Original article

Evaluation of soluble CD40L in children with type 1 diabetes mellitus and its relation to diabetes associated vasculopathy

**Background:** Type 1 diabetes Mellitus (T1DM) is a dynamic autoimmune disorder characterized by retrogressive insulin production as a consequence of autoimmune-mediated destruction of insulin producing pancreatic β-cells. This study aims at evaluating serum levels of soluble CD40 Ligand (sCD40L) as biomarker for early detection of complications associated with T1DM in children. **Methods:** A comparative cross-sectional study was conducted on 120 children with T1DM attending the Pediatric Diabetes Clinic of Beni-Suef University Hospital, in the period from April 2019 to January 2021. They were classified into 2 groups each with 60 patients, according to the presence or absence of microvascular diabetic complications. A group of 60 age and sex-matched healthy subjects were also included as a control group. Enrolled patients were subjected to detailed clinical evaluation, fundus examination, nerve conduction velocity, in addition to laboratory testing including fasting lipid profile, mean 2-hours postprandial blood glucose, Glycated hemoglobin (HbA1c), serum levels of sCD40L using enzyme linked immunosorbent assay (ELISA) and urinary albumin-to-creatinine ratio by an immuno-nephelometric method. **Results:** Patients’ group comprised 68 males (56.7%) and 52 females (43.3%). The most common complication encountered in the studied patients with T1DM was micro albuminuria in 44 patients (73.3%), followed by peripheral neuropathy in 18 (30%) and retinopathy in two (3.3%). Serum sCD40L levels were significantly elevated in patients with complicated T1DM (695.6±2025 pg/ml) compared to non-complicated group (547.7±125.4 pg/ml) and healthy controls (205.7±55.3 pg/ml) [p < 0.001]. A cut-off level of sCD40L more than 315 pg/mL could discriminate complicated from non-complicated case with 96 % sensitivity and 90 % specificity, area under the curve 0.96 (p<0.001). Serum sCD40L levels were positively correlated with HbA1C and urinary albumin excretion (p < 0.001). **Conclusion:** Serum sCD40L could be a helpful biomarker for monitoring microvascular complications of T1DM. Wider scale longitudinal studies might help to assess the value of sCD40L in following-up diabetic patients and predicting complications so that preventive measures can be timely taken.

Keywords: Microalbuminuria, Soluble CD40 ligand, Type 1 Diabetes Mellitus, Biomarker, Inflammation, Vascular complications.

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**INTRODUCTION**

Type 1 diabetes mellitus (T1DM) is a dynamic autoimmune disorder characterized by retrogressive insulin production that is a consequence of autoimmune-mediated destruction of insulin producing pancreatic β-cells. Patients can be diagnosed with T1DM at any age, but the most common age of presentation is at early childhood years peaked at 5-7 years old. Patients with T1DM most often have lost approximately 80% to 90% of β-cell mass at the time of diagnosis, so they depend on exogenous insulin therapy for a steady blood glucose level. With the impossibility to regenerate destructed beta-cells or cease the deterioration of T1DM at this late stage, the main target in management remains to supply adequate insulin and monitor for and prevent complications as much as possible.

Complications of T1DM have been defined as one of the foremost causes of morbidity and mortality worldwide. Such complications may occur as a result of microvasculopathy (i.e., retinopathy, nephropathy and/or neuropathy) or macrovasculopathy (i.e., cardiovascular disease, cerebrovascular accidents and/or peripheral vascular disease). In addition, children with T1DM display higher rates of disobedience to treatment, which make the situation more complicated.

All of these considerations necessitate a competent, rapid and decisive method for early
detection and monitoring of disease progression for a long-term avoidance or minimizing of T1DM complications at the early stages.

Serum or plasma biomarkers could be ideal candidate for diagnosis and follow-up of T1DM, especially if characterized by availability, feasibility, high specificity and low cost. However, the development of a particular marker for detection of T1DM is puzzling due to the numerous phases compulsory for biomarker discovery, unpredictability using classical metabolic tests and the need for long term studies to validate the value of a specific biomarker and the reliability and feasibility of its use in clinical practice.

CD40 ligand (CD40L) is a co-stimulatory cell surface glycoprotein that is expressed on the surface of B cells, T lymphocytes, monocytes and dendritic cells. It is also identified on the surface of non-immune cells, including platelets, fibroblasts, endothelial cells, pancreatic ductal cells and pancreatic islet β-cells. CD40L/CD40 interactions exert profound effects on many cells of the hematopoietic and non-hematopoietic compartments and promotes cytokine production by inflammatory cells, trigger T-cell activation and differentiation, promotes germinal center formation, immunoglobulin (Ig) isotype switching and somatic hypermutation. CD40-CD40L interaction also produces many angiogenesis-associated factors, such as vascular endothelial growth factor (VEGF). Plasma levels might also be elevated in patients with various inflammatory disorders, such as obesity, cancer and hepatic steatosis. We sought in our study to evaluate sCD40 L as a potential biomarker for T1DM disease complications in a group of Egyptian children.

Methods
This comparative cross-sectional study was conducted on 120 children and adolescents diagnosed with T1DM. They were recruited consecutively from the regular attendants of the Pediatric Diabetes Clinic of Beni-Suef University Hospital from April 2019 to January 2021. Another group of 60 age and sex-matched healthy subjects were enrolled as a control group.

Patients were divided into two groups according to the presence of microvascular complications:

Group 1 (complicated cases) included 60 patients with type 1 diabetic microvascular complications. Patients included in this group had either one or more of these complications: diabetic nephropathy, retinopathy and/or neuropathy.

Group 2 (non-complicated cases) included 60 children with type 1 diabetes who have no clinical, physiological or laboratory evidence of the aforementioned microvascular complications at enrollment and in their records.

For the diagnosis of complications, medical records of patients were reviewed retrospectively for the presence of microvascular complications starting from time of diagnosis of diabetes. The presence of complications was further verified at time of the study by clinical examination and investigations to avoid overestimation.

All patients received three injections of insulin per day of short and intermediate acting insulin. Exclusion criteria were the presence of any clinical evidence of infection, history of allergies, collagen disease, recent trauma, surgery, physical inactivity, liver dysfunction and malignancy.

All patients were subjected to detailed medical history and thorough clinical examination with special emphasis on age of onset of diabetes, disease duration, insulin therapy, and diabetic microvascular complications (neuropathy, nephropathy, retinopathy or cardiovascular ischemic events). Anthropometric measurements (height, weight) were recorded. Body mass index (BMI) was calculated.

Fundus examination was performed using direct and indirect ophthalmoscopy, as well as 90-diopter slit lamp examination through dilated pupils for assessment of diabetic retinopathy indicated by presence of one or more of the followings: traction detachment of the macula, vitreous hemorrhage, phthisis, or macular edema or enucleation secondary to proliferative diabetic retinopathy.

Neuropathy was defined by the presence of clinical features and confirmed by nerve conduction velocity test. Clinical features of neuropathy included two or more of the following: features of sensory neuropathy, abnormal autonomic function (postural hypotension with a fall in systolic blood pressure of ≥20 mmHg, loss of heart rate variability with a ratio of <1.04 or both), the absence of two or more reflexes of the ankle or knee tendons and/or a vibration perception threshold that was abnormal for the patient’s age.

Blood sampling
Peripheral blood (PB) samples were collected on ethylene diamine tetra-acetic acid (EDTA) for analysis of HbA1c. Blood samples were left for clotting at room temperature and the serum was separated by centrifugation for 15 minutes. Samples were deposited at -20°C for assessment of serum sCD40L levels or immediate analysis.
Medical records were revised for fasting lipid profile, measurement of mean fasting blood glucose (FBG), and mean 2-hours postprandial blood glucose (PPBG) levels in the last 3 months prior to the study. Mean fasting and 2-hours postprandial blood glucose levels were calculated based on the patients’ glucose meter records. Dyslipidemia was defined if at least one of the following was present; serum total cholesterol ≥ 200 mg/dL, low-density lipoprotein (LDL) cholesterol ≥ 130 mg/dL, high-density lipoprotein cholesterol (HDL) < 40 mg/dL, or serum triglyceride ≥ 110 mg/dL. Assessment of mean glycated hemoglobin (HbA1c) % in the year preceding the study was performed on Beckman Coulter AU480.

Diabetic nephropathy was assessed by measuring urinary albumin excretion in an early morning urine sample as albumin-to-creatinine ratio by an immuno-nephelometric method on prime photometer (BCP BioSed, Rome, Italy). Microalbuminuria and macroalbuminuria were present if urinary albumin excretion in at least two out of three consecutive urine samples, 2 months apart was 30–299 mg/g creatinine and ≥ 300 mg/g creatinine, respectively. Potential factors affecting urinary albumin excretion as exercise, fever, posture were excluded at time of enrollment.

Serum levels of sCD40L were assessed using enzyme linked immunosorbent assay (ELISA). Samples were taken at time of enrollment and measurement was done using the Quantikine human soluble CD40L immunoassay (R&D systems, Minnesota, USA) according to the manufacturer instructions on a Beckman Coulter, Inc.(2505, Kraemer Bivd, Brea, CA92821, USA).

**Statistical analysis**

Analysis of data was done using GraphPad Software v8.1 (San Diego, CA 92108, USA). Quantitative variables were described as mean, SD, median and range and qualitative variables were described in numerical form and percentage. Parametric variables were compared between 3 groups using the student t-test and for the non-parametric Mann-Whitney and Kruskal-Wallis tests were used. Receiver operating characteristic (ROC) curve was used to determine the cut-off value of sCD40L that best combined sensitivity and specificity in discriminating complicated from non-complicated diabetic cases. The area under the curve (AUC) and 95% confidence interval (CI) were calculated for each plot. Significance of results was considered with p values < 0.05.

**RESULTS**

Patients’ group comprised 68 males (56.7%) and 52 females (43.3%), with their age ranging between 6 and 17 years with mean ± SD 11.55 ± 3.11 years. Among patients with diabetic microvascular complication, the most common complication encountered was microalbuminuria in 44 patients (73.33%), followed by peripheral neuropathy in 18 patients (30%) and retinopathy in two patients (3.33%).

Upon comparison of the clinical and laboratory data between patients with and without microvascular complications and control subjects (Table 1), patients with type 1 diabetes in both groups had significantly higher blood pressure standard deviation score (SDS), fasting blood glucose (FBG), 2 hours post prandial blood glucose (2-h PPBG), serum total and LDL cholesterol, triglycerides, HbA1c and urinary albumin creatinine ratio while serum HDL cholesterol was lower than control subjects (p < 0.05). Patients with microvascular complications were older with longer disease duration in comparison to those without. They had significantly higher blood pressure SDS, FBG, HbA1c, urinary albumin excretion, serum lipids (except HDL), and insulin dose compared with non-complicated cases (p < 0.05).

Serum sCD40L levels were significantly elevated in all patients with type 1 diabetes compared with control group (p < 0.001). sCD40L levels were also significantly increased in both diabetic groups when compared separately with healthy control subjects (p<0.001). Notably, complicated cases had significantly higher serum sCD40L concentrations compared with patients without microvascular complications.

ROC curve analysis revealed that a cutoff level of sCD40L more than 315 pg/mL could differentiate complicated from non-complicated cases with a sensitivity of 96% and specificity of 90% (area under the curve, 0.96; confidence interval, 0.922–0.999; p< 0.001) (Figure 1). When this value was assigned in diabetic patients, it was found that 96% of patients above this level presented with complications (p<0.001) [Table 2].

Analysis of the clinicopathological characteristics in relation to sCD40L revealed that patients with microalbuminuria or peripheral neuropathy had significantly higher levels of sCD40L compared with patients without any of these complications (p<0.05). Patients with diabetic retinopathy had higher sCD40L levels than those without, although the difference did not reach statistical significance (p > 0.05) [Table 3].
Stepwise regression analysis (Table 4) revealed that HbA1c and urinary albumin excretion were independently related to sCD40L levels (p < 0.001). Serum sCD40L levels were positively correlated with HbA1c and urinary albumin excretion (p < 0.001).

Table 1. Demographic data, disease duration and insulin dose and Clinical and laboratory variables of patients with type 1 diabetes and healthy controls.

|                      | Complicated (60) | Non-Complicated (60) | Control (60) | P-value |
|----------------------|------------------|----------------------|--------------|---------|
| Sex                  |                  |                      |              |         |
| M                    | 40 (66.7%)       | 28 (46.7%)           | 30 (50%)     | NS      |
| F                    | 20 (33.3%)       | 32 (53.3%)           | 30 (50%)     |         |
| Age (yr) [Mean±SD]   | 11.92±3.28       | 11.44±2.37           | 11.27±2.14   | 0.3869  |
| Disease duration (yr)| 9.96±3.01        | 4.9±2.8              | -----        | <0.001  |
| BMI SDS              | 0.63 ± 0.3       | 0.60 ± 0.2           | 0.59 ± 0.3   | 0.752   |
| SBP (mmHg) [Mean±SD] | 105.0±6.76       | 99±7.06              | 103.6±7.74   | <0.001  |
| DBP (mmHg) [Mean±SD] | 72.23±6.60       | 67.8±2.82            | 80.6±2.72    | <0.001  |
| Puberty              |                  |                      |              |         |
| Normal               | 34 (56.7%)       | 56 (93.3%)           | 60 (100%)    | <0.001  |
| Delayed              | 26 (43.3%)       | 4 (6.7%)             | 0 (0%)       |         |
| FBS (mg/dl)          |                  |                      |              |         |
| Normal               | 128.3±32.8       | 97.9±21.2            | 100.2±10.3   | 0.001   |
| Delayed              | 194.5±18.7       | 118.3±15.7           |              |         |
| HbA1c (%)            | 9.8±1.2          | 6.99±0.4             | 4.5±0.3      | <0.001  |
| Triglycerides (mg/dL)| 186.9±29.8       | 122.7±16.9           | 100.2±10.3   | <0.001  |
| Total cholesterol (mg/dL)| 200.4±32.3 | 160.4±27.2           | 118.3±15.7   | <0.001  |
| HDL cholesterol (mg/dL)| 39±13.5   | 63±21.5              | 70.8±25.7    | <0.001  |
| LDL cholesterol (mg/dL)| 139±31.7 | 89.7±20.6            | 80.7±11.4    | <0.001  |
| UACR (mg/g creatinine)| 225.3±37.4       | 77.2±20.9            |              | <0.001  |
| sCD40L (pg/ml)       |                  |                      |              |         |
| Mean±SD              | 6956±2025.7      | 547.7±125.4          | 205.7±55.3   | <0.001  |
| Range (min-max)      | 3500-11500       | 280-740              | 120-300      | <0.001  |
| Median               | 6900             | 560                  | 210          |         |
| Insulin dose (IU/kg/d)[Mean±SD] | 49.3±24.3 | 37.8±9.082         | <0.001†††   |

BMI, body mass index; BP, blood pressure; FBS, fasting blood glucose; HbA1c, hemoglobin A1c; HDL, high-density lipoprotein; LDL, low-density lipoprotein; PPBG, postprandial blood glucose; sCD40L, soluble CD40 ligand; SDS, standard deviation score; UACR, urinary albumin creatinine ratio.

Data are presented as mean±SD. The student’s unpaired t-test and ordinary one-way ANOVA test were used. *Indicates P < 0.05 and **indicates P < 0.001 in comparison between complicated cases and controls.
†Indicates P> 0.05 and †††indicates P< 0.001 in comparison of complicated to non-complicated patients. SBP: systolic blood pressure; DBP: diastolic blood pressure.

Table 2. The best cut-off value (315 pg/ml) of serum sCD40L in relation to micro-vascular complications

| Variable                | Non-complicated patients | Complicated patients | p value | Sig. |
|-------------------------|--------------------------|----------------------|---------|------|
| sCD40L (pg/ml)          |             |                      |         |      |
| <315 (pg/ml)            | 54 (90)      | 2 (4)                | <0.001  | HS   |
| ≥315 (pg/ml)            | 6 (10)       | 58 (96)              |         |      |

sCD40L: serum soluble CD40 ligand.

Table 3. sCD40L levels in relation to clinico-pathological characteristics of diabetic patients

| Variable                        | sCD40L (Median) | Test value | p value | Sig. |
|---------------------------------|-----------------|------------|---------|------|
| Micro-albuminuria (+ve/-ve)     | 9000/250        | 3.10       | 0.002   | ***  |
| Retinopathy (+ve/-ve)           | 10000/290       | 1.33       | 0.18    | NS   |
| Peripheral neuropathy (+ve/-ve) | 7000/300        | 2.96       | 0.003   | ***  |

Data are medians (+ve/-ve). Mann Whitney test was performed. ***indicates P < 0.001 compared to non-complicated patients.
Table 4. Multiple regression analysis of the relation of sCD40L to clinical and laboratory variables

| Variable          | sCD40L (pg/ml) | Standardized coefficients | P-value |
|-------------------|----------------|---------------------------|---------|
| Age (years)       | 0.1796         | 0.35                      |         |
| Duration (yr)     | 0.3202         | <0.05                     |         |
| SBP SDS           | 0.13           | 0.41                      |         |
| DBP SDS           | 0.02           | 0.93                      |         |
| FBG (mg/dl)       | 0.01084        | 0.43                      |         |
| HbA1C %           | 0.60           | <0.001                    |         |
| UACR (mg/g creatinine) | 0.41       | <0.001                    |         |

BP: blood pressure; FBG: fasting blood glucose; HbA1c: hemoglobin A1c; SDS: standard deviation score; UACR, urinary albumin creatinine ratio.

Figure 1. Receiver Operating Characteristic (ROC) curve analysis of sCD40L for discrimination of complicated from non-complicated diabetic cases

**DISCUSSION**

T1DM is an autoimmune disease characterized by a selective destructive phenomenon of pancreatic beta cells. Clinical presentation of T1DM varies as regard to age, sex, onset, duration and immune response of the patients. The role of CD40-CD40L interactions in diabetic patients was assessed in several studies and proved its role in development of vascular diseases. Other studies showed elevated serum levels of sCD40L in diabetic patients, regardless the type of diabetes compared to healthy groups suggesting a key role of this molecule in the development of diabetic complications regardless of the disease form.

In this study, sCD40L serum levels showed significant elevation in all studied patients with T1DM compared to healthy individuals. The increase in serum sCD40L concentrations was more evident in patients with microvascular complications than non-complicated patients and sCD40L levels were found to be related to microvascular complications independently of other risk factors. Increased low grade inflammation plays a major role in diabetes-related vascular pathologies. Researchers reported a robust association amid elevated serum levels of sCD40L and expression of platelet CD40 ligand. Increased levels of serum sCD40L were also noticed in patients with metabolic disorder than healthy
individuals. In addition, pre-diabetics had increased levels of sCD40L indicating that patients with higher levels of sCD40L are under ongoing inflammatory stress. Raised levels of sCD40L is a subclinical sign of primary stage atherosclerosis related T1DM. This explains the close relation of sCD40L as an inflammatory biomarker with diabetic status and vascular pathologies that nominate that molecule as a good marker for prognosis of diabetic complications. Other studies reported elevation of serum levels of sCD40L in diabetic patients without any presented vascular complications than healthy subjects. This may be explained by environmental status, the socioeconomic conditions, age, ethnicity and glycemic control among the studied subjects. The method of evaluating presence of complication also might differ with a subsequent difference in sensitivity of detection of complication. Microvasculopathy in some patients might have already started for a while before being evident in clinical and laboratory aspects.

In our study, children with microvascular complications were older in age with longer disease duration, higher blood pressure, urinary albumin excretion, FBG, 2-h PPBG, HbA1c and lipid profile as compared to the non-complicated diabetic children. ROC curve analysis revealed that the cutoff value of sCD40L more than 315 pg/mL could differentiate complicated from non-complicated cases with a sensitivity of 96% and specificity of 90%. These results were concordant with previous publications. Microalbuminuria is the principal clinical phenotype of diabetic nephropathy. Thus, higher levels of sCD40L in first years of diabetes could help as a prognostic indicator for predicting upcoming vascular complication in the future.

In our study, microalbuminuria come on the top of the microvascular complications followed by peripheral neuropathy and retinopathy. The frequency of diabetic microvascular complications varies between studies depending on several factors including disease duration and glycemic control, the medical care provided and the access to antidiabetic treatment and blood glucose monitors and the type of diabetes itself.

In our study, factors that remained significantly related to sCD40L after regression analysis were HbA1c and urinary albumin excretion. The significant relation between sCD40L and HbA1c may explain why poor metabolic control may play a role in the pro-inflammatory state found in patients with T1DM. Some studies link poor glycemic control to increased inflammation and atherothrombotic risk in patients with diabetes mellitus and showed that improved metabolic control was associated with a significant reduction in sCD40L biosynthesis while others found no significant association between the degree of glycemic control and plasma sCD40L levels in both types of diabetes.

**CONCLUSIONS**

Serum sCD40L levels are elevated in children and adolescents with T1DM, particularly those with microvascular complications and are positively correlated with HbA1c suggesting a significant link with the glycemic control. The current study highlights the role of sCD40L as a potential marker for diabetic microangiopathy.

**STUDY LIMITATIONS**

Our study was limited by several points, the main of which was the assessment of complications based on clinical and basic laboratory test and through a mix of retrospective and cross-sectional methods. Cases with starting complications might be missed or not counted as complicated. We did not measure other parameters including the corresponding levels of TNF and other interleukins. Macrovascular complications were not included in the analysis as well. Further longitudinal prospective analysis might provide more accurate results and might elucidate a possible predictive role for sCD40L in anticipating the occurrence of diabetic complications before being clinically evident.

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