Evaluation of some Inflammatory Cytokines in Children with Type1 Diabetes Mellitus

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Abstract: Objectives: Recent evidence favors primary role of cellular autoimmunity and its humoral mediators in pathogenesis and following Type I diabetes mellitus (IDDM) The present study is carried out to investigate serum concentration of TNF-α, IL-6 and sIL-2 R in children with IDDM. Potential role of glycemic control, body mass index and disease duration were evaluated. Design and Methods: Thirty five children with IDDM and 30 age and sex matched non diabetic healthy subjects were recruited for this study from the out patients Clinic of diabetes of National Institute of Diabetes and Endocrinology. Results: Circulating level of TNF-α IL-6 and sIL-2R were elevated in children with type I DM (39.91±17.46 pg/ml, 14.89 ± 10.69 pg/ml and 779.0 ± 467.06 pg/ml respectively). Compared with nondiabetic controls (5.67 ± 1.88 pg/ml, 6.23 ± 2.78 pg/ml and 254.33± 173.6 pg/ml respectively). These differences were statistically highly significant (<0.0001). Glycemic control, Insulin dose and disease duration were not significant predictors of cytokine concentration in children with IDDM. A significant negative correlation was obtained between TNF -α with age, weight, BMI and sIL-2R in diabetic patients. However there was a significant positive correlation between IL-6 with weight and BMI in those children. Conclusion: Circulating levels of inflammatory cytokines were elevated in patients with IDDM suggesting activation of the inflammatory immune response system. Their levels were not affected by glucose level , insulin dose or duration of the disease.
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1. Introduction:

Diabetes Mellitus is a primary error of carbohydrate metabolism characterized by hyperglycemia with or without glycosuria with secondary disturbance of protein and fat metabolism, which is believed to result from autoimmune destruction of B-cells of the pancreas. (Hussain *et al.*, 1988).

Cytokines are regulatory proteins produced and secreted by lymphocytes and monocytes. Those produced by lymphocytes are called lymphokines. (Karlsson et al., 2004).

According to increasing experimental and clinical evidence, proinflammatory cytokines may play important roles alone or in combination with the pathogenesis of type I diabetes mellitus.

Interlukin-2 is a lymphokine produced by T-helper cells after its stimulation by interlukin-1 (IL-1). The action of IL-2 is mediated through its binding to specific IL-2 receptors (IL-2R) that are variably present on T-cells depending on their degree of activation by antigen. The lymphocytes can shed their IL-2 R in soluble form, so many investigators have proposed that measurements of IL-2R concentration may be useful in assessing immunological function in autoimmune disorders. (Blandino 2008 et al).

Plasma concentration of (IL-6) was found to be elevated in diabetic patients. IL-6 through its effects on soluble intercellular adhesion molecule-1 (s ICAM-1) and tumour necrosis factor (TNF-alpha), may promote vascular adhesion adding to vascular disease risk.

Aim of the work

- To investigate serum concentrations of interleukin-6 (IL-6), soluble interleukin-2 receptor (sIL-2R) and tumour necrosis factor alpha (TNF-alpha) in children with IDDM.
- To evaluate potential role of glycemic control, body mass index (BMI) and disease duration on serum levels of these cytokines.

2. Subjects and Methods:

Patient Group

This group included 35 children suffering from IDDM. They were selected from the out patients Diabetes Clinic of International Institute of

Diabetes and Endocrinology. Their ages ranged from 7-17 years, with a mean ages of 12.03 ± 2.2 years.

Healthy subjects

30 apparently healthy children with no family history of diabetes were taken as a control group. They were free from any systemic disease. Their ages ranged from 7-16 years with a mean age of 12.77 ± 2.76 years.

Methods

All participants were subjected to the following :

1- Full history taking laying stress on the age, the duration of the insuline dose.
2- Thorough clinical examination.
3- Anthropometric measurements weight, height and BMI (kg/m^2) measurement were done
4- Laboratory assessment of random blood sugar glycohemoglobin and serum, sIL-2R, serum IL-6 and TNF-α.

Sampling

Five milliliters of venous blood were collected from each child 4 ml of blood in a clean dry tube without the addition of any anticoagulant. It was left to clot for 15 minutes, centrifuged and serum was separated. Assessment of random blood sugar was done immediately and the rest of the serum was kept frozen at –20°C for the subsequent assay of IL-2R, IL-6 and TNF -α and 1 ml was added in tube with anticoagulant as EDTA to made glycotheamoglobin.

Analytical Methods

A- Random Blood sugar:

Assay was done by quantitative determination of glucose IVD using a kit provided by SPINRE ACT Inc.

B- Glycohemoglobin:

Assay was done by quantitative colorimetric determination of glycohemoglobin in whole blood using a kit provided by STANBIO LABORATORY INC cat. No 0350.

c- Tumour Necrosis factor- α:

This was done by sandwich enzyme immunoassay using a Kit provided by INSTRUCTION for the quantitative determination of human TNF-α in plasma, serum, and culture supernatant fluids. cat. No. 1121

D- IL-6 assay:

This was done by a solid phase sandwish enzyme linked immunosorbent assay using a kit provided by Biosource International, Inc. cat No KHC0062

E- SIL-2R assay:

This was done by a salid phase sandwich enzyme linked immunosorbent assay using a kit provided by Biosource International, Inc. cat. No. KHR0022

Statistical analysis

Data were analyzed by computer using the statistical program SPSS v12 for Windows. Group was compared by the Student's test for normally distributed data or by the Mann-Whitney test otherwise. The linear relationship between variables was assessed by Pearson's correlation coefficient (r). For all tests, P values less than 0.05 were considered statistically significant. Sensitivity, specificity and ROC curves were done for all inflammatory cytokines.

3. Results

Clinical and physical characteristics of children with IDDM and healthy subjects were demonstrated in table (1). There was a significant change in weight and BMI in diabetic patients as compared with healthy group. However, Age and height of diabetic patients didn't have significant change than healthy one.

Comparison of serum glucose levels and glycosylated hemoglobin (Hb A_1c) between children with diabetes mellitus and healthy subjects were shown in table (2). The results indicated that serum glucose and Hb A_1c levels were highly significant in diabetic patients than healthy subjects (P<0.001).

As can be seen from table 3 & Fig. 1, the mean values of serum inflammatory cytokins [TNF-α , IL-6 and sIL-R] levels were highly significant in diabetic patients than healthy one (P<0.001). The recorded increase was 603.9, 139 and 206.3 respectively.

Table 1: Physical characteristics of children with IDDM and control [Mean ± SD (Range)]

	Diabetics (n=35)	Control. (n=30)	% of change	P value
Age (years)	12.03 ± 2.2 (7-17)	12.77± 2.76 (7-16)	-5.8	n.s
Weight (kg)	41.69±11.31 (22-64)	52.93±13.94 (29-95)	-21.2	0.0006
High (cm)	146.57±12.12 (125-168)	150.57±16.54 (125-175)	-2.7	n.s
BMI (Kg/m^2)	19.04±2.94 (14.1-26.2)	23.06±2.98 (18-32.1)	-17.4	<0.0001

Table 2: Serum glucose levels and glycosylated haemoglobin level in patients and control [Mean ± SD (Range)]

	Diabetics (n=35)	control. (n=30)	% of change	P value
Glucose (mg/dl)	224.34 ± 118.78 (90 – 516)	86.0 ± 7.97 (72-107)	160.9	<0.0001
Hb A_1c (%)	9.01 ± 1.61 (6.6-15)	6.6±0.34 (6.0-7.1)	36	<0.0001

Table 3: Serum inflammatory cytokines levels in patients and control [Mean ± SD (Range)]

	Diabetics (n=35)	control. (n=30)	% of change	P value
TNF - α (Pg/ml)	39.91 ± 17.46 (12 – 80)	5.67 ± 1.88 (2.5-10)	603.9	<0.0001
IL-6 (Pg/ml)	14.89± 10.69 (6-48)	6.23 ± 2.78 (3-15)	139.0	<0.0001
sIL-2R (Pg/ml)	779.0 ± 467.06 (100-1750)	254.33±173.06 (50-700)	206.3	<0.0001

Table 4: Inflammatory cytokines in children with newly diagnosed (≤ 1 year) and long Standing (>1 year) type 1 diabetes mellitus [Mean ± SD (Range)]

	Duration≤ 1 year (n=12)	Duration>1 year (n=23)	P value
TNF - α (Pg/ml)	43.0 ± 19.12 (22 – 80)	38.3 ± 16.74 (12-80)	>0.05
IL-6 (Pg/ml)	12.17± 5.69 (6-23)	16.3± 12.43 (6- 48)	>0.05
sIL-2R (Pg/ml)	677.5 ± 467.28 (100-1700)	831.96± 468.41 (150-1750)	>0.05

On trying to elucidate the effect of duration of illness on the inflammatory markers we compared serum level of TNF-α_1 IL-6 and SIL-2R in Diabetic patients with duration of illness less than one year and those in patients with duration of illness greeter than one year (table 4).

The relationships between serum inflammatory cytokines and all clinical and biochemical indices in children with children with IDDM and healthy subjects were obtained in Table 5. A significant negative correlation was obtained between TNF-α with and age, weight, BMI and sIL-2R in diabetic patients. However, there were significant positive correlations between IL-6 with weight and BMI in both diabetic children and healthy subjects. Insulin dose and glycosylated hemoglobin were not correlated to any of the inflammatory markers.

Table (5): Correlation coefficient between inflammatory cytokines and all clinical and biochemical characteristic in children with IDDM and healthy subjects.

Group	Diabetic patients (n=35)			Healthy group (n=30)		
Variables	TNF-α (pg/ml)	IL-6 (pg/ml)	sIL-2R (Pg/ml)	TNF-α (pg/ml)	IL-6 (pg/ml)	SIL-2R (Pg/ml)
Age (year)	-0.37*	0.45**	0.17	-0.08	0.24	0.13
Weight (kg)	-0.39*	0.47**	0.01	0.05	0.52**	0.28
Hight (cm)	-0.29	0.30	0.02	-0.01	0.31	0.10
BMI ($kg/m^{2)}$	-0.42*	0.50**	0.02	0.08	0.46**	0.33
Duration	-0.15	0.19	0.13	-	-	-
Glucose (mg/dl)	0.28	-0.11	-0.12	-0.04	0.20	0.22
Hb A_1c (%)	-0.12	0.08	-0.02	-0.07	0.30	-0.02
Insulin dose (Iu)	-0.28	0.14	-0.05	-	-	-
TNF-α	-	-0.09	-0.39*	-	0.04	0.21
IL-6	-0.09	-	0.31	0.09	-	0.30
sIL-2R	-0.39*	0.31	-	0.21	0.30	-

* P <0.05 ** P <0.01

Roc analysis was used to determine the accuracy of each serum cytokines as a marker for diagnosis of children with IDDM in 35 patients and 30 healthy subjects. The data obtained in tables 6, 7 and fig. 1, proved that TNF-α test was the best test to discriminate children with diabetes mellitus from healthy subjects with an area under the curve of 1 and cut-off value of 10 Pg/ml. The sensitivity and specificity were 100 %. IL-6 showed an area under the curve of 0.86 for cut-off value of 6pg/ml, the sensitivity was 88.6% and specificity was 66.7% (tables 6, 7 and fig. 2). The data illustrated in tables 6, 7 and fig. 3, proved that sIL-2R had an area under the curve 0.87.for cut-off value of 330 pg/ml. The sensitivity was 85.7% and specificity was 86.7%.

Multivariate analysis using logistic regression indicated that all tested inflammatory cytokines [TNF-α, IL-6 and sIL-2R] were found to be independently associated with diabetes mellitus (P<0.001) fig 4.

Table (6): Results of ROC analysis to differentiate between patients and healthy subjects .

	Cut-off	AUC	± SE	95% Cl	Sensitivity	Specificity
TNF	> 10pg/ml	1.00	0.00	0.94-1*	100%	100%
IL-6	>6 pg/ml	0.86	0.05	0.75-0.94*	88.6%	66.7%
SIL-2R	>330 pg/ml	0.87	0.04	0.77-9.94*	85.7%	86.7%

* Significant (P<0.05) AUC = area under the curve SE = standard error
95% Cl=95% confidence interval for AUC.

Table (7): Chi-square analysis using determined cut-offs

		Patients		Controls		Chi	P
		N	%	n	%	square	value
TNF	>10	35	100%	0	0%	65.00	<0.001
	<=10	0	0%	30	100%		
IL-6	>6	31	88.6%	10	33.3%	21.16	<0.001
	<=6	4	11.4%	20	66.7%		
SIL-2R	>330	30	85.7%	4	13.3%	33.92	<0.0001
	<=330	5	14.3%	26	86.7%		

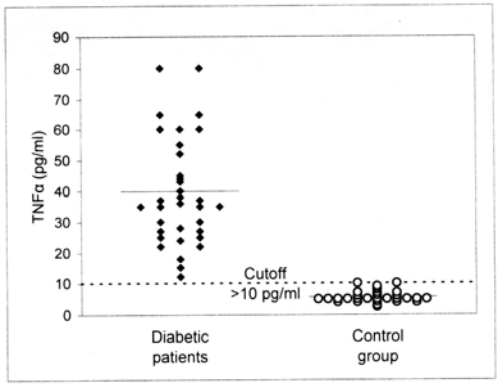

Fig. 1: Scatter diagram of TNF-α (pg/ml) level in diabetic patients and healthy group.
The horizontal line represents the cut-off value of 10 pg/ml. The sensitivity is 100% at specificity 100%.

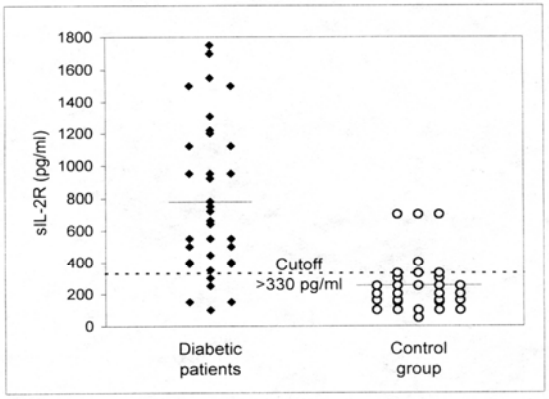

Fig. (2): Scatter diagram of sIL-2R (pg/ml) level in diabetic patients and healthy group.
The horizontal line represents the cut-off value of 330pg/ml. The sensitivity is 85.7% at specificity 86.7%.

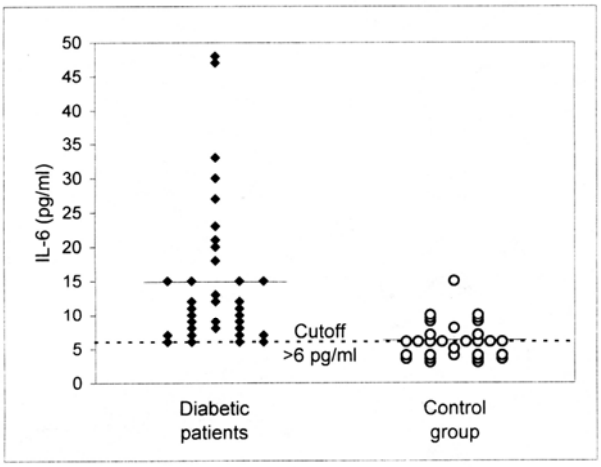

Fig. (3): Scatter diagram of IL-6(pg/ml) level in diabetic patients and healthy group.

The horizontal line represents the cut-off value of 6pg/ml. The sensitivity is 88.6% at specificity 66.7%.

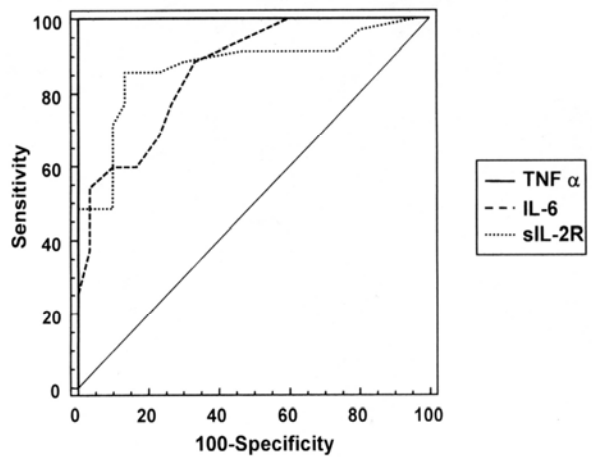

Fig. (4): Roc curve testing the ability of TNF-α, IL-6 and sIL-2R.

Differentiate between patients with type (1) diabetes mellitus and healthy group. Areas nder the curve (AUC) are 1.0, 0.86 and 0.87 for TNF-α, IL6 and sIL-2R respectively P<0.0001 for comparison of the markers.

4. Discussion:

Insulin dependent diabetes mellitus is the effect of T cell dependent autoimmune destruction of insulin production beta cells in the pancrease. Insulin is one of the islet autoantigens responsible for activation of T-lymphocyte functions, inflammatory cytokine production and development of IDDM (Tchorzewski et al., 2001).

Information about the inflammatory state of an individual can become of clinical relevance since factors that determ inflammation can be modified,(Chatz et al,2010).

The proinflammatory cytokines TNF-α and IL-6 play an important role in the pathogenesis of insulin- development diabetes mellitus (*Alexandrak et al.*,2008) while TNF-α is also involved in promoting insulin resistance, development or progression of IDDM (Shbaklo *et al.*, 2003).

Data of the present study revealed that serum cytokines (TNFα, IL-6 and sIL-2R) levels were significantly higher in patients than healthy control. These results are in agreement with those of Wasmuth *et al.*, 2004, Dondona *et al.*, 2004, Glowinska & Urban, 2003 and Miranda *et al.*, 2003 who reported that the inflammatory activity is increased in individuals with type-1 diabetes, may be due to hyperglycemia and the formation of advanced glycation end products. Another report indicated that TNF-α is identified as the uniting principle linking the pathogenesis of insulin-dependent diabetes mellitus and non insulin-dependent diabetes mellitus. Elevated TNF-α initially increases and then inhibits, the activity of a number of key enzymes involved in energy metabolism and major histocompatibility (MHC) class I molecule expression. These enzymes include protein-tyrosine kinase (PTPase). Enzymes involved in energy metabolism, cell proliferation and stimulation of MHC class I molecule path way (*Foss et al.*,2007) and concomitant destruction of pancreatic beta cells. So, TNF-α can be implicated as indicator of continuing autoimmune aggression against beta-cells before the development of extensive beta – cell distruction (*Haller* and Schatz 2008).

It is well documented that TNF-α can be cytotoxic, ctyostatic since it inhibits insulin synthesis and secretion (rabinovitch 2002 & Suarez-Pinzon, 1998). Addionally TNF-α and IL-6 mediated damage to micro-and macrovascular tissues, altered insulin secretion directly or through stimulation of free fatty acids production and altered glucose homoestasis (Peraldi & Spiegelman, 1998, Schmidt *et al.*, 1999, Corbett *et al.*, 1997 and Unger, 1995).

About the molecular mechanisms for increased IL-6 under hyperglycemia, Igarashi *et al.* (1999) found that high glucose has been shown to activate p38 MAPK (Mitogen Activated Protein kinase) which regulate the production of inflammatory cytokines such as TNF-α and IL-6 (Yamakawa *et al.*, 1999). On the same line, Sridevi *et al.* (2005) reported that under high glucose, monocytes secrete increased amounts of IL-6, via upregulation of protein kinase (PKC-α- and β), P38 MAPK and Nuclear factor (NF~B) activity leading to increased IL-6 transcription and release .

There is a discrepancy between the data obtained by our work and that of *Haller and Schatz.* (2008) who found non significant change in serum TNF-α levels of diabetic children when compared with age-matched healthy controls. Similarly,) Todd *et al.* (2005), indicated that serum TNF-α and IL-6 levels were comparable in diabetic and non diabetic groups. However, newly diagnosed (<1yr) cases had higher TNF-α and IL-6 levels compared with larger standing DM_1. Another study obtained by *Chatzi et al.* (2010) found that the duration of diabetes was associated with TNF-α and general scores of inflammatory markers. Unlike previous reports, our data indicated that the elevation of cytokine markers were comparable in diabetic patients with a duration of disease more than one year as well as in diabetic patients with a shorter duration of diabetes (≤1 yr).

This results are in agreement with that given by Blandino et al. (2008). ON the other hand, another study indicated that IL-6 concentrations were statistically higher at onset diabetes than in diabetic patients with long-term disease (Wedrychowicz *et al.* 2004) and *Fosset al.* 2007. We have tested the correlation between cytokine levels and duration of disease to confirm whether these difference observed in cytokine levels have any pathophysiological consequences. Inflammatory, diabetes preventing or diabetes promoted effects have been suggested for altered cytokine (Rabinovitch *et al.,2002*). On the other hand there is increasing evidence that a number of acute phase proteins such as CRP and IL-6 itself are antiinflammatory and immuno suppressive being involved in the resolution of the inflammatory response (Haller and Schatz 2008).

Adipocytes can produce IL-6 and TNF-α and many studies in non diabetic (Onate *et al.,* 2001, & Yamada *et al.* 2001) and diabetic (Festa *et al.,* 2000, Saraheimo *et al.,* 2003) individuals, have shown an association between estimates of body fat and inflammatory activity. Our data revealed that BMI was negatively correlated with TNF-α levels ($r=-0.42$, $P<0.05$) in diabetic children and positively correlated with IL-6 levels ($r=0.50$, $P<0.01$) in the same group. BMI showed also positive correlation with IL-6 levels in non diabetic children. The data obtained by *Karlsson et al.* 2004 suggesting that BMI was associated with all inflammatory markers (TNFα, IL-6) in diabetic children that support our data. On the other hand, there is no evidence correlation between these cytokines and BMI. was reported in other reports (Ayse *et al.* 2001).

Many cytokines play an important role in the etiopathogenesis of type 1-DM, among these is IL-2 which induces its action through its binding to specific interleukin-2 receptors (IL-2Rs) that are present on the surface of T-cells (Zhenge *et al.,* 1999 & Kretowski *et al.,* 1999).

Interleukin-2 system which involves IL-2 production, IL-2 receptor expression and response to IL-2, is associated with autoimmune phenomena. Immunological abnormalities including autoimmune phenomena are believed to contribute to the pathogenesis of IDDM (*Blandino et al.,*2008). It was found that the percentage of IL-2 receptor positive circulating T-cells was significantly increased in diabetic children than non diabetic group (Gartner *et al.,* 1995). Similar results were obtained by previous study of *Chatz and his co-workers (*2010) who indicated that there was no correlation between sIL-2R and any metabolic parameters in type-1 diabetic patients. This results are in accordance with our data that revealed serum sIL-2R levels were significantly higher in diabetic children than non diabetic group. On the other hand, correlation was not detected between level of sIL-2R and any clinical or biochemical parameters in diabetic patients except TNF-α that showed significant negative correlation with sIL-2R ($r=-0.39$, $P<0.05$). Supportive to our results, is the evidence that TNF-α cytokine regulate production of other soluble factors (*Foss et al.,*2007). Furthermore, the TNF-α gene is located on chromosome 6 in close proximity to the MHC class II region. Thus further studies must address the question as to whether there is an association between TNF-α and the abnormalities of cytokine production and its soluble receptors such as IL-2R as observed in the present study.

Previous data indicated that 46% of diabetic patients, 40% of their parent and 55% of their sibling had sIL-2R levels exceeding the highest normal value. Moreover, the authors found increased levels of TNF-α and IL-6 in the diabetic patients and their healthy relatives. They explained such spectrum of immunological abnormalities in diabetic patients and their family members by heightened immune response in these individuals in which activated T-lymphocytes express IL-2R with shedding of these receptors in the serum (Hussain *et al.,* 1998).

Contradictory results, indicating decrease in the concentration of sIL-2R in pre diabetic and diabetic patients with newly diagnosed type-1-DM. when compared with age-matched control subjects). Such inconsistency and contradiction might be due to a difference in stages of the autoimmune process (Alexandraki et al., 2008).

Regarding our study that revealed level of IL-2R were not correlated with disease duration, random blood sugar or age in the diabetic patients or non diabetic group. These results are in agreement with other previous studies (Rabinovitch *et al.,* 2002, Kukrega *et al.,* 2002 & *Haller and Schatz et al.,*2008).

In order to determ if the inflammatory cytokines (TNF-α, IL-6 and sIL-2R) were useful as markers in screening for early IDDM and in monitoring immunological treatment, diagnostic reliability was performed to choose the best cut-off value within the patient group (calculated from control group). Our data proved that TNF-α was the best to discriminate type 1 DM. and its cut-off value of 10 Pg/ml and at this value, the sensitivity was 100% and specificity was 100%. The next most useful test for predicating type-1 DM, among our patients, was IL-6 with cut-off value of 6 pg/ml at which the sensitivity was 88.6% and specificity was 66.7%. the data obtained also, proved that sIL-2R with cut-off value of 330 pg/ml had a sensitivity of 85.7% and specificity of86.7%.

In conclusion, circulating levels of tumor necrosis factor, interleukin-6 soluble interleukin-2 receptor are significantly increased in patients with IDDM as compared to healthy subjects and their levels are not affected by glucose level, insulin dose or disease duration. This is highly suggestive of the availability of these non invasive indices to help in further examining Type 1DM pathophysiology and monitoring pharmacological interventions to interfere with disease development and progression.

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