyl adducts (Wautier and Schmidt 2004), the fluorescent compounds with an excitation maximum at 370 nm and emission at ≈445 nm (Galler *et al.* 2003, Kumar *et al.* 2004). They can be formed in short- and long-lived proteins such as albumin, immunoglobulins, skin collagen, ocular, renal and vascular tissues (Thorpe and Baynes 2003, Januszewski *et al.* 2008). Many AGEs have not been well characterized, but a few AGE-structures have been identified. According to Januszewski *et al.* (2008) AGE levels may have a role in the identification of Type 1 diabetic patients at high risk for complications and also provide a tool for monitoring therapeutic interventions. Measurement of serum AGEs is hence of great importance for clinicians and researchers concerned with the management and prevention of diabetic vascular diseases (Sampathkumar *et al.* 2005). Cohen *et al.* (2003, 2008) have dealt with glycation gap (GG) of HbA1c calculated from FAM and have found a relation of gap to nephropathy and retinopathy. We have dealt not only with glycation gap of HbA1c, but also with GG of serum AGEs from HbA1c.

The aim of the present study was to evaluate if monitoring of circulating FAM, HbA1c and serum AGEs in patients with type 1 diabetes could help to predict the diabetic microvascular complications development. We aimed also to find a relationship of GG of HbA1c and GG of serum AGEs to DC.

Materials and Methods

Patients and the sample collection

The study was approved by Local Ethical Committee. Blood, serum and urine were obtained from 76 diabetic patients regularly attending the First Department of Pediatrics, Children Diabetological Center of the Slovak Republic, University Hospital, Faculty of Medicine, Comenius University, Bratislava. Patients were from 7 to 18 years old except two subjects 19 years of age and had Type 1 diabetes with duration of at least 5 years. No microvascular complications (nephropathy, retinopathy, neuropathy) were observed in 35 subjects including both 19-year-old patients (–DC), whereas 41 subjects were with complications (+DC). Urinary albumin excretion rate (UAER) in the microalbuminuric range and its tracking (i.e. annual increase) are still considered reliable markers for prediction of later overt diabetic kidney disease. Irrespective of the procedure used, at least two samples over 3-6 months should be tested positively before microalbuminuria is confirmed and 'persistent microalbuminuria' defined (Chiarelli *et al.* 1997). The urine samples were collected three times overnight, microalbuminuria was considered present when the UAER was between 20 and 200 μg/min (Mogensen 1995, Mojto and Tisoň 2004) in two samples (according to Galler *et al.* 2003). No changes (fundus diabetic retinopathy) were found by the ophtalmologist examining the eyes in subject without retinopathy. Diabetic neuropathy was confirmed by EMG exploration using the conductivity assessment of sensor and motor fibers of peripheral nerves. 59 patients had long-term poor glycemic control (mean of HbA1c during last two years >8.5 %) and poor short-term glycemic control (actual data of HbA1c>8.5 %) was found in 52 patients. 30 children without diabetes or other metabolic disease from 0 to 17 years old were used as controls.

Methods

The values of FAM were determined by spectrophotometric method at wavelength 530 nm (LS 500, SRN). We used 1-deoxy-1-morpholino-fructose (Sigma Aldrich, USA) as the standard.

HbA1c was measured by LPLC (DiaSTAT, USA). The HbA1c values were obtained by the National Glycohemoglobin Standardization Program (NGSP) calibrated method (John *et al.* 2007). Values of total cholesterol (TC), high density lipoproteins (HDL) and triacylglyceroles (TAG) were evaluated enzymatically, low density lipoproteins (LDL) was obtained using Friedewald formula. UAER was determined turbidimetrically (Integra 400 Plus, Roche, Switzerland).

Serum AGEs were determined as AGE-linked specific fluorescence, serum was diluted 20-fold with deionized water, the fluorescence intensity was measured after excitation at 346 nm, at emission 418 nm using a spectrophotometer Perkin Elmer LS-3, USA. Chinine sulphate (1 μg/ml) (Merck, Germany) was used to calibrate the instrument. Fluorescence was expressed as the relative fluorescence intensity in arbitrary units (A.U.).

Glycation gap

CG was determined according to Cohen *et al.* (2003, 2008). GG was defined as the difference between

Table 1. Clinical and biochemical parameters in diabetic patients and controls.

	Children and adolescents with DM1	n	Children and adolescents DM1 -DC	n	Children and adolescents DM1 +DC	n	Controls	n
Age (years)	15.06±2.66	76	14.19±3.17	35	15.81±1.81	41	9.25±4.85	30
Diabetes duration (years)	8.68±3.02	76	7.54±2.51[2]	35	9.64±3.08[1]	41		
HbA1c (%)	9.53±1.88[3]	76	8.41±1.19[2,3]	35	10.48±1.83[1,3]	41	5.0±0.38	20
FAM (mmol/l)	2.850±0.495[3]	75	2.614±0.430[2,3]	34	3.043±0.459[1,3]	41	1.62±0.34	29
Serum AGEs (U.A.)	69.4±12.4[3]	70	65.8±9.05[2]	35	73.0±14.09[1,3]	35	60.17±13.78	30
TC (mmol/l)	4.24±0.77[3]	76	4.3±0.69[3]	35	4.19±0.83	41	3.91±0.72	30
HDL (mmol/l)	1.65±0.59[3]	76	1.72±0.45[3]	35	1.58±0.67[3]	41	1.29±0.32	30
LDL (mmol/l)	2.60±0.67	76	2.53±0.68	35	2.66±0.65	41	2.43±0.64	30
TAG (mmol/l)	1.20±0.86	76	0.90±0.47[2]	35	1.45±1.02[1]	41	1.37±0.67	29
Creatinine (μmol/l)	58.52±11.27[3]	75	57.09±11.04[3]	34	59.1±11.33[3]	41	42.43±12.25	28
UAER (μg/min)	38.88±119.69	71	7.95±4.03[2]	35	67.34±160.98[1]	36		

Data are presented as means ± S.D. [1] significantly different compared with diabetic group –DC. [2] significantly different compared with diabetic group +DC. [3] significantly different compared with controls.

the measured HbA1c and HbA1c predicted from the FAM based on the HbA1c-FAM regression equation:

$$GG = (HbA1c\ measured - HbA1c\ predicted)$$

There were used FAM values of all diabetic subjects for calculating of predicted HbA1c values using the regression line equation. By the definition, GG is negative if measured HbA1c is less than HbA1c predicted from FAM and positive if measured HbA1c is greater than predicted. For example, HbA1c at the lower limit of normal and FAM above the upper limit of normal would result in a negative GG.

In this study the GG of HbA1c expressed in % was calculated using the formula:

$$GG = 100 \times (HbA1c\ measured - HbA1c\ predicted)/ /HbA1c\ measured$$

GG of serum AGEs was established. The expected values of serum AGEs were predicted from the actual HbA1c values and were based on the serum AGEs - HbA1c regression.

The GG was expressed in % using the formula:

$$GG = 100 \times (s\text{-}AGEs\ measured - s\text{-}AGEs\ predicted)/ /s\text{-}AGEs\ measured$$

Statistics

Unpaired Student's t-test (by Excel 2000) and Pearson linear correlation (by Origin 3.83) were used for statistical calculation. χ^2-tests were performed using Excel 2000. $P<0.05$ value was defined as statistically significant.

Results

Comparison of clinical and biochemical parameters

The FAM levels are significantly higher in both diabetic groups in comparison with controls (+DC vs. controls: 3.043±0.459 vs. 1.620±0.340 mmol/l, –DC vs. controls: 2.614±0.430 vs. 1.620±0.340 mmol/l, in both p<0.001) and in group +DC vs. –DC (FAM 3.043±0.459 vs. 2.614±0.430 mmol/l; p<0.001) (Table 1).

We observed significantly higher HbA1c levels in both diabetic groups in comparison with controls (+DC vs. controls: 10.48±1.83 vs. 5.0±0.38 %, –DC vs. controls: 8.41±1.19 vs. 5.0±0.38 %, in both p<0.001) (Table 1) and in group +DC vs. –DC (10.48±1.83 vs. 8.41±1.19 %, p<0.001) (Table 1).

The serum AGEs levels were significantly higher in subjects +DC than in controls (73.0±14.09 vs. 60.17±13.78 A.U., p<0.001) (Table 1, Fig. 1), but the difference between –DC patients and controls was not significant. The levels of serum AGEs were also significantly higher in group +DC vs. –DC (73.0±14.09 vs. 65.8±9.05 A.U., p=0.02) (Fig. 1).

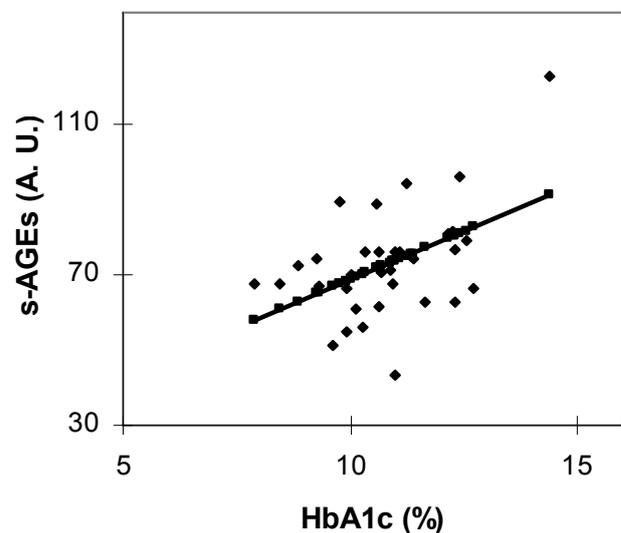**Table 2.** Correlations between clinical and biochemical parameters of diabetic subjects.

Group		HbA1c x FAM	HbA1c x s-AGEs	FAM x s-AGEs	HbA1c x TAG	Age x HDL	Age x TAG	Age x creatinine	DM duration x creatinine
+DC	r	0.374	0.478[1] 0.366[2]	0.396	0.433			0.542	0.452
	p	0.002	0.003[1] 0.03[2]	0.02	0.005	NS	NS	<0.001	0.003
	n	41	35	35	41			41	41
-DC	r	0.719				-0.386	0.352	0.684	0.407
	p	<0.001	NS	NS	NS	0.02	0.04	<0.001	0.02
	n	34				35	35	34	34

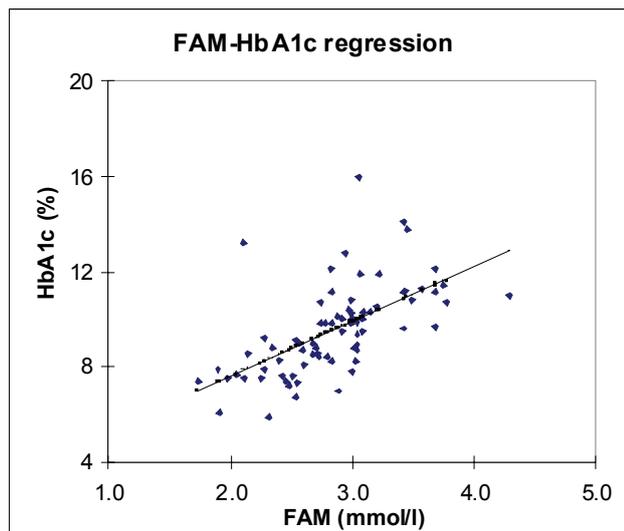[1] mean HbA1c values in previous two years, [2] actual HbA1c values. NS - p>0.05
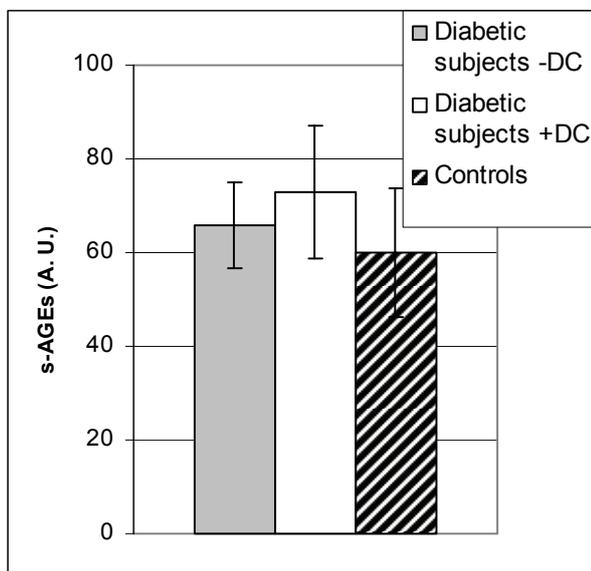
Fig. 1. Comparison of serum AGEs levels of diabetic subjects and controls (p_1<0.001, p_2>0.05, p_3<0.05, p_1: +DC vs. controls, p_2: -DC vs. controls, p_3: +DC vs. −DC).

Fig. 2. Correlation between serum AGEs and HbA1c (mean of the last 2 years) in diabetic subjects with complications (r=0.478, p<0.01).

HDL levels were significantly higher in all diabetic groups in comparison with controls (+DC vs. controls: p<0.001, –DC vs. controls: p=0.02). Lower HDL levels in controls compared with diabetic patients could be explained by significantly lower age of controls. The levels of TAG are lower in diabetic group –DC than in controls (p=0.03) and in –DC vs. +DC (p=0.003). The levels of creatinine were higher in all diabetic groups than in controls and the differences were extremely significant (+DC vs. controls: 59.1±11.33 vs. 42.43±12.25 μmol/l, –DC vs. controls: 57.09±11.04 vs. 42.43±12.25 μmol/l, p<0.001).

The significant correlations between creatinine and age were observed in both –DC (r=0.684, p<0.001) and +DC diabetic subjects (r=0.542, p<0.001) (Table 2). Creatinine was correlated with diabetes duration in both diabetic groups (–DC: r=0.407, p=0.02, +DC: r=0.452, p=0.003). TAG correlated with age in group –DC (r=0.352, p=0.04) (Table 2) and with HbA1c in group +DC (r=0.433, p=0.005). HDL correlated inversely with age in –DC group (r = –0.386, p=0.02). The serum AGEs were correlated with HbA1c (mean levels in previous two years) in +DC group (r=0.478, p=0.004) (Fig. 2), but not in –DC group and with FAM in +DC group (r=0.396, p=0.02). Serum AGEs also significantly correlated with

Fig. 3. Correlation between FAM and HbA1c.

Fig. 4. Mean glycation gap of HbA1c (in %) calculated from FAM in diabetic subjects.

Table 3. Occurrence of diabetic complications in relation to GG of HbA1c.

Occurrence of DC	+DC	-DC	Sum
Number of subjects with positive GG of HbA1c	26	7	33
Number of subjects with negative GG of HbA1c	15	27	42
Sum	41	34	75

actual HbA1c levels in +DC group (r=0.366, p=0.03), although not as significantly as with mean levels, but not in –DC group or controls. There were observed significant correlation between HbA1c and creatinine (r=0.494, p=0.03) and creatinine and age (r=0.782, p<0.001) in controls.
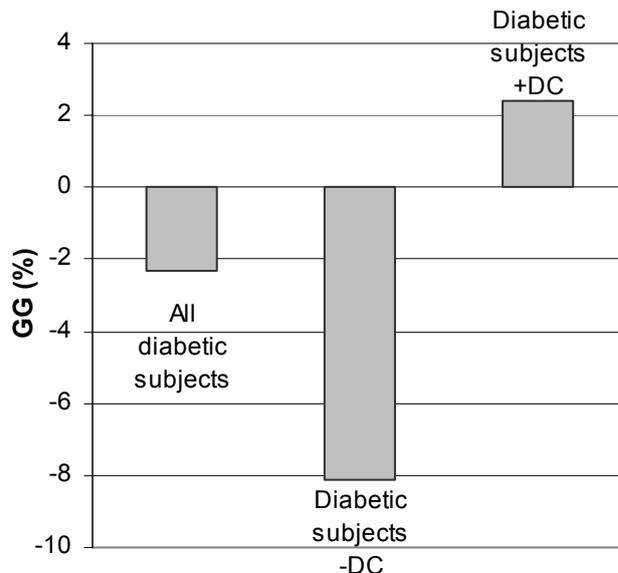
Glycation gap

From HbA1c-FAM regression (Fig. 3) we obtained the equation for calculation of expected HbA1c values: HbA1c = 2.29 + 3.02 x FAM, r=0.601 (p<0.001).

The mean GG of HbA1c expressed in % in diabetic subjects was negative in all diabetic patients (–2.3±14.6 %) and in –DC group (–8.1±11.4 %) and positive in +DC group (2.4±15.4 %) (Fig. 4). The χ^2 test have shown the significant relation of GG of HbA1c to DC occurrence (p=0.0003) (Table 3).

The equation for calculation of expected serum AGEs values was obtained from serum AGEs - HbA1c regression: s-AGEs = 2.92 + 41.64 x HbA1c, r=0.443, p<0.001. The GG of serum AGEs was calculated in %. Fifteen diabetic subjects –DC (from 35) and 19 diabetic subjects +DC (from 35) had positive GG, but χ^2 test have not shown the relation of GG of serum AGEs to DC occurrence. We have tried to evaluate a relation between GG of serum AGEs and GG of HbA1c in diabetic patients), but no relation was found.

Discussion

Vascular complications of diabetes, including retinopathy, neuropathy, nephropathy, and macrovascular disease, are the major cause of morbidity and mortality in diabetic patients, with macrovascular disease being a major cause of premature death. Dysfunction of the key cells responsible for vascular function, including endothelial cells, pericytes, and vascular smooth muscle cells, can be induced by increased cellular concentrations of glucose during hyperglycemia. This can activate multiple pathways of biochemical dysfunction leading to increased glycation of proteins (Ahmed *et al.* 2005) and to enhanced oxidative and carbonyl stress with subsequent direct tissue damage. According to Galler *et al.* (2003) HbA1c does not seem to be directly involved in the development of vascular complications and therefore 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.

We have found only a few studies investigating clinical, biochemical and glycation parameters of patients with type 1 diabetes with regard to the presence of DC.

In our study TC and HDL levels were significantly higher in all diabetic subjects (regardless to DC presence) than in controls and in –DC group vs. controls. HDL levels were significantly higher also in +DC group vs. controls. Merzouk et al. (2004) observed the similar TC levels in all diabetic groups, TAG and LDL were higher in +DC vs. controls and HDL levels were significantly lower in +DC than those in controls.

The significantly higher levels of FAM were found in all diabetic subjects than in controls in accordance with Ajabnoor et al. (1990), Martinez et al. (1994b), Schalkwijk et al. (1999), Jakuš et al. (2000). FAM levels were significantly higher in diabetic group +DC compared to –DC similarly to the findings of Schalkwijk et al. (1999) who presented the higher levels of FAM in diabetic group with nephropathy than without it. Cohen et al. (2008) presented the similar results in patients with and without retinopathy. Martin-Gallan et al. (2003) have not found significant differences in FAM and HbA1c levels of diabetic subjects +DC compared with –DC, but we have found significantly elevated FAM and HbA1c levels in +DC group compared with –DC one. In accordance with Merzouk et al. (2004) the higher HbA1c levels were observed in both +DC and –DC diabetic group compared with controls.

In accordance with some other studies (Berg et al. 1997b, Chiarelli et al. 2000, Jakuš et al. 2001) we detected significantly higher serum AGEs levels in diabetic subject on the whole (regardless of DC presence) compared to controls. In this study the serum AGEs levels in diabetic subjects +DC were significantly higher in comparison with group –DC, but not in –DC group compared with controls. Chiarelli et al. (2000) investigated serum AGEs in patients with nephropathy and retinopathy and without angiopathies. Authors concluded that the severity of diabetic angiopathy is related to serum AGE levels.

In the present study the age and the duration of disease were associated with creatinine in both diabetic groups, whereas the age was associated with HDL (negative correlation) and with TAG in group –DC. These results suggest the eventual association of lipid disorders with complications in relation with age and duration of diabetes. Good management of diabetes seems to be of paramount importance in controlling dyslipidemia.

No correlation was found between FAM and any of lipoproteins in all studied subjects. Many studies presented significant correlations between HbA1c and lipoproteins levels in serum. According to Ladeia et al. (2006) HbA1c correlated with TC or LDL and TAG including LDL also correlated with duration of diabetes in young diabetic patients. Martinez et al. (1994a) have found significant correlation between HbA1c levels and each of TC, LDL and TAG serum concentrations in diabetic patients. In our study HbA1c was correlated with TAG in subjects +DC. Glycemic control and lipid levels are independently associated in youth (Petitti et al. 2007), but lipid disorders may be present regardless presence of complications in young subjects and may be affected by food.

Some studies provided correlations between FAM and HbA1c in diabetic patients (Baker et al. 1983, 1985a, Koskinen et al. 1987, Ajabnoor et al. 1990, Schalkwijk et al. 1999). In our study there was significant correlation between FAM and HbA1c in both diabetic groups (+DC, –DC). Baker et al. (1983, 1985b) considered FAM as a reliable indicator of glycemic control, but Jerntorp et al. (1988) reported that the value of FAM in clinical practice is unclear. Ajabnoor et al. (1990) have found poor correlation of FAM with duration of diabetes, but we did not find any similar relationship. The significant correlation was found between serum AGEs and HbA1c only in +DC diabetic patients. In our study neither FAM nor HbA1c have correlated with age or duration of disease. Serum AGEs correlated with FAM and HbA1c only in subjects +DC. No similar associations were found in –DC subjects.

According to Berg et al. (1997a) and Chiareli et al. (2000) serum AGEs predict the progression of early morphological kidney damage in patients with type 1 diabetes.

Cohen et al. (2003, 2008) investigated glycation gap of HbA1c calculated from FAM in patients with and without nephropathy and with and without retinopathy. The authors present negative GG in subjects without nephropathy or retinopathy and with microalbuminuria and positive GG in subjects with advanced nephropathy and with retinopathy. Similarly, in our study we have found positive GG of HbA1c in +DC and negative in –DC group. Using χ^2 test we have also found a significant relation of GG positivity to occurrence of DC. Cohen et al. (2003, 2008) proposed that patients with high GG and high risk of nephropathy and retinopathy had a low FAM

in relationship to the HbA1c. They demonstrated a correlation between GG and nephropathy and retinopathy, but there was no prediction so that they suggest the necessity of prospective studies of this issue. McCarter *et al.* (2000) believe that the differences between extracellular and intracellular measures of glycemic control may precede nephropathy and that the hemoglobin glycation index, an analogous measure of discordance between self glucose-monitoring and HbA1c, predicts retinopathy and nephropathy.

We have also calculated GG of serum AGEs predicted from measured actual HbA1c values in our study. Fifteen out of 35 –DC diabetic subjects and 19 out of 35 +DC diabetic subjects had positive GG of serum AGEs. Because hemoglobin is an intracellular protein and FAM and serum AGEs reflect extracellular proteins, the GG could result from differences between the ambient glucose concentrations or rates of glycation in the intracellular and extracellular compartments or individual differences in the turnover/metabolism of underlying proteins. We have found surprisingly high number of positive GG also in –DC group. Unfortunately, despite these findings no relation between GG of serum AGEs and occurrence of DC was proved. The GG of serum AGEs have never been studied. Our idea was to try

if GG of serum AGEs could be a predictor of DC development, but this was not confirmed. However, we assume that this issue requires a longitudinal study.

Because the elevated serum AGEs levels occur not only in subjects with complications, the serum AGEs could predict a development of microangiopathies, but the role of serum AGEs as a marker of later complications development is to be confirmed by long-term studies and the consequent study should be focused on subjects without complications.

We assume that serum AGEs not only reflect the presence of DC, but also predict the development of them. This issue requires further investigation of glycemic control impact on the presence of enhanced serum AGEs levels in –DC diabetic subjects.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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