

# Association Between Serum Levels of the Soluble Receptor (sRAGE) for Advanced Glycation Endproducts (AGEs) and their Receptor (RAGE) in Peripheral Blood Mononuclear Cells of Children with Type 1 Diabetes Mellitus

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## ABSTRACT

**Aim:** The binding of AGEs to RAGE is involved in diabetic vascular complications. We studied sRAGE levels and RAGE protein expression (P) together with N-carboxymethyl lysine (CML), a major AGE, in 74 patients with type 1 diabetes mellitus (DM1) and 43 healthy (C) children.

**Methods:** sRAGE and CML levels were determined by ELISA and RAGE P was evaluated in mononuclear cells by Western immunoblotting.

**Results:** Serum sRAGE was higher in DM1 than in C ( $1430 \pm 759$  vs  $1158 \pm 595$  pg/ml,  $p = 0.047$ ), inversely correlated to diabetes duration ( $r = -0.265$ ,  $p = 0.037$ ) and directly correlated to LDL-cholesterol levels ( $r = 0.224$ ,  $p = 0.039$ ). Diabetes duration correlated independently with sRAGE ( $p = 0.034$ ). Circulating CML levels were not significantly different between DM1 and C groups ( $3.51 \pm 1.49$  vs  $3.59 \pm 1.83$  ng/ml,  $p > 0.05$ ) and RAGE P was lower in DM1 than in C ( $61 \pm 46$  vs  $102 \pm 63\%$ ,  $p = 0.0001$ ).

**Conclusions:** Increased serum sRAGE in children with DM1 may provide temporary protection against cell damage and may be sufficient to eliminate excessive circulating CML.

## KEY WORDS

type 1 diabetes mellitus, advanced glycation end-products, RAGE, sRAGE

## INTRODUCTION

The development of vascular complications (micro- and macro-angiopathy) is common in patients with poorly controlled type 1 diabetes mellitus (DM1) and over time it may bring about significant morbidity and early mortality to some affected patients<sup>1</sup>. Recent observations suggest an emerging role for advanced glycation endproducts (AGEs) in the pathogenesis of diabetic complications<sup>2</sup>.

The glycation reaction (glycosylation without enzymatic intervention) is driven by hyperglycemia. Protein glycation is initiated by the reaction between a free amino group and the carbonyl group of a sugar to form a reversible Schiff base in a period of hours. The latter can rearrange into a stable ketoamine or Amadori product over a period of days. The Amadori product can be transformed in a period of weeks into reactive dicarbonyl products to form AGEs<sup>3</sup>. Other pathways for AGE formation have also been described, as AGEs are a heterogeneous group of proteins, the best characterized of which is N<sup>ε</sup>-(carboxymethyl)lysine (CML), one of the major AGEs *in vivo*<sup>4</sup>.

The formation and accumulation of AGEs have been known to progress at an accelerated rate in

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**Abbreviations:** AGEs = advanced glycation endproducts; RAGE = receptor for AGEs; sRAGE = soluble receptor for AGEs.

diabetes mellitus. Non-crosslinking AGEs may accumulate in the tissues in diabetes mellitus and exert key influences on the vasculature by engagement of distinct cell surface receptors, such as RAGE (receptor for advanced glycation end-products)<sup>5</sup>. Many studies have shown that RAGE is expressed at low levels in the adult in a range of cell types, such as endothelial cells, peripheral blood-derived monocytes, lymphocytes, vascular smooth muscle cells, glomerular epithelial cells or podocytes and neurons<sup>6</sup>.

RAGE is a member of the immunoglobulin superfamily of cell surface molecules<sup>7</sup>. It is composed of an extracellular region containing one V-type and two C-type immunoglobulin domains, a hydrophobic transmembrane domain and a highly charged short cytosolic tail, which is essential for post-RAGE signalling<sup>8</sup>. RAGE acts as a signal transduction receptor for CML, although it is also likely to interact with other AGEs<sup>7</sup>. Interaction of AGEs with RAGE on macrophages causes oxidative stress and activation of nuclear factor- $\kappa$ B (NF- $\kappa$ B), which modulates gene transcription for various pro-inflammatory molecules<sup>9</sup>. In addition, the binding of AGEs to RAGE is known to cause phenotypic changes in various cells, such as endothelial cells, smooth muscle cells, pericytes, and renal mesangial cells, leading to the pathogenesis of diabetic retinopathy, nephropathy, and macroangiopathies<sup>6</sup>.

Soluble forms of RAGE (sRAGE) were previously shown to appear in human blood<sup>10,11</sup>. Plasma sRAGE consists of an endogenous splice variant of RAGE lacking the transmembrane domain of the receptor (esRAGE)<sup>12</sup> as well as proteolytically cleaved forms shed into the bloodstream by the action of extracellular metalloproteinases<sup>13,14</sup>. Administration of a recombinant sRAGE has been shown not only to suppress the development of atherosclerosis but also to stabilize established atherosclerosis in diabetic apolipoprotein E-null mice<sup>15</sup>. Both sRAGE and esRAGE were shown to act as decoys capturing inflammatory RAGE ligands extracellularly, thereby protecting cells from AGE-induced injury. However, very little information has been available about the relationship between AGE ligands, RAGE and

sRAGE in DM1, especially in children.

In the present study, we examined differences in the expression of the total circulating sRAGE of children and adolescents with DM1, compared with non-diabetic healthy children and adolescents. We also studied the association between plasma sRAGE levels and plasma CML levels in these children. Moreover, we examined differences in RAGE protein expression in peripheral blood mononuclear cells (PBMCs) between patients with DM1 and non-diabetic healthy children.

## PATIENTS AND METHODS

The study population consisted of a total of 117 children and adolescents, 74 with DM1 and 43 healthy non-diabetic children matched for age, sex and pubertal stage (Table 1). Patients with DM1 had undergone periodic follow-up examinations every 4-6 months at the Outpatient Clinic of the Division of Pediatric Endocrinology and Diabetes of the University Hospital of Patras, Greece. The control group was selected from healthy children and adolescents who also visited the outpatient clinic. The Patients with DM1 had no micro- or macro-angiopathy and were moderately controlled (glycosylated hemoglobin A<sub>1c</sub> [HbA<sub>1c</sub>] =  $8.0 \pm 1.8\%$ ). Controls were recruited according to the following criteria: (a) absence of DM1, (b) absence of metabolic or other diseases, and (c) no treatment with drugs known to affect the AGE-RAGE axis. In all participants, age, duration of diabetes (in DM1), body mass index (BMI), BMI percentile for age and sex, Tanner stage, systolic blood pressure (SBP), diastolic blood pressure (DBP), waist circumference, hip circumference and waist to hip ratio were obtained together with measurements of HbA<sub>1c</sub> (in the DM1 group), total cholesterol, high density lipoprotein-cholesterol (HDL-C), low density lipoprotein-cholesterol (LDL-C) and triglyceride concentrations (Table 1). Informed parental consent and children's assent were obtained in all cases. The study was approved by the Ethics Committee of the University Hospital of Patras (Patras, Greece).

**TABLE 1**  
Clinical and biochemical parameters in the children and adolescents  
with type 1 diabetes mellitus (DM) and controls

	<b>Controls</b>	<b>Type 1 DM</b>	<b>p</b>
<b>n</b>	43	74	–
<b>Sex</b>	23 M/20 F	42 M/32 F	NS*
<b>Age (years)</b>	13 ± 6	13 ± 5	NS
<b>Tanner I (n)</b>	13	17	NS*
<b>Tanner II (n)</b>	5	7	NS*
<b>Tanner III (n)</b>	6	8	NS*
<b>Tanner IV (n)</b>	6	25	0.047*
<b>Tanner V (n)</b>	13	17	NS*
<b>BMI %</b>	50 ± 23	59 ± 24	NS
<b>SBP (mm Hg)</b>	106 ± 6	102 ± 10	NS
<b>DBP (mm Hg)</b>	63 ± 7	63 ± 10	NS
<b>WC (cm)</b>	64.6 ± 4	69.7 ± 10	0.006
<b>HC (cm)</b>	82.7 ± 9	83.4 ± 14	NS
<b>Waist to hip ratio</b>	0.78 ± 0.07	0.84 ± 0.06	0.003
<b>TC (mg/dl)</b>	154 ± 30	168 ± 27	0.043
<b>TG (mg/dl)</b>	78 ± 36	82 ± 80	NS
<b>LDL-C (mg/dl)</b>	88 ± 29	96 ± 25	NS
<b>HDL-C (mg/dl)</b>	51 ± 11	57 ± 12	0.036

Data are means ± SD or percentages unless otherwise indicated. Student's t-test was performed except as indicated. \* Chi-squared test.

NS = not significant; BMI % = body mass index percentile; SBP = systolic blood pressure; DBP = diastolic blood pressure; WC = waist circumference; HC = hip circumference; TC = total cholesterol; TG = triglycerides; HDL-C = high-density lipoprotein-cholesterol; LDL-C = low-density lipoprotein-cholesterol.

### Measurement of circulating sRAGE

The serum and plasma levels of sRAGE were determined using a specific sandwich ELISA kit (Quantikine, R & D Systems, Minneapolis, MN, USA). Measurements were performed in duplicate and the results were averaged.

### Determination of circulating CML levels

Serum and plasma CML levels were determined using a specific competitive ELISA kit [CircuLex CML/N<sup>c</sup>-(carboxymethyl)lysine ELISA Kit (CycLex Co., Ltd, Nagano, Japan)]. Measurements were performed in duplicate and the results were averaged.

### Isolation of human peripheral blood mononuclear cells

Twenty ml of venous blood from the patients with DM1 and healthy non-diabetic children were obtained in a heparinized syringe (heparin, 90 IU/10 ml blood). The blood was overlaid on Ficoll-Paque (Histopaque 1077, Sigma, Aldrich Company Ltd, Dorset, UK) at a 2:1 ratio and centrifuged at 400 *g* for 35 minutes at 20°C. PBMCs were collected from the interface and washed with RPMI (Roswell Park Memorial Institute) media 1640 (GIBCO Invitrogen Corporation, Carlsbad, CA, USA) three times to remove the plasma and the Ficoll, according to the manufacturer's instructions.

### Western immunoblotting

Protein samples of isolated PBMCs were lysed with laemmli sample buffer (2 x SDS sample buffer). Cell lysates were separated by SDS-PAGE and proteins were transferred to nitrocellulose membranes (Amersham Biosciences plc, Buckinghamshire, UK). The membranes were blocked for 1 h at room temperature, washed and incubated with appropriate primary antibodies followed by protein A/G conjugate secondary antibodies. The following dilution and incubation times were employed for IgG: anti-RAGE (Biotechnology, Santa Cruz, CA, USA) 1:250, overnight at room temperature (RT), and for IgG: anti- $\beta$ -tubulin (Cell Signaling Technology, Inc., Danvers, MA) 1:500, overnight at RT. Protein content in the samples was

quantified with electrophoresis and stained with Coomassie Blue and corrected with densitometric measurement of  $\beta$ -tubulin protein expression. Band detection was performed with chemiluminescence reagent ECL (GE Healthcare Bio-sciences AB, Sweden). The Western blot signals (complexed protein bands) were quantified by densitometry with scion image (version 4.0.3.2; Scion Corporation).

### Statistical analysis

Data are given as means  $\pm$  SD. Means or proportions for clinical characteristics were computed for patients with DM1 and control children. Data between the two groups were compared by a two-tailed unpaired Student's *t*-test or by chi-squared test. Single linear univariate correlations (Pearson's or Spearman's correlation coefficients) and forward and backward stepwise multivariate regression analyses were performed to evaluate the relationship between sRAGE, CML or RAGE and the following variables: age, Tanner stage, duration of diabetes, BMI %, SBP, DBP, HbA<sub>1c</sub>, total cholesterol, triglycerides, LDL-C, HDL-C, waist circumference, hip circumference and waist to hip ratio. These statistical analyses were performed using the SPSS computer program (version 15.0 for Windows). The threshold of statistical significance was defined as  $p = 0.05$ .

## RESULTS

### Baseline characteristics of the study population

Clinical and biochemical characteristics of the study population are presented in Table 1. Total cholesterol, HDL-C levels, waist circumference and waist to hip ratio were significantly higher in patients with DM1 than in non-diabetic children ( $p < 0.05$ ). There was no significant difference between the two groups regarding the other clinical and biochemical parameters, including sex, age, Tanner stage, triglycerides, LDL-C, SBP, DBP, BMI % and hip circumference.

### Circulating sRAGE levels

To explore whether sRAGE has a protective action in the patients with DM1, the total soluble form of sRAGE in serum and plasma was measured in diabetic and non-diabetic children and adolescents and was correlated with their clinical and biochemical characteristics.

Circulating sRAGE levels were significantly higher in patients with DM1 than in non-diabetic children ( $1430 \pm 760$  vs  $1158 \pm 595$  pg/ml,  $p = 0.047$ ) (Fig. 1A) and they were significantly higher in diabetic patients under 13 years of age compared to patients older than 13 years of age ( $1620 \pm 869$  vs  $1214 \pm 570$  pg/ml,  $p = 0.021$ ). sRAGE levels were inversely correlated with diabetes duration ( $r = -0.265$ ,  $p = 0.037$ ) (Fig. 1B), and more specifically they were significantly higher in the patients with DM1 who had a duration of DM1 less than 5 years ( $1605 \pm 708$  vs  $1167 \pm 758$  pg/ml,  $p = 0.022$ ). Patients with DM1 who were under 13 years of age showed no significant difference in sRAGE levels in relation to the duration of DM1, whereas those patients who were older than 13 years of age had higher sRAGE levels if the duration of DM1 was less than 5 years ( $1471 \pm 636$  vs  $1011 \pm 526$  pg/ml,  $p = 0.036$ ).

Stepwise multivariate regression analysis showed that diabetes duration correlated independently with sRAGE ( $p = 0.034$ ).

Circulating sRAGE levels were directly correlated with LDL-C levels ( $r = 0.224$ ,  $p = 0.039$ ) (Fig. 1C). Specifically, sRAGE levels were significantly higher in patients with DM1 who had LDL-C serum concentrations more than 100 mg/dl ( $1704 \pm 991$  vs  $1209 \pm 524$  pg/ml,  $p = 0.034$ ). There was no significant difference found in sRAGE concentrations between the patients with DM1 with HbA<sub>1c</sub> <7% and those with HbA<sub>1c</sub> >7%.

### Circulating CML levels

To explore whether increased sRAGE in children and adolescents with DM1 could possibly eliminate circulating CML, this major AGE was measured in serum and plasma of diabetic and non-diabetic children.

Circulating CML levels were not significantly different between diabetic and non-diabetic groups

( $3.51 \pm 1.49$  vs  $3.59 \pm 1.83$ ,  $p >0.05$ ) (Fig. 2). There was no significant correlation with clinical and biochemical parameters studied. Although there was a weak inverse correlation between circulating CML levels and LDL-C, it did not reach statistical significance ( $r = -0.204$ ,  $p = 0.059$ ).

### RAGE protein expression in PBMCs

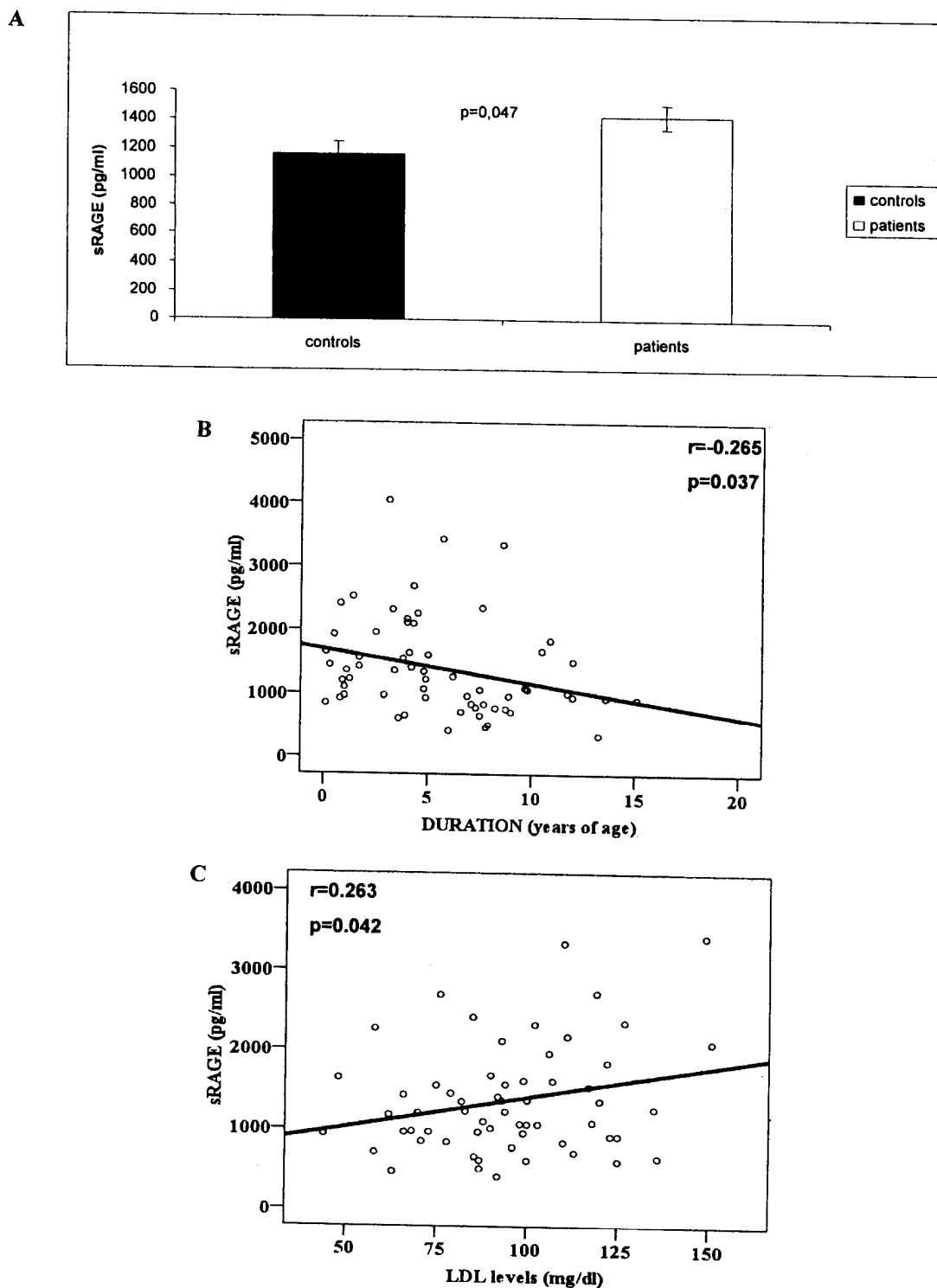
To explore whether increased sRAGE in the diabetic children and adolescents could possibly be derived from RAGE, isolated PBMCs were examined for the protein expression of RAGE by Western immunoblotting of cell lysates.

The protein expression of RAGE was significantly lower in patients with DM1 than in non-diabetic children ( $61 \pm 46\%$  vs  $102 \pm 63\%$  relative expression,  $p = 0.0001$ ) (Fig. 3). However, there were no significant correlations with the clinical and biochemical parameters studied.

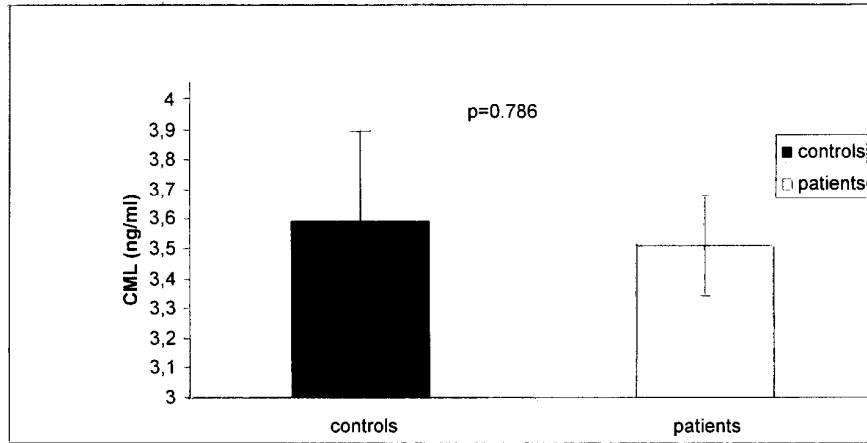
## DISCUSSION

In our study, circulating sRAGE levels were significantly higher in patients with DM1 than in non-diabetic children with an even greater significant difference between the DM1 and control groups in children <13 years of age. Furthermore, circulating sRAGE levels were inversely correlated with diabetes duration in the children >13 years of age whereas there was no significant correlation with the duration in children <13 years old. Interestingly, no correlation was found in any age group between the sRAGE levels and HbA<sub>1c</sub> concentrations.

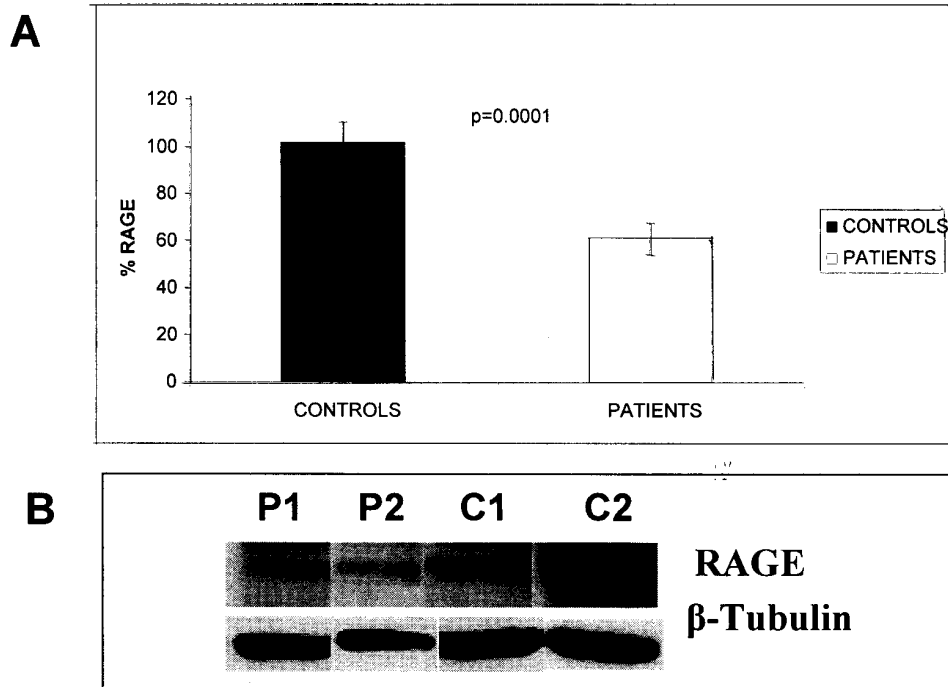
Very few studies have looked at sRAGE levels in patients with DM1 in comparison to non-diabetic individuals. Challier *et al.*<sup>10</sup> reported that serum levels of sRAGE were significantly increased in adult patients with DM1 compared with non-diabetic individuals. Also, Katakami *et al.*<sup>16</sup> found that circulating sRAGE levels were higher in adult patients with DM1 compared to non-diabetic individuals, although there was no significant difference. These results more closely correlate with ours even though their studies were performed in adults.



**Fig. 1:** A. Comparison of circulating sRAGE levels between patients with type 1 diabetes mellitus and non-diabetic controls. Circulating sRAGE levels were significantly higher in patients with type 1 diabetes than in non-diabetic controls ( $p < 0.05$ ). Bars represent mean sRAGE  $\pm$  SEM. B. Association between circulating sRAGE levels and diabetes duration. sRAGE levels were inversely correlated with diabetes duration ( $r = -0.265$ ,  $p = 0.037$ ). C. Association between circulating sRAGE levels and LDL-cholesterol in patients with diabetes. sRAGE levels were directly correlated with LDL-cholesterol concentrations ( $r = 0.263$ ,  $p = 0.042$ ).



**Fig. 2:** Comparison of circulating N-carboxymethyl lysine (CML) levels between patients with type 1 diabetes mellitus and non-diabetic controls. Circulating CML levels were not significantly different between the patients with diabetes and non-diabetic controls (3.51 vs 3.59 ng/ml, p NS). Bars represent means  $\pm$  SEM.



**Fig. 3:** A. Densitometry evaluation of expression levels of RAGE by Western immunoblotting showed that the protein expression of RAGE in peripheral blood mononuclear cells (PBMCs) was significantly lower in patients with type 1 diabetes mellitus than in non-diabetic controls (61% vs 102% relative expression,  $p = 0.0001$ ). Bars represent means  $\pm$  SEM. B. Representative pictures of Western immunoblotting of RAGE and  $\beta$ -tubulin proteins in PBMCs. RAGE protein expression was significantly lower in patients with type 1 diabetes mellitus than in non-diabetic controls. P1: patient 1, P2: patient 2, C1: control 1, C2: control 2.

Of great interest, univariate analysis showed a direct correlation between circulating sRAGE and LDL-C levels, implying that one of the possible stimuli for the sRAGE increase in our study may be increase in LDL-C levels. Specifically, sRAGE levels were significantly higher in patients with DM1 who had LDL-C serum levels greater than 100 mg/dl, even though this value is still within the limits of normal for LDL-C serum concentrations of healthy children and adolescents<sup>17</sup>. This could be explained by the fact that sRAGE, apart from the AGEs and other ligands, binds with high affinity to atherogenic HOCl-LDL, i.e. LDL modified by hypochlorous acid (HOCl), the major oxidant generated by the myeloperoxidase-H<sub>2</sub>O<sub>2</sub>-chloride system of phagocytes activated during inflammation, which is abundantly present in human atherosclerotic lesions. Most importantly, sRAGE blocks CD36 scavenger receptor-mediated uptake of HOCl-LDL, inhibits subsequent lipid accumulation and reduces foam cell formation, indicating a protective reaction against atherosclerotic plaque formation in patients with diabetes<sup>18</sup>.

Furthermore, univariate regression analysis showed an inverse correlation between circulating sRAGE and diabetes duration in the older children. Consequently, endogenous sRAGE levels could be elevated in diabetes, acting as a negative feedback agent against the AGE-elicited vascular injury. In our study this possibly protective reaction seems to be stronger in diabetic patients with shorter diabetes duration and those under 13 years of age.

As previously suggested, sRAGE may act as a decoy receptor for potentially inflammatory AGEs. This scenario is based on the finding that exogenously administered sRAGE actually blocked the harmful effects of AGEs in animals by acting as a decoy receptor<sup>6</sup>. The observed increase in sRAGE expression in the patients with DM1 can be viewed as a possible protective reaction to counterbalance, at least partly, the increase in AGE formation in these patients.

In our study, we demonstrated that circulating CML levels do not differ significantly between diabetic and non-diabetic children, possibly showing that total sRAGE levels in our young patients may be sufficient to efficiently eliminate excessive levels of circulating CML, one of the major AGEs,

in children and adolescents. This does not seem to happen in adults or diabetic patients who have already developed micro- or macro-angiopathy<sup>11</sup>. It should be emphasized that in our study the patients with DM1 were children and adolescents with moderate metabolic control, (HbA<sub>1c</sub> concentrations of  $8 \pm 1.8\%$ ), who had not yet developed any diabetic complications. However, it may be necessary to also examine the relationship between other types of AGEs, besides CML, and circulating sRAGE.

Galler *et al.*<sup>19</sup> also found no difference in the concentration of CML between children and adolescents with and without DM1. These patients were also well metabolically controlled. In contrast, Hwang *et al.*<sup>20</sup> reported that serum levels of CML were significantly higher in children and adolescents with DM1 with poor metabolic control than in the control group. The fact that our patients had better metabolic control than those in the study by Hwang *et al.* could possibly explain why the circulating CML levels did not differ significantly in our study between diabetic and non-diabetic children.

In addition, our study showed that the RAGE protein expression of PBMCs is significantly lower in diabetic patients than in non-diabetic children. It has been suggested that sRAGE is mainly generated by proteolytic cleavage of membrane-bound RAGE, whereas esRAGE represents a quantitatively minor isoform<sup>21</sup>. This finding coincides with our results, assuming that sRAGE is higher in our patients with DM1 due to increased cleavage from RAGE protein by action of metalloproteinases intramembranely with consequent reduction in RAGE levels intracellularly in the patients with DM1<sup>22</sup>. However, more studies are needed to confirm this hypothesis.

Taken together, our present observations suggest that serum levels of sRAGE may be increased in younger children (<13 years old) with DM1 and in the older children (>13 years old) with a duration of DM1 <5 years as a possible counter-regulatory system against cell damage, and may reflect enhanced cleavage of RAGE protein by metalloproteinases, resulting in sRAGE release in plasma. Our cohort of patients had not developed

any micro- or macro-angiopathy, probably due to their young age and moderately good metabolic control. These advantages of our study population may explain why circulating CML levels did not differ between the patient and control groups. Perhaps in young patients and in those with a duration of DM1 <5 years increased levels of sRAGE are sufficient to act as a decoy for pro-inflammatory ligands, such as CML, thus blocking diabetic vascular complications. Also, in our patients a slight increase in LDL-C could be a stimulus for an increase in sRAGE, leading finally to CML capture and a decrease in serum CML levels.

The relationship between diabetes duration, LDL-C, circulating sRAGE and CML levels or RAGE protein levels in patients with DM1 warrants further investigation so as to be confirmed. Additional studies are required to elucidate the potential relevance of AGEs in the pathogenesis of the complications of diabetes mellitus in children and adolescents.

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