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# Evaluation of Some Immunological Biomarkers (TGF-β, IL-35, CD68) as Prediabetes Markers for the Early Diagnosis of Type 2 Diabetes

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**HOMA-IR, HbA1c, C-peptide, TGF-β, IL-35, CD86**

## ABSTRACT

Type 2 diabetes is a complicated metabolic disorder that leads to resistance to insulin, high levels of blood sugar and inflammation that doesn't produce pain. In this matched case-control study, metabolic, hormonal and immunological parameters have been analiced in T2DM subjects vs healthy controls taking into account IR and immune factors. Levels of insulin, glucose, HbA1c and C-peptide were all recorded under fasting conditions as well as following a fast assessing insulin resistance through the internal equilibrium equation for evaluating insulin resistance. TGF-β, IL-35, and CD86 in the blood were examined by ELISA. Results for controls were statistically compared to those for patients. Type 2DM people exhibited significantly higher levels of insulin, HbA1c, C-peptide and HOMA-IR as compared to healthy ($P < 0.0001$). Additionally, there were significantly higher serum levels of TGF-β and CD86 but lower levels of IL-35 in patients than in HC. These results reflect an increased immune activation and inflammatory response in the patient set. In sum, T2DM is characterized not only by profound dysregulation of glucose homeostasis and insulin resistance but also by profound disturbance in immune activation and inflammatory pathways. Concurrent upregulation of T cell activation and metabolic biomarkers reveals a strong marriage between immune function and disordered metabolism in T2DM. This means they may be helpful as a measure of the disease, or in deciding how best to treat it.



## INTRODUCTION

As a metabolic disorder, local and systemic RAS system have been associated in the development of type 2 diabetes mellitus (T2DM). Recent data, however, underscore the involvement of immune system in its pathogenesis (Hayden, 2023). Chronic subclinical inflammation with an imbalance of proinflammatory to anti-inflammatory cytokines is involved in the pathogenesis and development of T2DM (Velikova *et al*., 2021). Adipose tissue, previously considered as a passive fat-storing organ, is now known to be an active endocrine and immune organ that secretes a variety of cytokines and adipokines modulating glucose metabolism and insulin sensitivity (Al-Msaid and Aljazaeri, 2025).

Transforming growth factor-β (TGF-β) is a pleiotropic cytokine involved in immune regulation, inflammation and cell differentiation (Chandiran and Cauley, 2023). It has a double effect in glucose homeostasis, being an antiinflammatory mediator under physiological circumstances but with exaggerated expression its function is redirected to fibrosis, chronic inflammation and insulin insensitivity (Shi *et al*., 2022). Higher TGF-β concentration systems have previously been observed in obese and T2DM patients, therefore indicating the implication of TGF-β in the shift from MHO to metabolically unhealthy (Mussa *et al*., 2021). In addition, compromised TGF-β responses have been demonstrated to prevent the insulin receptor pathway and peripheral tissue glucose uptake (Wang *et al*., 2022).

Interleukin-35 (IL-35) belonging to IL-12 cytokine family is anti-inflammatory molecules mainly

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produced by regulatory T cells (Tregs) and B cells (Goleij *et al*., 2025). Functionally, IL-35 inhibits the activation of effector T cells and diminishes pro-inflammatory cytokine production such as IL-6 and tumor necrosis factor alpha (TNF-α). Dysregulated IL-35 production has also been found in metabolic diseases, including obesity and T2DM, suggesting its implication in the pathogenesis of metabolic inflammation (Wang and Liu, 2025). Few reports indicate decreased IL-35 is a parameter of insulin resistance and greater inflammation, but others describe increasing IL-35 in response to metabolic inflammation as a counter-regulatory response (Hu *et al*., 2021).

Lysosomal glycoprotein CD68 is broadly employed as a marker for macrophages. In diabetes, macrophages infiltrate adipose tissue and pancreatic islets and release inflammatory mediators which induce insulin resistance and β-cell dysfunction. Upregulated CD68 in AT is associated with higher fasting glucose, insulin resistance and BMI. Furthermore, CD68 positive macrophages have been reported to alter their phenotypes from M2 (anti-inflammatory) to M1 (pro-inflammatory) during the development of insulin resistance and play a key role in chronic low-grade inflammation associated with T2DM (Hussein *et al*., 2025; Nguyen *et al*., 2025).

These three markers, namely TGF-β, IL-35 and CD68 may thus be considered to reflect a central immune-inflammatory network of T2DM. Deciphering the complex interrelationship between these factors, and amongst others erythrocyte ferric reduction capacity and antioxidant parameters (e.g., superoxide dismutase) may not only explain the immunopathological basis of diabetes but can also identify new predictive markers for early recognition as well as treatment. This case–control study was conducted to assess the serum concentrations of TGF-β, IL-35, and the expression of CD68 as potential immunological biomarkers linked to the development of T2DM.

## MATERIALS AND METHODS

*Study population*

A total of 80 participants were divided into two groups each of 40 namely: Group I (Patients) and Group II (Control). Group I comprised recently diagnosed type 2 diabetes mellitus patients as per the diagnostic criteria for American Diabetes Association (American Diabetes Association, 2021), based on fasting blood glucose, HbA1c and c-peptide while group the Group II apparently healthy volunteers matched for age and sex with normal FBS without positive family history of DM.

*Inclusion criteria:* Adults newly diagnosed (untreated) T2DM patients 30–60 years. No prior use of insulin or anti-diabetic medications.

*Exclusion criteria:* Patients with type 1 diabetes or other endocrine disorders. Individuals with autoimmune diseases, chronic infections, liver or kidney disease. Pregnant or lactating women. Subjects receiving corticosteroids or immunosuppressive drugs.

*Sample collection*

Five ml venous blood was drawn aseptically from all subjects. Two mL of blood was aseptically collected in EDTA tube for the hemogram and flow cytometry study for ascertaining CD68 expression. 3 mL into a plain tube; the serum was separated following clotting by centrifugation at 3000 rpm for 10 min and kept at -20 °C until cytokine assays were performed.

*Measurement of immunological markers*

Serum TGF-β, CD68, and IL-35 levels were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits (company name, country), according to the manufacturer's instructions.

*Other biochemical parameters*

Fasting blood glucose, glycated hemoglobin, and fasting insulin were measured using standard laboratory methods. Insulin resistance was estimated using the Homeostasis Model Assessment of Insulin Resistance formula:

$$\text{HOMA} - \text{IR} = \frac{\left(\text{Fasting glucose}\left(\frac{\text{mg}}{\text{dL}}\right) \times \text{Fasting insulin}\left(\frac{\mu\text{U}}{\text{mL}}\right)\right)}{405}$$

*Statistical analysis*

All statistical analysis were conduct by SPSS software version 26.0 (IBM Corp., USA). Data for continuous variables are reported as mean ± standard err. To compare 2 independent groups either the Student's test or Mann–Whitney U test were used, as appropriate. Correlation analyses were further employed by calculating Pearson's or Spearman's correlation coefficients with respect to the variables. A p value of less than 0.05 was considered indicative of statistical significance.

## RESULTS AND DISCUSSION

There were a very statistically significant differences between patients and controls in all the studied metabolic parameters ($P < 0.0001$). (i) Fasting level of insulin: The patient group demonstrated the elevation of fasting insulin in comparison with the normal controls (28.05 ± 0.48 µU/mL vs. 14.11 ± 0.42 µU/mL). (ii) Blood sugar (fasting): The level of fasting blood sugar was observed to be sig-

nificantly very high in the patients (207.9±3.04 mg/dl) as compared to control values (99.10±1.33 mg/dl). (iii) Homeostatic Model Assessment for Insulin Resistance (HOMA-IR): HOMA-IR index was highly raised in patients (14.41± 0.34) than control group (3.45±0.11), denoting severe insulin sensitivity. (iv) HbA1c: The level of HbA1c was significantly higher in the patients (6.66±0.13%) than in controls (4.247±0.087%). (v) C-peptide: A remarkable higher level of C peptide concentration was observed in the patient group (6.248±0.2338 ng/mL) as compared to that of control (1.69±0.065 ng/mL). (vi) TGFβ level: A marked increment in C-peptide amount was observed between patient (11.95±0.22 ng/mL) and healthy participants (3.94±0.21 ng/mL). (vii) Interleukin-35 concentration: C-peptide level was significantly decreased in patients (17.88±0.51 ng/mL) than that of controls (71.31±2.42 ng/mL). (viii) cluster of differentiation 68 (CD68) concentration: The level of CD68 was markedly higher in the patients group (5.43±0.14 ng/ml) than that in control group (2.10 ± 0.13 ng/ml).

**Table I. Immunological biomarker as predictor markers for the early diagnosis of type 2 diabetics.**

| S. | | Control (n=40) | Patients (n=40) | P |
|---|---|---|---|---|
| 1 | Fasting insulin (μU/mL) | 14.11±0.42 | 28.05±0.48 | <0.0001 |
| 2 | FBS (mg/dL) | 99.16±1.32 | 207.9±3.03 | <0.0001 |
| 3 | HOMA-IR | 3.44±0.11 | 14.41±0.34 | <0.0001 |
| 4 | HbA1c levels (%) | 2.47±0.06 | 6.66±0.12 | <0.0001 |
| 5 | C- Peptide (mg/mL) | 1.69±0.06 | 6.24±0.23 | <0.0001 |
| 6 | TGFβ (ng/mL) | 3.94±0.21 | 11.95±0.22 | <0.0001 |
| 7 | Interleukin-35 (ng/mL) | 71.31±2.42 | 17.88±0.51 | <0.0001 |
| 8 | CD68 (ng/mL) | 2.10±0.12 | 5.43±0.14 | <0.0001 |

FBS, fasting blood sugar; HOMA-IR, homeostatic model assessment for insulin resistance; TGFβ, transforming growth factor beta; CD68, cluster of differentiation 68

## DISCUSSION

The current findings also show distinct metabolic, hormonal and immunological changes in patients relative to healthy controls, indicating a complex pathophysiology of IRI and T2DM. The much higher fasting insulin values that were seen in patients imply a compensatory hyperinsulinemia, a pattern frequently found as a result of peripheral insulin resistance. This is also justified by the extremely high HOMA-IR values, which demonstrate severe insulin resistance in patients. These modifications represent a sign of decreased insulin sensing in target tissues, commonly seen during type 2 diabetes evolution. Potential of the triglyceride-glucose (TyG) index and the homeostasis model assessment 2 (HOMA2) in identifying insulin resistance in non-diabetic patients with acromegaly: A pilot study and literature review. The study was conducted on 50 acromegaly patients and 50 age- and sex-matched control subjects. In patients with acromegaly there was evidence of profound metabolic derangements compared to the healthy controls including higher TyG levels and a significantly increased frequency of diabetes despite similar HOMA-IR values. This is in a good agreement with what have been found in the present study (Karadeniz *et al*.,2025; Samavarchitehrani *et al*., 2025).

Concomitantly, the patients presented with fasting blood glucose and HbA1c levels that were significantly higher than normal values (chronic hyperglycemia and poor long-term glycemic control). Increased HbA1c levels indicate prolonged exposure to high glucose levels and are closely related to the development of diabetic complications such as cardiovascular and microvascular complications. This study was based on a study by Cui *et al*. (2023) in which 5308 patients with AMI were examined including 2081 patients with diabetes and without (3227). The presence of stress-induced hyperglycemia was correlated to the deterioration of condition of patients with SH AMI, along with an elevation in HbA1c values. other research indicate same results (Kushwaha *et al*., 2022; Cui *et al*., 2023).

The marked increase in patients C-peptide levels indicates enhanced endogenous insulin production from pancreatic β-cells. This response probably is an early compensatory action to insulin resistance. In the long term, however, chronic β-cell overactivity may lead to β-cell dysfunction and depletion reflecting progressive disease. A study conducted by Samavarchitehrani *et al*. (2025) entitled "The bidirectional association of C-peptide with cardiovascular risk in nondiabetics" reported similar results to our results, and the significance of high C-peptide was greatly reduced. In line with this, a high level of serum C-peptide was also demonstrated in another study "Effects of serum C-peptide on blood lipids and cardiovascular and cerebrovascular injury in patients with type 2 diabetes mellitus" (Huang *et al*., 2022; Qin *et al*., 2022; Yan *et al*., 2022).

Besides metabolic alterations, the significant shifts of inflammatory and immunoregulatory markers were also observed. The elevated TGF-β in the patients may, thus, indicative of its dual function in immune regulation and tissue repair. While the TGF-β is anti-inflammatory, its chronic elevation has been associated with fibrosis, endothelial impairments as well as insulin resistance. Increased TGF-β levels were detected as well in a study on the role of fibroblast-specific TGF-β signaling mediates

cardiac dysfunction, fibrosis and hypertrophy in obese diabetic mice, which is similar to our observation. Type 1/2 TGF-β isoforms in the pathogenesis also make it more difficult to compare studies, e.g., a study that examines the role of TGF-β signaling pathways in large vessel arteriopathy and diabetic retinopathy, which also found increases in similar assayable TGF-β (Callan *et al*., 2024; Tuleta *et al*., 2024).

In addition, we observed a significant reduction of IL-35 that indicates activation of immune-regulatory circuits due to sustained metabolism stress and inflammation. IL-35 is a well-characterized immune suppressor, which might reflect as a compensatory mechanism to cut inflammation it diabetes excessive. A reduction in GCF belonged with IL-35 among diabetic generalized periodontitis subjects was found in a study of IL-39 and IL-35GCF levels. This is consistent with our results as well as another study by Systematic Review of Interleukin-35 in Endothelial Dysfunction: A New Target for Therapeutic Intervention who also observed a diminished concentration of IL-35 (Hassan *et al*., 2024; Li *et al*., 2025).

In addition, the approach also reveals a robust induction of CD86 expression reflecting heightened immune activation in patients. CD86 is a costimulatory molecule present on the T-cell activation, and its high expression also indicates that type 2 diabetes is not simply an endocrine connection but rather chronic low-grade inflammation with the participation of immune system. This was a cross sectional (case control) study that included 61 T2DM patients and 48 healthy controls. PAR levels in purified peripheral blood mononuclear cells (PBMCs) were measured by ELISA. Plasma and PBMC oxidative stress was determined, as well as plasma reactive oxygen metabolites (d-ROMs) and ferric reducing ability of the plasma (FRAP). Antioxidant enzymes; the levels of SOD1, GPX1 and CAT gene expressions were measured by quantitative real time-polymerase chain reaction (qPCR) in PBMCs. The mRNA levels of IL-6, TNF-α, CD68 and MCP-1 in PBMCs were also examined by qPCR. These observations are CONSISTENT with our data, in which elevated expression of CD68 was found in patients with type 2 diabetes mellitus (Zampieri *et al*., 2024; Obaid *et al*., 2025).

Taken together, these findings suggest that type 2 diabetes is orchestrated by marked abnormalities of glucose metabolism, insulin action and immune-inflammatory pathways. Joint elevation of metabolic and immunological biomarkers emphasizes the crosstalk between insulin resistance and immune disturbance. A deep understanding of the interactions may be useful to identify new diagnostic markers and therapeutic targets for type 2 diabetes.

## CONCLUSION

Combined measurement of metabolic and immune biomarkers could provide a more comprehensive picture of disease sports, and may be used for better diagnosisand therapeutic perspective in patients with type 2 diabetes.

## DECLARATIONS

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*IRB approval and ethical statement*

This study was approved by the University of Kufa, Faculty of Science, Department of Biology Institute Review Board (IRB). All subjects provided their written informed consent to participate in this study.

*Generative AI and AI-assisted technology statement*

The authors declare that no generative AI and AI assisted technology was used in the creation of this manuscript.

*Statement of conflict of interest*

The authors have declared no conflict of interest.



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