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ORIGINAL ARTICLE

Transforming Growth Factor-β1 and Receptor for Advanced Glycation end Products Gene Expression and Protein Levels in Adolescents with Type 1 Diabetes Mellitus

Ninić A et al. Transforming Growth Factor-β1 in Type 1 Diabetes

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What is already known on this topic?
As the non-enzymatic glycation products arisen under the condition of chronic hyperglycemia, advanced glycation end products (AGE) by binding to its receptors (RAGE) activates a range of signaling pathways which play an important role in the pathogenesis of diabetic complications (nephropathy, retinopathy, neuropathy and atherosclerosis). Transforming growth factor-β1 (TGF-β1) as multifunctional cytokine, exerts pleiotropic effects from differentiation, development to cell growth and immunity regulation. Induced by many factors including hyperglycemia, TGF-β1 is the important mediator in the pathogenesis of diabetic nephropathy mainly stimulating the production of extracellular matrix components.

What this study adds?
The decrease in TGF-β1 gene expression in peripheral blood mononuclear cells significantly independently correlated with type 1 diabetes (T1D) and might be used as a potential biomarker for early cardiovascular risk assessment in adolescents with T1D by predicting early elevation of urinary albumin excretion rate. Decrease in RAGE gene expression, increase in TGF-β1 and soluble sRAGE concentrations could serve as biomarkers independently associated only with T1D presence.

Abstract
Objectives: Type 1 diabetes mellitus (T1D) is one of the most frequent autoimmune diseases in childhood. Chronic complications are the main causes of cardiovascular morbidity and mortality in T1D. Although interactions between advanced glycation end products (AGE) and their receptors (RAGE) and transforming growth factor-β1 (TGF-β1) are implicated in development and progression of diabetic micro- and macrovascular complications, they also have important roles in immune system regulation.
Methods: Blood samples were obtained from 156 adolescents with T1D and 80 apparently healthy controls. T1D patients diagnosed with any other autoimmune disease and receiving any kind of drugs except insulin therapy were excluded from this study. Exclusion criteria for controls were positive family history of T1D and drugs/supplements application. TGF-β1 and transmembrane full-length RAGE (flRAGE) messenger ribonucleic acid (mRNA) levels in peripheral blood mononuclear cells (PBMC) were obtained by quantitative polymerase chain reaction (qPCR) method. Circulating levels of biochemical markers, TGF-β1 and soluble RAGE (sRAGE) levels were also determined.
Results: TGF-β1 and flRAGE mRNA levels were significantly higher in controls compared to patients (P<0.001, for both). However, TGF-β1 and sRAGE levels were higher in patients than controls (P<0.001, for both). There were significant independent associations of all mRNA and protein levels with T1D. TGF-β1 mRNA was the only marker independently negatively associated with urinary albumin excretion rate in T1D adolescents (P=0.005).
Conclusion: Our results indicated gene expression downregulation of TGF-β1 and flRAGE in PBMC of T1D adolescents. TGF-β1 mRNA downregulation could be used to predict early elevation of urinary albumin excretion rate.
Keywords: Transforming growth factor-β1, receptor for advanced glycation end products, type 1 diabetes, urinary albumin excretion rate, quantitative polymerase chain reaction

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Introduction
Type 1 diabetes mellitus (T1D) is a chronic T lymphocyte mediated autoimmune disease leading to destruction of pancreatic Langerhans β-cells, endogenous insulin secretion decline and consequent hyperglycemia (1). Although, autoantibodies for β-islet

cells constituents are used for ongoing autoimmune process and T1D diagnosis (2), other markers that reflect T lymphocytes activities can be used (3). Growing knowledge about gene expression changes in regulatory and effector immune cells, as new biomarkers, could provide novel insights into the T1D pathogenesis (3,4). Autoreactive T cells together with immune cells, like monocytes and other lymphocytes, which target islet β-cells, are capable of inducing T1D development.

Chronic hyperglycemia, either good or poorly regulated, causes the formation of advanced glycation end products (AGEs), the large group of irreversibly modified proteins (5). Contrary to early glycated end products (Schiff bases and Amadori products e.g fructosamine) (5), AGEs express their effects by binding to transmembrane full-length receptor for AGEs (flRAGE) (6). flRAGE in interaction with AGE elicits disbalance in oxidants and antioxidant production and arise inflammatory and thrombogenic responses in endothelial cells (7). Accordingly, AGE-flRAGE interaction induce vascular stiffening, angiogenesis, and extracellular matrix (ECM) accumulation (5,8). By these mechanisms, flRAGE-AGE interaction expresses major effects in chronic micro- and macrovascular complications (nephropathy, neuropathy, retinopathy, and atherosclerosis) development. Also, enhanced flRAGE expression interacting with AGE in T cells may induce their inflammatory functions leading to β-cells injury during T1D progression (9). Opposite to this, flRAGE activation is, also, capable to suppress autoimmune responses by activation of regulatory T cells (Treg) (10).

Circulating RAGE isoforms, termed soluble RAGE (sRAGE), can bind AGEs and thus preventing their detrimental effects following flRAGE activation (11). In this manner, sRAGE demonstrates its protective role. By blocking flRAGE, sRAGE exerted anti-atherogenic capability inhibiting cells migration and enlargement of atherosclerotic lesions (12). However, other studies demonstrated that sRAGE induced vascular permeability by increasing production of proinflammatory and chemoattractant molecules thus maintaining vascular inflammation and progression of atherosclerotic process (13).

As multifunctional cytokine, transforming growth factor-β1 (TGF-β1) stimulates production of ECM proteins (e.g. collagen I and IV, laminin, and fibronectin) participating in extracellular remodelling in peripheral organs leading to development of micro- and macro- vascular chronic complications (14,15). TGF-β1 is likely to be an essential factor in diabetic nephropathy development. On the other hand, TGF-β1 secreted by Treg has role in autoreactive T cells suppression, induction of immune tolerance and inhibition of proinflammatory cytokine production (14). These TGF-β1 opposite roles make it an interesting biomarker for early evaluation of T1D and its chronic complications development.

The association of AGE-flRAGE and TGF-β1 exists during development of diabetes and diabetic nephropathy. TGF-β-activated kinase 1 stimulated by AGEs, plays an important role in innate immune responses and inflammation activating main proinflammatory pathways (mitogen-activated protein kinases - MAPKs and nuclear factor-κB), macrophage polarization from M2 to M1, and inflammatory cytokine production (16,17). Moreover, AGEs stimulate fibrotic process referring to appearance of myofibroblasts and the accumulation of ECM components via the TGF-β1-independent Smad3 signaling pathway (18).

Accordingly, the first aim of this study was to investigate whether changes in TGF-β1 and flRAGE gene expression in peripheral blood mononuclear cells (PBMCs) and TGF-β1 and sRAGE serum protein levels were associated to T1D autoimmunity presence. Our second goal was to contribute to current knowledge by identifying whether these gene expression and protein levels could be related to suboptimal/poor glycemic control and future diabetic chronic complication such as early elevation of urinary albumin excretion rate.

Materials and Methods

156 adolescents with T1D and 80 apparently healthy adolescents (the control group) were enrolled the study. The groups were matched by age, pubertal stage, and body mass index (BMI). The participants were recruited from the Mother and Child Health Care Institute of Serbia "Dr Vukan Čupić", Belgrade during regular follow in the outpatient clinic. Diabetes was diagnosed according to Serbian national and international guidelines of good clinical practice for diagnosis and treatment of diabetes mellitus (19,20). Patients with T1D were treated with intensive insulin therapy given as basal-bolus regimen. Good, suboptimal/poor glycemic control in T1D patients was defined according to glycated hemoglobin A1c (HbA1c) concentration cut-off of 7.5% (21). Exclusion criterion for T1D patients were medicaments usage (except insulin) and presence of cystic fibrosis and other autoimmune diseases such as thyroiditis and celiac disease. Exclusion criteria for the control group were positive family history of T1D and use of drugs and supplements.

In all study participants we performed these evaluations: demographic and clinical (age, body weight, body height, BMI, puberty staging, age at diabetes onset, diabetes duration, insulin dosage) and laboratory (glucose, creatinine, HbA1c, C-reactive protein (CRP), albumin in urine, glomerular filtration rate, TGF-β1 and RAGE gene expression and protein levels).

Body weight was measured to the nearest 0.1 kg by a portable electronic scale (Tanita, Amsterdam, Netherlands). Body height was measured to the nearest 0.1 cm using a portable wall-mounted stadiometer. BMI was calculated as body weight (kilograms) divided by square height (meters). Puberty stages were stratified by clinical examination according to Tanner (22).

Blood samples from all study participants were collected into two serum and one ethylenediaminetetraacetic acid (EDTA) containing vacutainers (BD Vacutainer®, New Jersey, USA). After venepunction, serum and plasma were immediately separated and stored at -80°C prior analyses.

Glucose and creatinine were assayed in serum samples using routine laboratory methods. HbA1c level was determined by competitive turbidimetric inhibition immunoassay. CRP was measured using immunoturbidimetric method. All the analyses were performed on Roche/Hitachi c501 automated analyser (Roche, Mannheim, Germany). Albumin in urine was determined in the timed overnight sample using nephelometer Siemens BN ProSpec® System (Siemens, Erlangen, Germany). Early elevation of albumin excretion rate cut-off was set as ≥ 7.5 μg/min because these values were shown to predict subsequent persistent microalbuminuria development later in life (23). Estimated glomerular filtration rate (eGFR) was assessed using Schwartz equation. The TGF-β1 protein levels were determined in serum samples and sRAGE protein levels were measured in plasma

using enzyme-linked immunosorbent assays (ELISA) according to the manufacturer's manual (DuoSet, R&D systems Wiesbaden, Germany).
PBMCs were isolated after plasma separation using the Ficoll-Paque® PLUS gradient-gel (GE Healthcare, Wisconsin, USA) according to the manufacturer's instructions. After isolation, but before freezing at -80°C, PBMC were suspended in 1 mL of TRIzol™ reagent (Invitrogen Life Technologies, Foster City, USA).
The total ribonucleic acid (RNA) from PBMCs was isolated using modified classic RNA isolation protocol, described by Chomczynski et al (24), which was optimized for the laboratory of the Department for Medical Biochemistry, University of Belgrade-Faculty of Pharmacy, in detail explained and published elsewhere (25).
Reverse transcription and quantitative real-time polymerase chain reaction (qPCR) experiments were performed on the 7500 Real-Time PCR System using Assays-on-Demand based on TaqMan™ chemistry (Applied Biosystems, Foster City, USA).
The qPCR reactions were performed using TaqMan™ 5'-nuclease gene expression assays (Applied Biosystems, Foster City, USA) for TGFβ1 (Hs00998133_m1) and flRAGE (Hs00153957_m1) genes. Relative standard curve method was used for gene expression quantification. Relative gene expression levels were expressed as a ratio between target gene and constitutively expressed gene as housekeeping gene (β-actin) messenger RNA (mRNA) using following equations:
Normalised TGF-β1 mRNA levels = TGF-β1 mRNA/β-actin mRNA
Normalised RAGE mRNA levels = RAGE mRNA/β-actin mRNA.
Negative controls for reverse transcription (no reverse transcriptase enzyme) and for qPCR (no complementary deoxyribonucleic acid) were included in the experiments.
The study was carried out in line with the principles of the Declaration of Helsinki and approved by Ethics Committees of Mother and Child Health Care Institute of Serbia "Dr Vukan Čupić" (protocol number: 8/8, date: April the 9th 2015) and University of Belgrade-Faculty of Pharmacy (protocol number: 2536/2, date: December 26th 2018). Written informed consent was obtained from all the participants and their parents.

Statistical analysis

All statistical testing was performed using a statistical program IBM® SPSS® Statistics version 22 (SPSS Inc., Chicago, USA). Distribution of continuous variables was tested by Kolmogorov-Smirnov test. Continuous normally distributed data were presented as arithmetic mean ± standard deviation (SD). If normal distribution was not achieved after logarithmic transformation, skewed distributed data were presented as median (interquartile range).
Comparisons between the tested groups were done by Student *t*-test for normally distributed data and by Mann-Whitney and Kruscal-Wallis tests for skewed distributed data. Categorical data were given as absolute frequencies and compared by Chi-square test for contingency tables. Associations between clinical data were tested by Spearman's bivariate correlation analysis. In-depth possible associations of tested markers with T1D and urinary albumin excretion rate were assessed by univariate and multivariate binary logistic and ordinal regression analyses, respectivly. Binary logistic regression analysis was used to find single predictors and models (mRNA and protein levels) as explanatory variables associated with T1D presence. Ordinal regression analysis was used as a statistical test to determine potential associations of single markers and models and T1D complication (early elevation of urinary albumin excretion rate). In multivariate ordinal regression analysis, there were no multicollinearity between independent variables (predictors) and all of them had an identical effect at each cumulative split of the ordinal dependent variable (urinary albumin excretion rate quartiles). Data from bivariate correlation were presented as correlation coefficient (ρ). Data from binary logistic and ordinal regression analyses were presented as odds ratio (OR) and 95% Confidence Interval (CI). Explained variations in T1D and urinary albumin excretion rate were assessed by the Nagelkerke R^2. The statistically significant level was set at $P < 0.05$.

Results

Clinical and laboratory data in tested groups

General anthropometric and biochemical data of tested populations were presented in the Table 1.
The median age for 156 T1D adolescents (49% females) was (12-16) years. The median T1D duration was 7 (5-8) years. 34 out of 156 adolescents had good glycemic control, 123 had suboptimal/poor glycemic control. In the control group of 80 participants (82% females) median age was 15 (13-17) years. No statistically significant di erences between T1D patients and the controls were seen in ages and BMI. However, significantly more females than males were in the control compared to patient group (P<0.001). Adolescents in the control group had significantly lower glucose, HbA1c and CRP than patients with T1D (Table 1).
TABLE 1
Patients with T1D had significantly lower TGF-β1 mRNA and flRAGE mRNA levels (Figure 1A and C), but higher TGF-β1 and sRAGE concentrations than controls (Figure 1B and D).
FIGURE 1
In order to examine whether discrepant gender distribution in tested groups could affect obtained results, firstly we compared clinical markers between males and females of the control group, secondly, we compared only females and finally we compared only males between patient and control groups. There were no significant differences in any clinical marker between genders (66 females *vs* 14 males) of the control group. The same trends for examined markers were found between females from control group (N=66) *vs* patient group (N=76) and males from control group (N=14) *vs* patient group (N=80) as we already obtained when we analysed both genders joined in each examined group (Tables 1 and 2, Figure 1). Briefly, glucose, HbA1c, CRP, TGF-β1, sRAGE levels were significantly higher in patients compared to controls for each gender. TGF-β1 mRNA and flRAGE mRNAs were significantly lower in patients compared to controls for each gender. Accordingly, we performed further statistical analysis in the initially formed groups.

Binary logistic regression of mRNA and protein levels for association with T1D

We further investigated whether TGF-β1 mRNA and flRAGE mRNA levels and TGF-β1, sRAGE and CRP levels were associated with T1D (Table 2). Significant ORs obtained in univariate binary logistic regression analysis were evident for all tested markers indicating their significant associations with T1D. Nagelkerke R^2 showed that each predictor TGF-β1 mRNA, flRAGE mRNA, sRAGE protein, TGF-β1 protein and CRP, in univariate analysis could explain the variation in T1D development by 24.1%, 17.8%, 14%, 8.2% and 6.6%, respectively. These predictors were further adjusted for demographic and laboratory variables which were significantly different between tested groups or implicated in T1D development, to assess their possible independent associations with T1D. Those variables were gender, age and CRP. As presented by the Model 1 TGF-β1 mRNA and flRAGE mRNA levels were independently negatively associated with T1D (OR=0.284, $P<0.001$ and OR=0.396, $P<0.001$, respectively). On the other hand, CRP, TGF-β1 and sRAGE protein levels was independently positively associated with T1D (OR=1.438, $P=0.018$, OR=1.037, $P=0.002$ and OR=3.552, $P<0.001$, respectively) (Table 2).

TABLE 2

Correlation analyses of mRNA and protein levels with other clinical and laboratory markers in T1D patients

Next, we conducted Spearman's correlation analysis to test bivariate associations between TGF-β1 mRNA, flRAGE mRNA, TGF-β1 and sRAGE protein levels with other markers in patients with T1D (Table 3). We found that lower TGF-β1 mRNA levels were associated with older age of diabetes onset and higher urinary albumin excretion rate. Lower flRAGE mRNA levels were related to higher serum creatinine levels and lower eGFR. sRAGE and TGF-β1 protein levels correlated positively with diabetes duration and negatively with eGFR. Also, TGF-β1 protein correlated positively with age and creatinine level (Table 3). Furthermore, TGF-β1 mRNA and flRAGE mRNA levels were in mutual positive correlation, as well as sRAGE and TGF-β1 protein concentrations. There were no significant correlations either between TGF-β1 mRNA and its protein levels or between flRAGE mRNA and sRAGE levels.

TABLE 3

mRNA and protein levels according to glycemic control in T1D patients

Additionally, we wanted to test whether good or suboptimal/poor regulated T1D could influence TGF-β1 mRNA and flRAGE mRNA, TGF-β1 and sRAGE protein levels, as well as CRP concentration. Consequently, we divided group of adolescents with T1D into two subgroups (0 – HbA1c<7.5%; 1 – HbA1c≥7.5%). No significant differences in mRNA and protein levels, according to glycemic control were determined. Also, no significant correlations were evident between HbA1c and mRNA and protein levels in T1D patients (Table 3). However, adolescents with suboptimal/poor glycemic control (median: 0.85 mg/L, interquartile range: 0.40-2.30 mg/L) had significantly higher CRP concentration ($P=0.020$) than those with good glycemic control (median: 0.50 mg/L, interquartile range: 0.30-0.75 mg/L).

mRNA and protein levels according to urinary albumin excretion rate quartiles in T1D patients

Our further intention was to determine whether TGF-β1 mRNA and flRAGE mRNA and TGF-β1 and sRAGE protein levels were associated with early elevation of urinary albumin excretion rate in T1D patients. To achieve this, urinary albumin excretion rate values were divided into quartiles. Each quartile consisted of 39 participants. Early elevation of albumin excretion defined as ≥ 7.5 μg/min corresponds to the fourth quartile. TGF-β1 mRNA levels were significantly different between quartiles ($P=0.025$) being lower in the fourth than in the first quartile group ($P=0.005$) (Figure 2A). There were no significant differences in other tested markers between urinary albumin excretion rate quartile groups (Figure 2B, 2C, 2D). Also, we were not able to determine significant differences in CRP concentration between quartile groups ($P=0.967$) (data not presented in Figure 2).

FIGURE 2

Ordinal regression analysis of TGF-β1 mRNA levels for association of early elevation of urinary albumin excretion rate in T1D patients

Due to the significantly high negative correlation between TGF-β1 mRNA and urinary albumin excretion rate in T1D patients (Table 3), our further intention was to determine whether in-depth association between them exists. To achieve this, univariate and multivariate ordinal regression analysis was performed. TGF-β1 mRNA levels showed significant ORs for urinary albumin excretion rate in univariate analysis (OR=0.278, 95% CI: 0.126-0.612, $P=0.001$). Nagelkerke R^2 for TGF-β1 mRNA levels was 0.140. Multivariate analysis revealed independent association of TGF-β1 mRNA levels with early elevation of urinary albumin excretion rate when tested with other clinical variables which might be implicated in its elevation. Those variables were age, diabetes duration, CRP and HbA1c. Decrease in TGF-β1 mRNA levels, increased the probability for elevation of urinary albumin excretion rate (OR=0.309, 95% CI: 0.136-0.698, $P=0.005$). Nagelkerke R^2 of 0.162 indicated that multivariate regression model could explain 16.2% variation in urinary albumin excretion rate.

Discussion

The present study demonstrated that decrease in TGF-β1 mRNA levels were independently associated with T1D and early elevation of urinary albumin excretion rate, while CRP, TGF-β1 and sRAGE protein concentrations were independently associated with T1D only. Also, adolescents with T1D expressed lower flRAGE mRNA levels than controls which were independently associated to T1D, but not to early elevation of urinary albumin excretion rate. None of tested markers were related to glycemic control in T1D adolesents.

As autoimmune disease accompanied by chronic inflammation, T1D tends to develop in childhood (1). Regardless, vascular complications can be detected in adolescents after 5-years duration of T1D indicating their faster development than in adults (26). Such complications are one of the most important causes of mortality in T1D patients. Although autoantibodies assessment in blood is gold standard for identification of patients with T1D or at-risk patients (27), new biomarkers are required to point at β-cells destruction and monitor the T1D progression in clinical practice (4).

It is very difficult to obtain samples of body organs, e.g. pancreas, blood vessel endothelium or kidneys in adolescents. However, PBMC, lymphocytes and monocytes, can serve as surrogate cells for RNA isolation and gene expression determination (28). Gene

expression profiles in peripheral blood immune cells may provide new insights into the T1D pathogenesis (4,28). Still there are contradictory results and conflicting explanations on RAGE and TGF-β1 gene expression in PBMC of T1D patients published in scientific literature so far (3,29-32,33).

TGF-β1 as multifunctional cytokine, produced by virtually all cells in human organism, has opposite roles in certain body tissues (14,15). Demonstrated by Saxena and colleagues (14), TGF-β1 plays a dual role during the development and progression of systemic autoimmune-inflammatory disease in mice. Reduced TGF-β1 synthesis by immune cells indicated autoimmune onset in early life. Also, TGF-β1 produced by Treg cells was shown to inhibit autoantibody synthesis. On the other hand, increased TGF-β1 synthesis in other tissues predisposes local fibrogenesis and likely leads to organ damage, e.g. kidney injury (14). Results from our study supported these findings. We found out significantly lower TGF-β1 gene expression in PBMC from T1D patients compared to healthy adolescents (P<0.001). On the other hand, TGF-β1 protein concentration in T1D patients was significantly higher than in controls (P<0.001). This was expected because TGF-β1 production is enhanced in other peripheral organs in children with T1D (34). Furthermore, although they were not in mutual correlation, downregulation in TGF-β1 gene and increase in TGF-β1 protein levels were found to be independently associated with T1D development (P<0.001 and P=0.002, respectively). Adolescents with lower TGF-β1 mRNA levels were 71.6% more likely to exhibit T1D than those with higher levels. However, odds of having T1D was 1.037 times greater in adolescents with higher TGF-β1 concentration.

TGF-β1 downregulation has been previously reported in PBMC of children with T1D indicating depressed immunity in patients with long-term T1D (29). Tolerance against self-antigens can be maintained through activation of Treg cells which produce TGF-β1 (14). However, in our adolescents with T1D downregulation of TGF-β1, reflected as lower mRNA levels than in controls was evident. This apparently suggested maturation of T helper 1 lymphocytes which could have been implicated in destruction of pancreatic β-cells (35). Nevertheless, dysregulation of TGF-β1 expression by Treg cells occurs even during the pre-diabetic stage (29). Children positive to islet cell antibodies had significantly lower TGF-β1 mRNA levels than controls (29). In our study, another confirmation of immune cell dysregulation was demonstrated by flRAGE mRNA levels which were lower in T1D patients than in controls (P<0.001). Membrane RAGE also modulates Treg cells function and suppresses the autoimmune response and its downregulation has been implicated T1D development (10,36). Decrease in flRAGE mRNA levels was found to be independently associated with T1D development (P<0.001). Adolescents with lower flRAGE mRNA levels were 60.4% more likely to exhibit T1D than those with higher levels. Additionally, positive correlation between TGF-β1 mRNA and flRAGE mRNA levels (P<0.01) was evident in T1D cohort suggesting potential interplay of their signalling pathways in T1D pathogenesis.

In contrast to our and other studies, Jin and associates (33) published the study on gene expression profiles in human PBMC of T1D patients using microarray technology. They analysed 18 genes involved in inflammation and immunity, among which TGF-β1 showed higher expression in T1D patients than in controls. These authors suggested that increase in TGF-β1 gene expression may have a role in T1D through stimulation of proinflammatory cellular pathways in PBMC (33).

T1D as chronic metabolic disease causes micro- and macro vascular complications in years to come (26). Disturbance in blood vessel integrity correlates with poor glycemic control and diabetes duration (37). Heier and co-workers demonstrated that after 5 years of diabetes onset, the accelerated atherosclerosis was evident in children with T1D (37). Though, microalbuminuria has been related to diabetic nephropathy development and progression (38), it has recently been recognized as independent predictor for endothelial dysfunction and cardiovascular disease (CVD) (39).

In our T1D study participants with average diabetes duration of 7 years, glycemic control defined by HbA1c seemed not to have any influence on the main tested markers. TGF-β1 mRNA, flRAGE mRNA, TGF-β1 and sRAGE protein levels did not differ between T1D adolescents with good *vs* suboptimal/poor glycemic control. Also, any correlations between them and HbA1c were not determined. However, when urinary albumin excretion rate values were divided into quartiles, TGF-β1 mRNA levels were lower in the fourth compared to the first quartile group (P=0.005). The fourth quartile corresponds to early elevation of urinary albumin excretion rate that could predict permanent micro- and later macro-albuminuria (23). Interestingly, our results indicated independent associations of lower TGF-β1 mRNA levels with elevated urinary albumin excretion rate in T1D adolescents (P=0.005). Adolescents having T1D with lower TGF-β1 gene expression were 69.1% more likely to have elevated urinary albumin excretion rate than those with higher expression. In our group of T1D adolescents with average diabetes duration of 7 years, TGF-β1 mRNA levels could be used as a potential biomarker for CVD risk assessment indicating not only dysregulation of immune response to autoantigens, but also predicting future cardiovascular complications. It was expected that TGF-β1 protein, as fibrogenic factor, correlates to urinary albumin excretion rate (14). However, this was not supported by our results. Yet, TGF-β1 protein levels in blood correlated significantly positively with creatinine (P<0.05) and negatively with eGFR (P<0.05) linking TGF-β1 with potential future renal function decline in T1D patients.

Miura and co-workers demonstrated that lower cell surface RAGE expression in monocytes of children with T1D could be partly explained by enhanced ligand binding, indicating the imbalance in receptors function on monocytes making them more prone to modification in subcellular space (30). Moreover, patients with incipient or clinical diabetic nephropathy showed significant decrease in monocyte RAGE mRNA levels compared to patients without nephropathy. Our results may support these findings. We did not determine AGE concentration in blood of our participants and were not able to explain lowering RAGE expression with AGE engagement like Miura's team, but obviously a relation of lowering flRAGE mRNA and future diabetic vascular complications was apparent in our study.

sRAGE, made by protein cleavage of extracellular ligand binding domain of transmembrane RAGE, has been suggested to be a biomarker for vascular diseases development (11-13). Our results indicated that higher sRAGE levels were independently associated with T1D (P<0.001). Odds of having T1D was 3.552 times greater in adolescents with higher sRAGE concentration. However, sRAGE were not related to urinary albumin excretion rate. It correlated significantly positively with diabetes duration

(P<0.01) and negatively with eGFR (P<0.05). Not only mRNA levels, but TGF-β1 and sRAGE protein levels correlated significantly positively (P<0.01), pointing out to one more evidence for TGF-β1 and sRAGE proteins probable mutual implication in T1D pathogenesis. These results could support sRAGE potent pro-atherogenic besides immunomodulating properties (11). Because T1D is immuno-inflammatory disease, CRP levels were expected to be increased in adolescents with T1D (37). According to our results, CRP levels were significantly higher in patients with T1D than in the control group and also were independently associated with T1D. These results are in line with the fact that not only autoimmune process, but inflammation is necessary for efficient destruction of islet β-cells and progression of diabetic chronic complications (40).

Changes in TGF-β1 mRNA and flRAGE mRNA, as well as in TGF-β1 and sRAGE protein levels were evident between patients and controls. Disturbances in TGF-β1 and flRAGE gene expression levels in PBMC start from pre-diabetic stage and persist during development of T1D (10,29,36,41). Therefore, mRNA measurement would be beneficial to perform at early age when there is a suspected onset of diabetes e.g. positive family history. The same can account for TGF-β1 and sRAGE protein levels. Also, TGF-β1 mRNA levels should be determined at T1D diagnosis for possible diabetes complications assessment (37,38) However, due to possible effects of acute and chronic hyperglycemia (5,41-43) on these markers, multiple measurements in the prospective manner would be strongly recommended.

Study Limitations

This study has several limitations. Firstly, this research was carried out as cross-sectional study which demonstrated significant associations between tested markers and T1D presence and urinary albumin excretion rate but not causal relationships between them. However, our findings need to be confirmed in prospective studies to determine whether progressive downregulation of TGF-β1 gene could follow microalbuminuria advancement. Secondly, TGF-β1 and RAGE protein concentration determination in PBMC of patients and controls should be addressed in future studies together with their mRNA levels to demonstrate whether their protein levels were lower like mRNA levels in T1D and to confirm immunomodulatory dysfunction in those cells of T1D patients. Finally, although presence of significantly more females than males in the control group did not skewed the results and conclusions of this study, inclusion of more males would have been more beneficial for our current findings. Nevertheless, our current study might present the basis for future research.

Conclusion

In conclusion, the T1D onset goes hand in hand with lower PBMC TGF-β1 mRNA and flRAGE mRNA levels together with higher secretions of the proinflammatory cytokines TGF-β1 and sRAGE by other cells in organism, as well as systemic low-grade inflammation. In addition, downregulation of TGF-β1 gene might be used as a potential biomarker for early CVD risk assessment in adolescents with T1D due to its independent significant negative association with the urinary albumin excretion rate.

Authorship Contributions

All authors have contributed to this article.

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Conflict of interest statement

The authors declare that they have no conflicts of interest.

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References

1. Atkinson MA, Maclaren NK. The pathogenesis of insulin-dependent diabetes mellitus. N Engl J Med 1994;331:1428–1436.
2. Bach JF. Insulin□dependent diabetes mellitus as a beta□cell targeted disease of immunoregulation. J Autoimmun 1995;8:439–463.
3. Han D, Leyva C, Matheson D, Mineo D, Messinger S, Blomberg B, Hernandez A, Meneghini L, Allende G, Skyler J, Alejandro R, Pugliese A, Kenyon S. Immune profiling by multiple gene expression analysis in patients at-risk and with type 1 diabetes. Clin Immunol 2011;139:290-301.
4. Jin Y, Sharma A, Bai S, Davis C, Liu H, Hopkins D, Barriga K, Rewers M, She JX. Risk of type 1 diabetes progression in islet autoantibody-positive children can be further stratified using expression patterns of multiple genes implicated in peripheral blood lymphocyte activation and function. Diabetes 2014;63:2506-2515.
5. Cooper ME. Importance of advanced glycation end products in diabetes associated cardiovascular and renal disease. Am J Hypertens 2004;17:31-38.
6. Xue J, Rai V, Singer D, Chabierski S, Xie J, Reverdatto S, Burz D, Schmidt AM, Hoffmann R, Shekhtman A. Advanced glycation end product recognition by the receptor for AGEs. Structure. 2011;19:722–32
7. Gebhardt C, Riehl A, Durchdewald M, Németh J, Fürstenberger G, Müller-Decker K, Enk A, Arnold B, Bierhaus A, Nawroth P, Hess J, Angel P. RAGE signaling sustains inflammation and promotes tumor development. J Exp Med 2008;205:275–85.
8. Brownlee M, Cerami A, Vlassara H. Advanced glycosylation end products in tissue and the biochemical basis of diabetic complications. N Engl J Med 1988:318:1315–21.
9. Durning S, Preston-Hurlburt P, Clark P, Xu D, Herold K. Type 1 Diabetes Trial Net Study Group. The Receptor for Advanced Glycation Endproducts Drives T Cell Survival and Inflammation in Type 1 Diabetes Mellitus. J Immunol 2016;197:3076-3085.

10. Wild CA, Bergmann C, Fritz G, Schuler P, Hoffmann T, Lotfi R, Westendorf A, Brandau S, Lang S. HMGB1 conveys immunosuppressive characteristics on regulatory and conventional T cells. Int Immunol 2012;24:485–494.
11. Ostendorp T, Weibel M, Leclerc E, Kleinert P, Kroneck P, Heizmann C, Fritz G. Expression and purification of the soluble isoform of human receptor for advanced glycation end products (sRAGE) from Pichia pastoris. Biochem Biophys Res Commun 2006;347:4-11.
12. Park L, Raman KG, Lee KJ, Lu Y, Ferran L, Chow W, Stern D, Schmidt AM. Suppression of accelerated diabetic atherosclerosis by the soluble receptor for advanced glycation endproducts. Nat Med 1998;4:1025-1031.
13. Liu Y, Liang C, Liu X, Liao B, Pan X, Ren Y, Fan M, Li M, He Z, Wu J. AGEs increased migration and inflammatory responses of adventitial fibroblasts via RAGE, MAPK and NF-κB pathways. Atherosclerosis 2010;208:34-42.
14. Saxena V, Lienesch D, Zhou M, Bommireddy R, Azhar M, Doetschman T, Singh RR.
Dual roles of immunoregulatory cytokine TGF-beta in the pathogenesis of autoimmunity-mediated organ damage. J Immunol 2008;180:1903-1912.
15. Border WA, Noble NA. Targeting TGF-beta for treatment of disease. Nat Med 1995;1:1000–1.
16. Xu X, Qi X, Shao Y, Li Y, Fu X, Feng S, Wu Y. Blockade of TGF-β-activated kinase 1 prevents advanced glycation end products-induced inflammatory response in macrophages. Cytokine. 2016;78:62-8.
17. Ma FY, Tesch GH, Ozols E, Xie M, Schneider MD, Nikolic-Paterson DJ. TGF-β1-activated kinase-1 regulates inflammation and fibrosis in the obstructed kidney. American Journal of Physiology-Renal Physiology. 2011;300:1410-21.
18. Li JH, Huang XR, Zhu HJ, Oldfield M, Cooper M, Truong LD, Johnson RJ, Lan HY. Advanced glycation end products activate Smad signaling via TGF□β□dependent and□independent mechanisms: implications for diabetic renal and vascular disease. The FASEB Journal. 2004;18:176-8.
19. Ministry of Health of Republic Serbia, The good clinical practice national guidelines on diabetes mellitus, In: Agency for the Accreditation of Health Care Institutions of Serbia, Belgrade, 2012.
20. Silverstein J, Klingensmith G, Copeland K, Plotnick L, Kaufman F, Laffel L, Deeb L, Grey M, Anderson B, Holzmeister LA, Clark N. American Diabetes Association. Care of children and adolescents with type 1 diabetes: a statement of the American Diabetes Association. Diabetes Care 2005;28:186–212.
21. American Diabetes Association. 6. Glycemic targets. Diabetes Care 2015;38:33–40.
22. Tanner JM. Growth at Adolescence. Oxford, England: Blackwell Scientific Publications; 1962.
23. Stone ML, Craig ME, Chan AK, Lee JW, Verge CF, Donaghue KC. Natural history and risk factors for microalbuminuria in adolescents with type 1 diabetes: a longitudinal study. Diabetes Care 2006;29:2072–2077.
24. Chomczynski P. A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples. Biotechniques 1993;15:532-537.
25. Vujovic A, Spasojevic-Kalimanovska V, Bogavac-Stanojevic N, Spasic S, Kotur- Stevuljevic J, Jelic-Ivanovic Z. Comparison of two RNA isolation methods for determination of SOD1 and SOD2 gene expression in human blood and mononuclear cells. Indian J Biotechnol 2013;12:468-474.
26. Amin R, Widmer B, Prevost AT, Schwarze P, Cooper J, Edge J, Marcovecchio L,
Neil A, Dalton N, Dunger DB. Risk of microalbuminuria and progression to macroalbuminuria in a cohort with childhood onset type 1 diabetes: prospective observational study. BMJ 2008;336:697–701.
27. Ziegler AG, Rewers M, Simell O, Simell T, Lempainen J, Steck A, Winkler C, Ilonen J, Veijola R, Knip M, Bonifacio E, Eisenbarth GS. Seroconversion to multiple islet autoantibodies and risk of progression to diabetes in children. JAMA 2013;309:2473–2479.
28. Kaizer E, Glaser C, Chaussabel D, Banchereau J, Pascual V, White P. Gene expression in peripheral blood mononuclear cells from children with diabetes. J Clin Endocrinol Metab 2007;92:3705-3711.
29. Halminen M, Simell O, Knip M, Ilonen J. Cytokine expression in unstimulated PBMC of children with type 1 diabetes and subjects positive for diabetes-associated autoantibodies. Scand J Immunol 2001;53:510–513.
30. Miura J, Uchigata Y, Yamamoto Y, Takeuchi M, Sakurai S, Watanabe T, Yonekura H, Yamagishi S, Makita Z, Sato A, Omori Y, Yamamoto H, Iwamoto Y. AGE down-regulation of monocyte RAGE expression and its association with diabetic complications in type 1 diabetes. J Diabetes Complications 2004;18:53-59.
31. Abbasi F, Amiri P, Sayahpour F, Pirmoradi S, Abolhalaj M, Larijani B, Bazzaz TJ, Amoli MM. TGF-β and IL-23 gene expression in unstimulated PBMCs of patients with diabetes. Endocrine 2012;41:430-434.
32. Łuczynski W, Stasiak-Barmuta A, Juchniewicz A, Wawrusiewicz-Kurylonek N, Iłendo E, Kos J, Krętowski A, Górska M, Chyczewskî L, Bossowski A. The mRNA expression of pro- and anti-inflammatory cytokines in T regulatory cells in children with type 1 diabetes. Folia Histochem Cytobiol 2010:48;93–100.
33. Jin Y, Sharma A, Carey C, Hopkins D, Wang X, Robertson D, Bode B, Anderson SW, Reed JC, Steed RD, Steed L, She JX. The expression of inflammatory genes is upregulated in peripheral blood of patients with type 1 diabetes. Diabetes Care 2013;36:2794-2802.
34. Zorena K, Raczyńska D, Wiśniewski P, Malinowska E, Myśliwiec M, Raczyńska K, Rachoń D. Relationship between serum transforming growth factor β 1 concentrations and the duration of type 1 diabetes mellitus in children and adolescents. Mediators Inflamm 2013;2013:849457. doi: 10.1155/2013/849457.

35. Bettelli E. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. Nature 2006;441:235–238.
36. Akirav E, Preston-Hurlburt P, Garyu J, Henegariu O, Clynes R, Schmidt A, Herold KC. RAGE Expression in Human T Cells: A Link between Environmental Factors and Adaptive Immune Responses. PLoS ONE 2012; 7(4): e34698. doi:10.1371/journal.pone.0034698.
37. Heier M, Margeirsdottir H, Brunborg C, Hanssen K, Dahl-Jørgensen K, Seljeflot I. Inflammation in childhood type 1 diabetes; influence of glycemic control. Atherosclerosis 2015;238:33-37.
38. Bogdanovic R. Diabetic nephropathy in children and adolescents. Pediatr Nephrol 2008;23:507–525.
39. Stehouwer C, Smulders Y. Microalbuminuria and risk for cardiovascular disease: Analysis of potential mechanisms. J Am Soc Nephrol 2006;17:2106-11.
40. Green E, Flavell R. The temporal importance of TNF expression in the development of diabetes. Immunity 2000;12:459–469.
41. Flores L, Näf S, Hernaez R, Conget I, Gomis R, Esmatjes E. Transforming growth factor β1 at clinical onset of type 1 diabetes mellitus. A pilot study. Diabetic medicine. 2004;21:818-822.
42. Sato Iwano M, Akai Y, Kurioka Kubo A, Yamaguchi T, Hirata E, Kanauchi M, Dohi K. Increased excretion of urinary transforming growth factor β1 in patients with diabetic nephropathy. Am J Nephrol 1998;18:490–494.
43. Perkins R, Miranda ER, Karstoft JK, Beisswenger PJ, Solomon TP, Haus JM. Effects of Acute Experimental Hyperglycemia on Oxidative Markers and AGE-RAGE Dynamics in Obese Humans. Diabetes 2018; 67(Supplement 1): https://doi.org/10.2337/db18-273-LB.

Figure 1

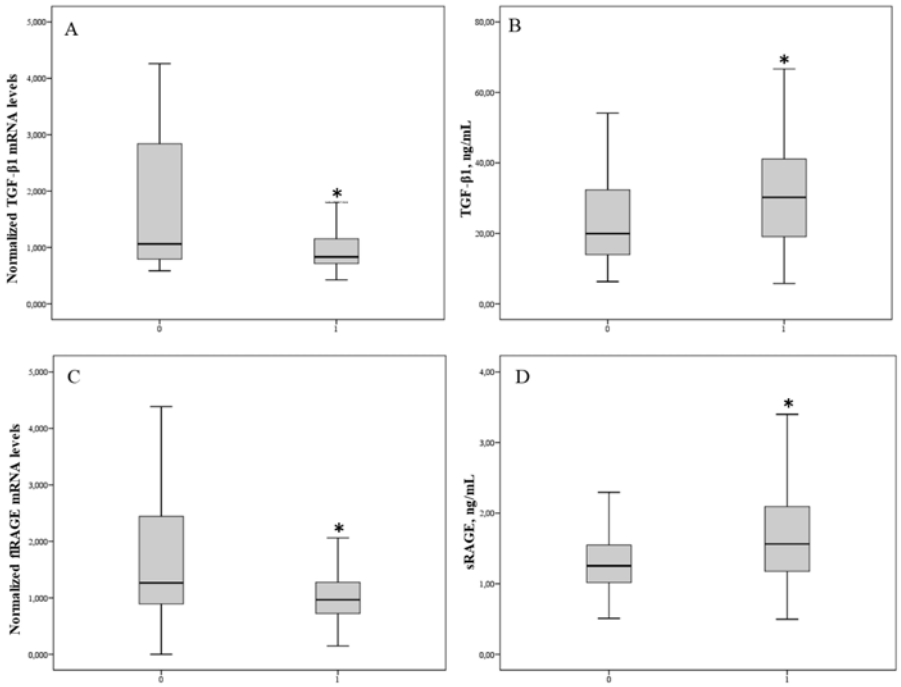

Figure 1. RAGE and TGF-β1 normalised mRNA levels and protein concentrations between tested groups

Data are presented as median (interquartile range) and compared by Mann-Whitney test.
*P<0.001
0-Control group; 1- T1D

T1D: Type 1 diabetes mellitus, TGF-β1: Transforming growth factor β1, flRAGE: full-length receptor for advanced glycation end product, sRAGE: solubile receptor for advanced glycation end products, mRNA: messenger ribonucleic acid

Figure 2

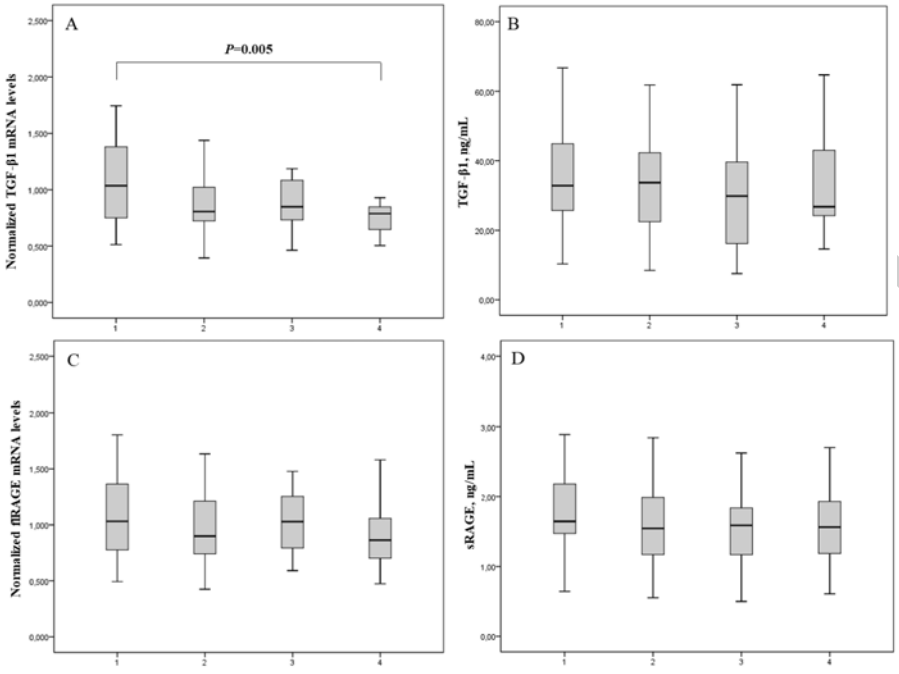

Figure 2. TGF-β1 and flRAGE mRNA levels, TGF-β1 and sRAGE protein concentration in adolescents with T1D according to urinary albumin excretion rate quartiles

Data are presented as median (interquartile range) and compared by Kruscal Wallis and Mann-Whitney tests.
1 - the first quartile (≤2.92 μg/min); 2 – the second quartile group (2.93-4.24 μg/min); 3 – the third quartile group (4.25-7.49 μg/min); 4 – the fourth quartile group (≥7.5 μg/min). Each quartile consisted of 39 participants.

TGF-β1: Transforming growth factor β1, flRAGE: full-length receptor for advanced glycation end product, sRAGE: solubile receptor for advanced glycation end products, mRNA: messenger ribonucleic acid

Table 1. General characteristics and biochemical markers of two study groups

	T1D	**Control group**	***P***
Sex, N	Males: 80 Females: 76	Males: 14 Females: 66	<0.001
Age, years	14 (12-16)	15 (13-17)	0.078
BMI, kg/m^2	20.62 (18.18-22.62)	20.50 (19.10-22.70)	0.064
Tanner stage, N			
Stage 1	17	8	
Stage 2	13	6	
Stage 3	25	9	0.367
Stage 4	26	8	
Stage 5	75	49	
Diabetes duration, years	7 (5-8)	/	/
Age at diabetes onset, years	7 (5-10)	/	/
Insulin dosage, U/kg/day	1.02 (0.86-1.21)	/	/
Glucose, mmol/L	10.72 (7.75-15.26)	4.81 (4.64-5.08)	<0.001
HbA1c, %	7.80 (7.10-8.77)	5.00 (4.80-5.10)	<0.001
Glycemic control, N Good / Suboptimal and Poor	33 / 123	/	/
Creatinine, μmol/L [a]	74.54 ± 13.47	76.18 ± 14.25	0.393
eGFR, mL/min/1.73 m²	79.78 (73.31-86.99)	78.00 (70.00-87.00)	0.305
Urinary albumin excretion rate, μg/mina	4.20 (2.90-7.60)	/	/
CRP, mg/L	0.70 (0.30-1.70)	0.40 (0.20-0.90)	0.001

Data are presented as median (interquartile range) and compared by Mann-Whitney test.
[a] Data are presented as arithmetic mean±SD and compared by Student *t*-test.
Categorical variables are presented as absolute frequencies and compared by Chi-squared test for contingency tables.
T1D: Type 1 diabetes mellitus, BMI: body mass index, HbA1c: glycated hemoglobin A1c, CRP: C-reactive protein, eGFR:estimated glomerular filtration rate

Table 2. Univariate and multivariate binary logistic regression analysis for the associations of tested markers and T1D development

Univariate	**Unadjusted OR (95%CI)**	**P**	**Nagelkerke R^2**
CRP, mg/L	1.421 (1.080-1.870)	0.012	0.066
TGF-β1 mRNA	0.347 (0.239-0.503)	<0.001	0.241

TGF-β1, ng/mL	1.040 (1.018-1.063)	<0.001	0.082
flRAGE mRNA	0.412 (0.291-0.585)	<0.001	0.178
sRAGE, ng/mL	3.969 (2.177-7.237)	<0.001	0.140
Multivariate	**Adjusted OR (95%CI)**	**P**	**Nagelkerke R^2**
CRP, mg/L [a]	1.438 (1.064-1.945)	0.018	0.213
TGF-β1 mRNA [b]	0.284 (0.176-0.457)	<0.001	0.394
TGF-β1, ng/mL [b]	1.037 (1.013-1-062)	0.002	0.264
flRAGE mRNA [b]	0.396 (0.264- 0.595)	<0.001	0.323
sRAGE, ng/mL [b]	3.552 (1.857- 6.792)	<0.001	0.314

[a] Adjusted for gender (categorical variable), age (continuous variable).
[b] Adjusted for gender (categorical variable), age and CRP (continuous variables).

T1D: Type 1 diabetes mellitus, OR: odds ratio, CI: Confidence Interval, CRP: C-reactive protein, TGF-β1: Transforming growth factor β1, flRAGE: full-length receptor for advanced glycation end product, sRAGE: solubile receptor for advanced glycation end products, mRNA: messenger ribonucleic acid

Table 3. Significant correlations between flRAGE and TGF-β1 mRNA and protein concentrations with demographic data and biochemical markers in adolescents with T1D

	TGF-β1 mRNA	TGF-β1, ng/mL	flRAGE mRNA	sRAGE, ng/mL
Age, years	-0.087	0.164*	-0.112	0.041
Tanner stage, N	-0.006	-0.097	-0.106	-0.077
Diabetes duration, years	0.069	0.243**	-0.086	0.210**
Age at diabetes onset, years	-0.176*	0.114	-0.012	-0.111
BMI, kg/m^2	0.067	0.045	-0.089	-0.098

Insulin dosage, U/kg/day	0.058	0.139	-0.025	0.006
Glucose, mmol/L	0.066	0.113	0.047	0.024
HbA1c, %	-0.057	0.109	0.070	-0.058
Creatinine, µmol/L	-0.061	0.181*	-0.186*	0.138
eGFR, mL/min/1.73 m²	0.032	-0.177*	0.162*	-0.163*
Urinary albumin excretion rate, µg/min	-0.292**	-0.052	-0.133	-0.098
CRP, mg/L	-0.090	0.079	-0.088	-0.109
TGF-β1 mRNA	-	0.125	0.273**	-0.060
TGF-β1, ng/mL	0.125	-	-0.047	0.221**
flRAGE mRNA	0.273**	-0.047	-	-0.107
sRAGE, ng/mL	-0.060	0.221**	-0.107	-

Data are presented as correlation coefficient (ρ).
*P<0.05, **P<0.01
TGF-β1: Transforming growth factor β1, flRAGE: full-length receptor for advanced glycation end products, sRAGE: solubile receptor for advanced glycation end product, mRNA: messenger ribonucleic acid, BMI – body mass index, HbA1c - glycated hemoglobin, eGFR – estimated glomerular filtration rate.