

## Original Research Article

# Serum Levels of Tumor Necrosis Factor Alpha and Interleukine-12 in Some Iraqi Diabetic Patients Type1

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## A B S T R A C T

### Keywords

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The cytokines produced by variety of cells of the innate and adaptive immune system. Their major functional activities are concerned with the regulation of the development and behavior of the immune effector cells. The serum levels of (TNF\_  $\alpha$ ) demonstrated a significant ( $p \leq 0.001$ ) increased in T1DM patients as compared to controls (117.6 vs. 97.06) and the serum levels of IL-12 also demonstrated a significant ( $P \leq 0.001$ ) increase in T1DM patients as compared to controls (58.02 vs. 28.05). The present study demonstrated that the increased serum level of TNF- $\alpha$  in T1DM patients was associated with increased poinsulin hormone levels and suggested that the high level of circulating TNF- $\alpha$  is an indicating of Hyperinsulinemia. Proinflammatory cytokines may be increased by hyperglycemia in subjects with impaired glucose tolerance, this result was confirmed, because all diabetics had elevated serum level of TNF\_  $\alpha$  and IL12 .However, the TNF\_  $\alpha$  and IL12 cytokine is stimulated by stress hyperglycemia and could play a role in acute coronary syndromes.

## Introduction

The term diabetes mellitus (DM) describes a metabolic disorder of multiple etiologies characterized by disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action or both (WHO, 1999). It is a major worldwide health problem predisposing to markedly increased cardiovascular mortality and serious morbidity and mortality related to the development of nephropathy, neuropathy and retinopathy (WHO, 2005). Cytokines are soluble

messenger molecules, eg. Lymphokines (produced by lymphocyte) and interleukins (made by other white blood cells) that facilitate communication between different compartment of the immune system. Examples include Interferons ,Tumor Necrosis Factor- alpha (TNF- $\alpha$ ), granulocyte -colony stimulating factor (G-CSF),granulocyte macrophage-colony stimulating factor (GM- CSF). These cause biological effects in destination cell population (e.g. activation, division or migration of destination cells) and often trigger inflammation (Gemmy,

2012). The cytokines produced by variety of cells of the innate and adaptive immune system. Their major functional activities are concerned with the regulation of the development and behavior of the immune effector cells (Swardfager, 2010). The cells regulated by cytokines must express a receptor for the factor. Thus, cells are regulated by the quantity and type of cytokines to which they are exposed and by the expression of up regulation and down regulation of cytokine receptor. Cytokines act in concert with one another to create synergistic effects that reinforce the other actions on a given cell. The interaction of multiple cytokines generated during atypical immune response are referred to cytokine cascade (Dowlati *et al.*, 2010).

#### **Tumor Necrosis Factor-alpha (TNF- $\alpha$ )**

Tumor Necrosis Factor- $\alpha$ , is a member of a group of cytokines that involved in systemic inflammation and it was discovered later independently as cachectin, a circulating mediator of wasting syndrome (cachexia) associated with chronic disease (Olszewski *et al.*, 2007).

The primary role of TNF- $\alpha$  is the regulation of immune cells because of its ability to induce fever, apoptotic cell death, inhibit tumorigenesis, viral replication, maintenance of secondary lymphoid organ structure, and host defense against various pathogens. So the TNF plays a critical role in bridging innate and adaptive immunity. However, its role in regulating the function of T regulatory cells or their impact on effect or cells is presently unknown. Deregulation of TNF production has been implicated in a variety of human diseases including Alzheimer's disease, cancer, major

depression, and Inflammatory disease (ID) (Swardfager *et al.*, 2010).

It is a 26 kilodalton transmembrane protein that is cleaved into a 17 kilodalton biologically active protein that exerts its effects via type I and type II TNF- $\alpha$  receptors. Within adipose tissue, TNF- $\alpha$  is expressed by adipocytes and stromovascular cells (Olszewski *et al.*, 2007).

Although initially suspected of playing a role in cachexia, TNF- $\alpha$  has now been implicated in the pathogenesis of obesity and insulin resistance (Hotamisligil, 2003). Adipose tissue expression of TNF- $\alpha$  is increased in obese rodents and humans and is positively correlated with adiposity and insulin resistance. Although circulating concentrations of TNF- $\alpha$  are low relative to local tissue concentrations, plasma TNF- $\alpha$  levels have been positively correlated with obesity and insulin resistance in some studies but not others (Fernandez-Real and Ricart, 2003). Chronic exposure to TNF- $\alpha$  induces insulin resistance both in vitro and in vivo (Ruan and Lodish, 2003).

Several potential mechanisms for TNF- $\alpha$ 's metabolic effects have been described. First, TNF- $\alpha$  influences gene expression in metabolically important tissues such as adipose tissue and liver (Ruan *et al.*, 2002). In liver, TNF- $\alpha$  suppresses expression of genes involved in glucose uptake and metabolism and fatty acid oxidation and increases expression of genes involved in synthesis of cholesterol and fatty acids (Ruan *et al.*, 2002). Second, TNF- $\alpha$  impairs insulin signaling, and this effect is mediated by activation of serine kinases that increase serine phosphorylation of insulin receptor substrate-1 and -2, making them poor

substrates for insulin receptor kinases and increasing their degradation (Hotamisligil, 2003).

### **Interleukin-12(IL-12)**

Interleukin-12 is a cytokine produced by antigen presenting cells like Dendritic Cells (DC), macrophages also by NK cells. It plays a critical role in cell-mediated immunity. It affects a variety of stages in the immune response; it prompts NK cells and T cells to produce pro-inflammatory cytokines, such as IFN- $\gamma$ , IL-2, IL-3 and TNF- $\alpha$ ; it contributes to NK cell maturation (Blazhev *et al.*, 2006); and, along with other pro-inflammatory factors, it stimulates CD4+CD25- T cell activation in the presence of regulatory T cells (Chuang *et al.*, 2012). Interleukin-12 also regulates naive T cell differentiation into T helper type 1 lymphocytes (Th1), and inhibits differentiation into T helper type 2 lymphocytes (Th2) (Kang *et al.*, 2005). It has been documented that increased systemic inflammatory activity in patients with coronary artery disease is associated with a prominent Th1 response (Tan *et al.*, 2013) Current data suggest that IL-12 plays a critical role in the pathogenesis of T1DM (Kang *et al.*, 2005; Skarsvik *et al.*, 2005), but the significance of IL-12 changes in the blood of patients with T2DM remains unclear. It has been observed that IL-12 plasma concentrations are elevated in T1DM (Winkler *et al.*, 1998), and that IL-12 contributes to the process of atherosclerotic plaque formation and probably accelerates the development of macrovascular complications in T2DM (Hauer *et al.*, 2005). Additionally, it has been noted that elevated glucose levels in diabetic animals stimulates inflammatory reactions related to IL-12 cytokine gene expression (Wen *et al.*, 2006). However, it is not known whether factors related to the course of

T1DM, such as metabolic compensation, beta cell secretory dysfunction, and insulin resistance affect IL-12 concentrations (Wegner *et al.*, 2008).

## **Materials and Methods**

### **Kit Contents**

- Microtiter Plate: 1 x 96 wells pre-coated with anti-human TNF- $\alpha$  monoclonal antibody or IL-12 (ready-to-use).
- Anti-human TNF- $\alpha$  and IL-12 antibody conjugated to biotin.
- Avidin conjugated to horseradish peroxidase (HRP).
- TNF- $\alpha$  and IL-12 standards.
- Calibrator diluents (animal serum with buffer).
- Washing buffer stock (buffered surfactant) (20X).
- Buffered solution with H<sub>2</sub>O<sub>2</sub> (A).
- Buffered solution with TMB (B).
- Stop solution (2N sulfuric acid solution; H<sub>2</sub>SO<sub>4</sub>).

### **Assay procedure for TNF- $\alpha$**

Before carrying out the assay procedure, the kit was left at room temperature (18-25°C) for 30 minutes to equilibrate, as suggested by the manufacturer. After that, the assay was carried out following the instructions in the kit's leaflet, which are summarized in the following steps:

- i. Serial concentrations (0, 31.25, 62.5, 125, 250, 500, 1000 and 2000) pg/ml of the standard was made using the diluent.
- ii. An aliquot (50 $\mu$ l) of (Biotin) was added to the pre-coated wells.
- iii. An aliquot (200  $\mu$ l) of the standard and serum was added into the appropriate well and then mixed well, covered and incubated for 120 minutes at room temperature.

iv. The wells were washed with five cycles of washing (350µl/well/wash) using the washing solution, with the aid of a microtiter plate washer. The washing buffer was prepared by diluting 60 ml of the washing buffer stock up to 1200 ml with distilled water.

v. An aliquot (100 µl) of avidin (HRP) was added to each well. After mixing the contents of wells, the plate was covered and incubated for 120 minutes at room temperature.

vi. The washing step was repeated (step iv).

vii. The substrate solution was prepared no more than 15 minutes before the end of incubation period (step v) by mixing equal volumes of A and B. Then, 200 µl of substrate solution was added to each well. After mixing the contents of wells, the plate was covered and incubated for 20 minutes at room temperature.

viii. An aliquot (100 µl) of stop solution was added to each well and the absorbance was read at a wave length of 450 nm using ELISA reader within 30 minutes.

### **Assay Procedure for IL-12**

Before carrying out the assay procedure, the kit was left at room temperature (18-25°C) for 30 minutes to equilibrate, as suggested by the manufacturer. After that, the assay was carried out following the instructions in the kit's leaflet, which are summarized in the following steps:

i. Serial concentrations (0, 31.25, 62.5, 125, 250, 500 and 1000) pg/ml for IL-12 of the standard were made using the diluent.

ii. An aliquot (50µl) of (Biotin) was added to the pre-coated wells.

iii. An aliquot (200 µl) of the standard and serum was added into the appropriate well and then mixed well, covered and incubated for 120 minutes at room temperature.

iv. The wells were washed with five cycles of washing (350µl/well/wash) using the washing solution, with the aid of a microtiter plate washer. The washing buffer was prepared by diluting 60 ml of the washing buffer stock up to 1200 ml with distilled water.

v. An aliquot (100 µl) of avidin (HRP) was added to each well. After mixing the contents of wells, the plate was covered and incubated for 120 minutes at room temperature.

vi. The washing step was repeated (step iv).

vii. The substrate solution was prepared no more than 15 minutes before the end of incubation period (step v) by mixing equal volumes of A and B. Then, 200 µl of substrate solution was added to each well. After mixing the contents of wells, the plate was covered and incubated for 20 minutes at room temperature.

viii. An aliquot (100 µl) of stop solution was added to each well and the absorbance was read at a wave length of 450 nm using ELISA reader within 30 minutes.

**Calculation:** The sample results were calculated by interpolation from a standard curve that was performed in the same assay as that for the sample (Figure 1-1) (Figure 1-2) using a curve fitting equation.

### **Results and Discussion**

The serum levels of (TNF<sub>a</sub>) demonstrated a significant ( $p \leq 0.001$ )

increased in T1DM patients as compared to controls ( 117.6 vs. 97.06) (Table 3-5) (figure 3.6) and the serum levels of IL-12 also demonstrated a significant ( $P \leq 0.001$ ) increase in T1DM patients as compared to controls (58.02 vs. 28.05) (Table 3- 6) (figure 3.7). Table.1-3 Serum levels of Tumor Necrosis Factor-alpha (TNF $\alpha$ ) and Interleukin12 (IL12) in Type 1 diabetic patients.

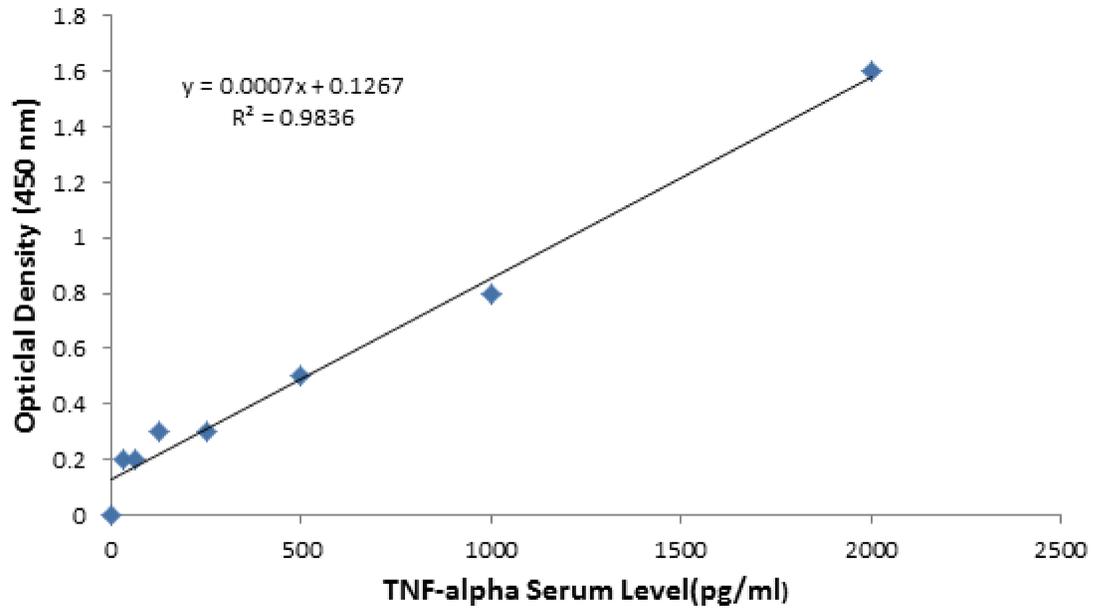
The results demonstrated an increased level of TNF- $\alpha$  in T1DM patients. This finding agrees with other investigators who reported similar findings (Rajala and Scherer, 2003; Hotamisligil *et al.*, 2003; Fernandez-Real and Ricart, 2003). Furthermore, Xu *et al.* (2002) and Kern *et al.* (1995) demonstrated a high expression of TNF- $\alpha$  in obese animals and obese human with T1DM, while Catalan *et al.* (2007) suggested that elevated pro-inflammatory cytokine level found in obese T1DM subjects is related mainly to obesity rather than to T1DM.

The present study demonstrated that the increased serum level of TNF- $\alpha$  in T1DM patients was associated with increased proinsulin hormone levels and BMI. Bruun *et al.* (2003) suggested that the high level of circulating TNF- $\alpha$  is an indicating of hyperinsulinemia. Furthermore Yaturu *et al.* (2006) demonstrated a positive correlation between TNF- $\alpha$  and high BMI. The production of TNF- $\alpha$  may limit the activity of the T regulatory cells and foster induction of immune reactivity and the effect or phase of lymphocyte responses. So a decrease in TNF production may result in enhanced T regulatory function that limits immune reactivity. In this way, TNF may play an important instructive role in controlling

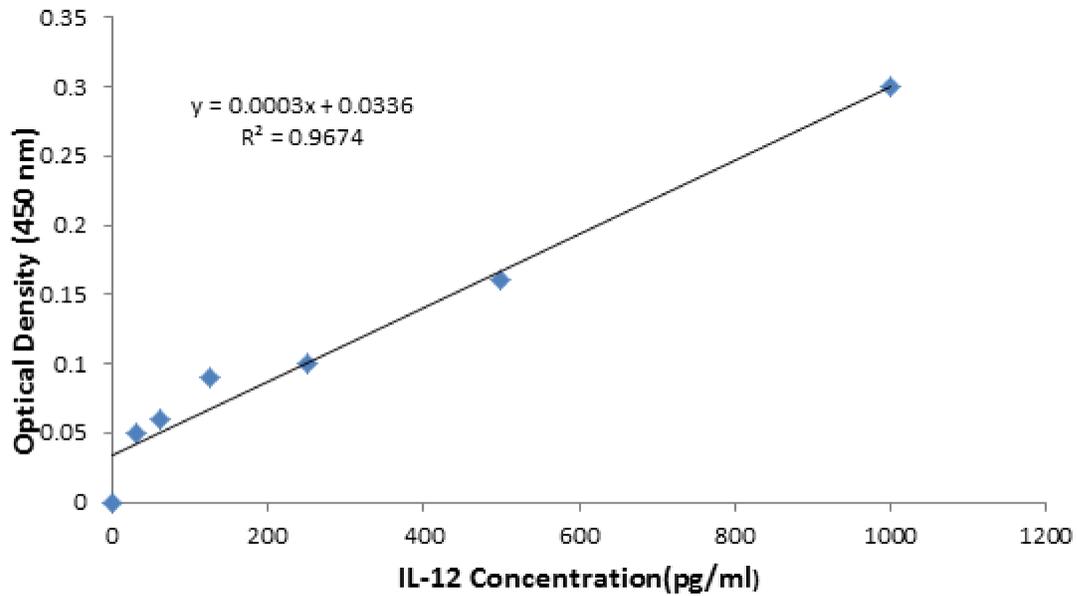
adaptive immunity (Valencia *et al.*, 2006). Interleukin-12 showed a significant increased level in T1DM patients as compared to controls. Wegner *et al.* (2008) demonstrated that elevated serum IL-12 levels in T1DM were related to the excessive proinsulin secretion. Therefore, this cytokine may play a critical role in the pathogenesis of T1DM, since IL-12 is important in immune response to infections. It has been shown that in the absence of infection, IL-12 induced autoreactive T cell responses might predispose to self-destructive immunity but the significance of IL-12 changes in the blood of patients with T1DM remains unclear. The IL-12 accelerates the development of macrovascular complications in the disease (Hauer *et al.*, 2005). Additionally, it has been noted that elevated glucose levels in diabetic animals stimulates inflammatory reactions related to IL-12 cytokine gene expression (Wen *et al.*, 2006). However, it is not known whether factors related to the course of T1DM, such as metabolic compensation, beta cell secretory dysfunction and insulin resistance affect IL-12 concentrations, but in a recent study, a multiple regression analysis revealed that the IL-12 serum level in T1DM primarily was dependent upon fasting pro-insulin concentration (Wegner *et al.*, 2008). It has been suggested that cytokines released by monocytes/macrophages, including IL-1beta, IL-12 and tumor necrosis factor alpha (TNF- $\alpha$ ) could have an initial role in islet B-cell damage (Blazhev *et al.*, 2006).

Proinflammatory cytokines may be increased by hyperglycemia in subjects with impaired glucose tolerance, this result was confirmed, because all diabetics had elevated serum level of TNF $\alpha$  and IL12.

**Figure.1-1** Standard curve of TNF-alpha



**Figure.1-2** Standard curve of IL-12



**Table.1-1** Age, Gender, Body Mass Index, Waist to Hip ratio, the duration of disease

P-Value	Type 1 Diabetic Patients (n=50)	Healthy controls (n=30)	Variables
N.S	21.48±1.03 24/26	22.40±1.31 18/12	Age Gender(F/M)
≤0.001	31.80±0.80	28.70±0.71	BMI(kg/m <sup>2</sup> )
≤0.01 –	1.02±0.02 9.7±2.1	0.95±0.01 –	Waist-to-Hip Ratio Duration of disease(years)

**Table.1-2** Biochemical profile of healthy controls and diabetic patients

p-value	Type 1 diabetic patients (n=50)	Healthy controls (n=30)	Variables
≤0.001	200.13±119.2	122.64±39.4	fasting blood glucose (mg/dl)
≤0.001	187.5±85.9	130.3±23.9	Cholesterol (mg/dl)
≤0.001	44.2±13.3	51.04±52.4	HDL Cholesterol (mg/dl)
≤0.001	80.06±27.7	64.06±37.9	LDL Cholesterol (mg/dl)
≤0.001	160.8±25.12	96.8±33.9	Triglyceride (mg/dl)
≤0.001	7.50±0.25	4.80±0.12	Hb A1C%
≤0.001	9.8±2.8	7.0±2.4	Proinsulin hormone (pmol/l)

**Table.1-3** Serum levels of Tumor Necrosis Factor-alpha (TNF<sub>a</sub>) and Interleukin12 (IL12) in Type 1 diabetic patient's

P_Value	Type 1 diabetic patients (n=50)	Healthy controls (n=30)	Parameters
≤ 0.001	116.6±33.9	93.06±22.61	TNF <sub>a</sub> (pg/ml)
≤ 0.001	48.02±14.43	22.05±21.4	IL12(pg/ml)

However, the TNF $\alpha$  and IL12 cytokine is stimulated by stress hyperglycemia and could play a role in acute coronary syndromes (Cheung *et al.*, 2012).

The cytokines (TNF- $\alpha$  and IL-12) have a positive correlation with BMI and obesity in patients with type 1 diabetic patients. These cytokine may be useful biomarker for early detection of diabetes.

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