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Unraveling the Role of CD4 and CD8 in Patients with Type-2 Diabetes Mellitus

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ABSTRACT

Diabetes mellitus is a serious metabolic disorder that causes considerable disease and mortality with micro- and macro-vascular complications. Long-lasting diabetes is poorly controlled and often leads to nephropathy and cardiovascular complications. To a greater extent studies indicate that T2DM is a protracted inflammatory disease associated with the immune system. The present study was therefore hypothesized to investigate and further investigate immune changes by evaluating CD4 and CD8 markers. Subjects were divided into two groups: healthy people (control group) and diabetic patients of type 2 (diabetic group). In comparison with the control group, our study shows a significant increase in the diabetic group in CD4 and CD8. Our study shows a significant increase in HbA1c in diabetic groups compared to the control group, shows significant rising in lipid profile (total cholesterol, LDL, and Triglycerides) in diabetic groups compared to the control group except for HDL which decreased in diabetic group compared to control group.

Keywords: DM, CD4, CD8, flow-cytometry.

1. INTRODUCTION

The prevalence of diabetes in adults has increased over recent decades and is one of the most common disorders for metabolic diseases in the world (Shaw et al., 2010). Diabetes Mellitus type 2 is a genetic disease. A lot of genes are involved, most of them not yet identified. These genes control several chemical steps in the action of β-cells, insulin secretion, and cell level insulin activity (Shoback et al., 2011). The common feature of all diseases which attitudinize to diabetes syndrome is a hormone insulin insufficiency. In the case of T2DM, there is also a problem with the impedance of insulin action. Insulin insufficiency is a combination problem with many probable reasons, not all of which are known. Here we will debate what we understand today, realizing that many of the basics discussed may change tomorrow (Yabe et al., 2015); achieved studies to investigate the effective role of T lymphocytes in adipose inflammation. A higher CD8 + effector T cell count entered obese epididymal adipose tissues in mice

feeding a high-fat regiment, while the CD4 + helper number, as well as the regulatory T cell counts, were reduced. The CD8 + T cell leakage phenomenon precedes the accumulation of macrophages (Nishimura et al., 2009).

The standard CD4 + and CD8 + T cells in the diabetic kidneys were much lower than the macrophages in Type 1 and Type 2 DN, which suggested that T cells interact with macrophages for inflammation and renal injury adjustment. Activated T cells can cause injury directly through cytotoxic effects and indirectly through the recruitment and activation of macrophages (Chow et al., 2006). The diabetic renal function may be performed by CD8 + cells. All cytokines and molecules strengthen inflammation and induce the expression of a colony-stimulating factor in macrophages (Navarro et al., 2011). Besides, Interact with the macrophages of renal tissue with T cells produces different reactive oxygen species, proinflammatory cytokines, metalloproteinase, and growth factors modifying local response and enhancing diabetic kidney inflammation (Yamagishi et al., 2007). Depleted CD8 + T cells could improve macrophage leakage, adipose inflammations, and systemic insulin resistance through specific antibodies. It is still indefinite whether CD8+T cells can be used for the treatment of DN. Medication for the treatment of DN in animal models was used for anti-inflammatory or immunosuppressive use; however, cure approaches to T cells remain finite (Turgut & Bolton, 2010). Flow Cytometry Standards were designed to describe both cellular characteristics (sizes, membrane potential, intracellular pH), and cellular content amounts (DNA, protein, surface receptors, calcium). Measurements which detect the allocation in cell populations of these parameters are crucial in biotechnology (Macey, 2007). Furthermore, flow cytometers can be integrated with a sorting unit which shows that the sub-population selected can be separated. The majority of the sorting units are set to fracture the cell stream to droplets. The cells are electrically charged before the droplets become formative. The resulting droplets are passed through an electric field into a collection vessel (Davey et al., 1999). CD4 is a glycoprotein found on the surface of immune cells like T-helper cells, macrophages, monocytes, and dendritic cells in molecular biology. It was found in the late seventies and first identified as leu-3 and T4 (Sakaguchi, 2004). CD4 is the T cell receptor (TCR) co-receptor, which facilitates communication with cells that present the antigen. The TCR complexes and CD4 connectivity are respectively; α1 / β1 and β2 within different areas of the MHCII molecule (Foti et al., 2002).

CD8 is a glycoprotein transmembrane, which serves the T cell receptor (TCR) as a co-receptor. CD8 binds to a major MHC molecule but is special to the MHC protein of class I. There are two protein isoforms, alpha, and beta, each with a separate gene encoding. In humans, the two genes are present in position 2p12 on chromosome 2 (Butler et al., 2011). CD8 forms a dimer made up of CD8 chains. The most common form of CD8 is the CD8-α and CD8-β chain with both a superfine stalk and an inner cell tail connected to the membrane, members of the immunoglobulin superfamily with a similar extracellular immunoglobulin variable (IgV) domain. Any cells are often expressed with less prevalent homodimers of the CD8-α series. The growing CD8 chain has a molecular weight of around 34 kDa (Gebhardt & Mackay, 2012). This study was conducted to assess and compared the CD markers (CD4 & CD8) among normal individuals, using flow cytometry, with untreated patients. Furthermore, we are determining changes in biochemical markers such as, blood glucose, HbA1c, HDL, LDL, cholesterol, and triglyceride.

2. SUBJECTS AND METHODS

The study was carried out -in October 2018- at the Clinical pathology department, Faculty of Medicine, Minia University. It was conducted on twenty newly diagnosed diabetic patients and twenty healthy individuals as control. The selected subjects included in the study were divided into two groups -

Group I (control group): It included twenty healthy individuals for age and sex, 13 males and 7 females, their ages ranged from 17 to 53 years old.

Group II (diabetic group): It included twenty newly diagnosed Diabetic patients; 14 males and 6 females, their ages ranged from 18 to 68 years old.

Exclusion criteria

Acute or chronic infection.

Acute or chronic inflammatory diseases.

Two groups were under fasting conditions for ten hours before collecting the lipid profile sample.

Patients were being subjected to a full assessment of the following;

Laboratory investigations

Routine investigations

Random blood sugar using SELECTIVE PRO XL (ELI TECH Group clinical systems).

Hba1c using SELECTIVE PRO XL (ELI TECH Group clinical systems).
Total lipid profile including total cholesterol, high-density lipoprotein cholesterol (HDL-C), Low-density lipoprotein cholesterol (LDL-C), and Triglycerides using SELECTIVE PRO XL (ELI TECH Group clinical systems).

Special investigations
Percentage of expression of CD4 and CD8 in peripheral blood by Flow Cytometry (BD-FACS FLOW Argon laser U.S.A).

Sampling
Blood was evacuated in Ethylene Diamine tetra acetic acid (EDTA) containing tube for Flow Cytometry analysis and HbA1c. 1 ml was evacuated on the plain tube. Blood was left to clot in the incubator then centrifuged. The expressed serum was used for the determination of serum glucose. The blood which was withdrawn under the fasting condition was evacuated on the plain tube. Blood was left to clot in the incubator then centrifuged. The expressed serum was used for the determination of serum cholesterol, serum LDL, serum HDL, and serum T.G.

Biochemical measurements
Random blood sugar
Blood glucose was determined by using a glucose kit purchased from SPINREACT Company, Egypt commercially available kit according to the instruction of the manufacturer.

HbA1c
Hemoglobin A1C (HbA1c) was determined by using the HbA1c kit purchased from SPECTRUM Company, Egypt commercially available kit according to the instruction of the manufacturer.

Total lipid profile
Total cholesterol
Cholesterol was determined by using a cholesterol kit purchased from HUMAN Company, Germany commercially available kit according to the instruction of the manufacturer.

HDL Cholesterol
HDL Cholesterol was determined by using an HDL cholesterol kit purchased from HUMAN Company, Germany commercially available kit according to the instruction of the manufacturer.

Triglycerides
Triglycerides were determined by using triglycerides kit purchased from HUMAN Company, Germany commercially available kit according to the instruction of the manufacturer

LDL Cholesterol
LDL Cholesterol concentration (LDL-C) is calculated from the total cholesterol concentration (TC), the HDL Cholesterol concentration (HDL-C), and triglycerides concentration (TG) according to (William T Friedewald et al., 1972).

Assessment of CD4 percentage by Flow Cytometry
This test is based on the ability of certain monoclonal antibodies to bind to the leucocyte-expressed antigenic determinants. The sample is incubated with the CD4-PC5 reagent to specifically stain the leucocyte. Lysis isolates the red cells and the flow cytometry analyses the leukocytes that are not affected by the process. The flow cytometer measures light dispersal as well as cell fluorescence. In the electronic window specified in a histogram, it enables a population of interest to be defined, which correlates orthogonal light scatter (Side scatter or SS) and narrow-angled light dispersal (Forward Scatter or FS). Depending on the application selected by the user, further histograms that combine two of the various parameters available for the cytometer can be used in the gating phase. To distinguish between positive and unstained ones, the fluorescence of the bounded cells is analyzed. The results are shown concerning all events acquired by the gate as a percentage of positive events.

Staining procedure

Whole blood was collected in evacuated tubes containing EDTA, For each sample, two tubes were prepared labeled 1&2 (Control and Test), add 10 µL of specific CD4-PC5 conjugated antibody to each test tube, and 10 µL of the isotopic control to each control tube then add 100 µL of the test sample to both tubes, Vortex the tubes gently then incubate for 15 to 20 minutes at room temperature (18 – 25°C) in the dark, cells were washed by PBS twice to remove any unbound antibodies then followed by red cell lysis using 2 ml of lysing solution then incubated for 10 minutes at room temperature in the dark, cells were centrifuged for 5 minutes, the supernatant was discarded and 2 ml of PBS were added then wash by PBS was repeated twice then the cells were re-suspended in 3 ml of PBS for final flow cytometry analysis.

Analysis of data

The analysis was carried out using a (BD-FACS FLOW Argon laser U.S.A) flow cytometry at 580 nm. Data processing was carried out with the software.

Assessment of CD8 percentage by Flow Cytometry

This test is based on the ability of certain monoclonal antibodies to bind to the leucocyte-expressed antigenic determinants. The sample is incubated with a CD8-APC reagent, which results in specific leukocyte staining. Lysis isolates the red cells and the flow cytometry analyses the leukocytes that are not affected by the process. The flow cytometer measures light Dispersion and cell fluorescence. It allows for the delimitation of the population of interest in a histogram specified in an electronic window which correlates the orthogonal light dispersion (Side Scatter or SS) and the narrow-angle light diffusion (Forward Scatter or FS). Depending on the application selected by the user, further histograms that combine two of the various parameters available for the cytometer can be used in the gating phase. To distinguish between positive and unstained ones, the fluorescence of the bounded cells is analyzed. The results are shown concerning all events acquired by the gate as a percentage of positive events.

Staining procedure

Whole blood was collected in evacuated tubes containing EDTA. For each sample, two tubes were prepared labeled 1&2 (Control and Test), add 10 µL of specific CD8-APC conjugated antibody to each test tube, and 10 µL of the isotopic control to each control tube then add 100 µL of the test sample to both tubes, vortex the tubes gently then incubate for 15 to 20 minutes at room temperature (18 – 25°C) in the dark, cells were washed by PBS twice to remove any unbound antibodies then followed by red cell lysis using 2 ml of lysing solution then incubated for 10 minutes at room temperature in the dark, Cells were centrifuged for 5 minutes, the supernatant was discarded and 2 ml of PBS were added then wash by PBS was repeated twice then the cells were re-suspended in 3 ml of PBS for final flow cytometry analysis.

Analysis of data

The analysis was carried out using a (BD-FACS FLOW Argon laser U.S.A) flow cytometry at 654 nm. Data processing was carried out with the software.

Statistical method

The collected data were coded, tabulated, and statistically analyzed using the SPSS program *(Statistical Package for Social Sciences software version 25)*. Descriptive statistics were done for parametric quantitative data my mean, standard deviation, and minimum & maximum of the range and non-parametric quantitative data by the median, while they were done for categorical data by number and percentage. Analyses were done for parametric quantitative data between the two groups using independent samples T-test and for non-parametric quantitative data using the Mann Whitney test between the two groups. Analyses were done for parametric quantitative data using the Chi-square test. Correlation between markers and laboratory data was done using Pearson's correlation coefficient. The level of significance was taken at (P-value ≤ 0.05)

3. RESULTS

Demographic Parameters

Demographic data records are summarized in (Table 1). There were no significant differences in gender distribution among the groups (P=0.7) with male predominance in the diseased group and they represented 14% in the Diabetic group. There was a significant difference in age regarding the control group.

Table 1 Comparison between patients and control groups regarding demographic data.

		Control N=20	Diabetic N=20	p
Age	Range Mean ±SE	17-53 34.3±2.1	18-68 47.8±2.8	0.001*
Gender	Male Female	13(56%) 7(35%)	14(70%) 6(30%)	0.7

Biochemical Laboratory investigations of subjects

Biochemical results are summarized in (Table 2,3 and Figure 1) that there is a highly statistically significant difference in patients' groups for FBG compared to the Control Group (P<0.001) (Fig.1). Furthermore, there is a significant difference between the patients' group for HbA1C (P-value = 0.001) (Fig.1). Also, there is a highly significant difference between all groups regarding lipid profiles compared to the Control group.

Table 2 Comparison between the studied groups regarding blood glucose and HbA1c levels.

Parameters / Groups		Control group N=20	Diabetic group N=20	p
Range	Blood glucose HbA1c	80-102 5-6	218-370 9-15	0.001*
Mean ±SE	Blood glucose HbA1c	89.5±1.3 5.4±0.1	282.3±9.01 10.5±0.3	

Table 3 Comparison between the studied groups regarding blood lipids

Parameters / Groups		Control group N=20	Diabetic group N=20	p
Range	Cholesterol TG HDL LDL	139-215 89-170 36-50 71-126	190-300 125-280 28-36 152-217	0.001*
Mean ± SE	Cholesterol TG HDL LDL	157.6±4.8 110.7±5.5 43.2±0.8 89.5±3.4	251.1±5.6 192.1±9.4 31.8±0.5 187.4±3.9	

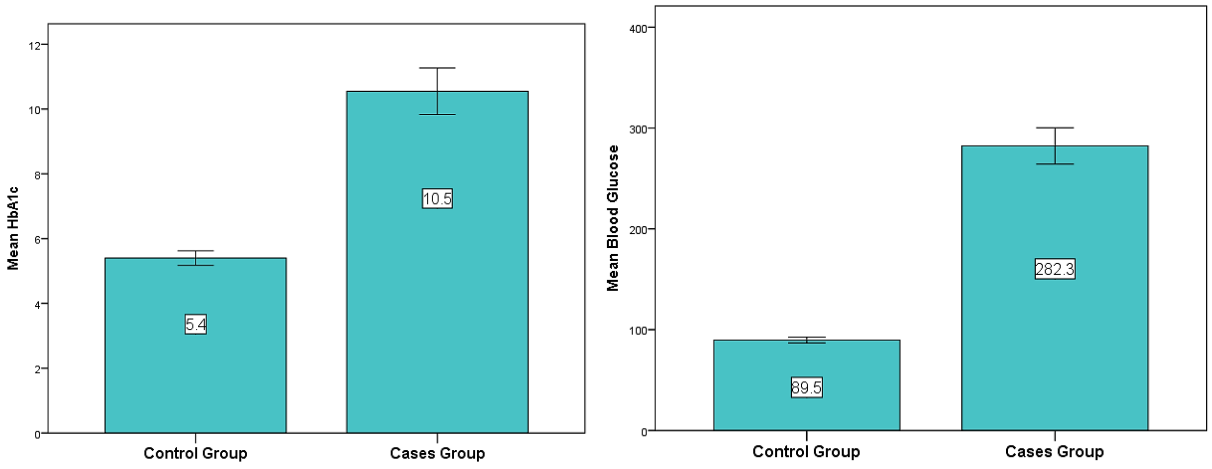

Figure 1 The average values of blood glucose and HbA_1c in the two groups which show higher blood glucose level in a diabetic group by 215% and higher HbA_1c level in a diabetic group by 94% than a control group.

Furthermore, CD4 and CD8 were significantly increased in the diabetic group as compared to the control group and also as shown in table (4) and figure (2). Despite the significant increases of CD4 and CD8 in the diabetic group as compared to the control group, the CD4/CD8 ratio was insignificantly increased in the diabetic group as compared to the control group (p-value =0.3) table (5).

Table 4 Comparison between the studied groups regarding CD4 and CD8 percentages

Parameters / Groups		Control group N=20	Diabetic group N=20	p
Range	CD4 CD8	32.8-39.3 20.9-3.5	49.4-53.1 35.9-42.1	0.001*
Mean ± SEM	CD4 CD8	35.2±0.46 25.1±0.58	56.8±0.61 38.7±0.42	

Table 5 Comparison between the studied groups regarding the CD4/CD8 ratio.

CD4/CD8 ratio	Control N=20	Diabetic N=20	p
1	9(45%)	6(30%)	0.3
2	11(55%)	14(70%)	

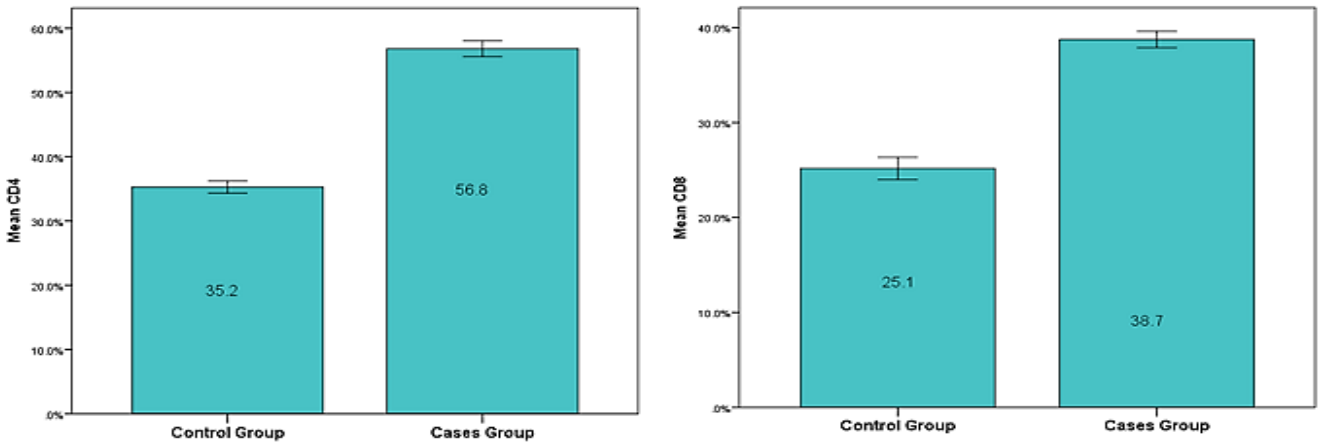

Figure 2 The average values of CD4 and CD8 in the two groups which show a higher CD4 level in the diabetic group by 61% and higher CD8 level in the diabetic group by 54% than the control group.

According to Spearman correlation coefficient, results showed a significant positive relationship between CD4 and CD8 levels with blood glucose level, HbA1C, TC, TG, and LDL as shown in (Figure 3) and both of them show an only negative correlation with HDL level as shown in (Table 6) and (Figure 4 and 5).

Table 6 Correlation between CD4 and CD8 levels with lipid profiles and Diabetic profiles in the Diabetic group.

Variables	CD4 (r)	CD8 (r)
	Pearson coefficient	Pearson coefficient
Blood glucose	0.008	0.15
Hba1c	0.10	0.09
Cholesterol	0.19	0.17
T.G	0.10	0.26
HDL	0.33-	0.08-
LDL	0.09	0.07

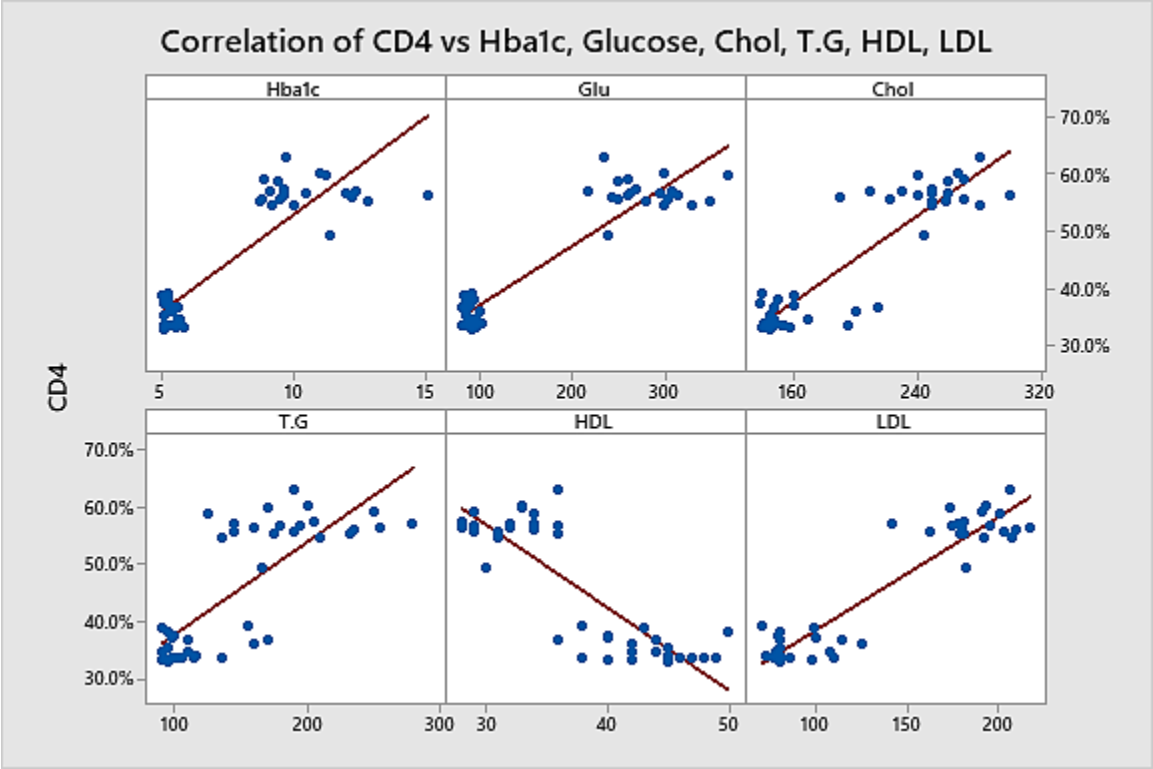

Figure 4 Correlation between CD4 levels with lipid profiles and Diabetic profiles in the diabetic group.

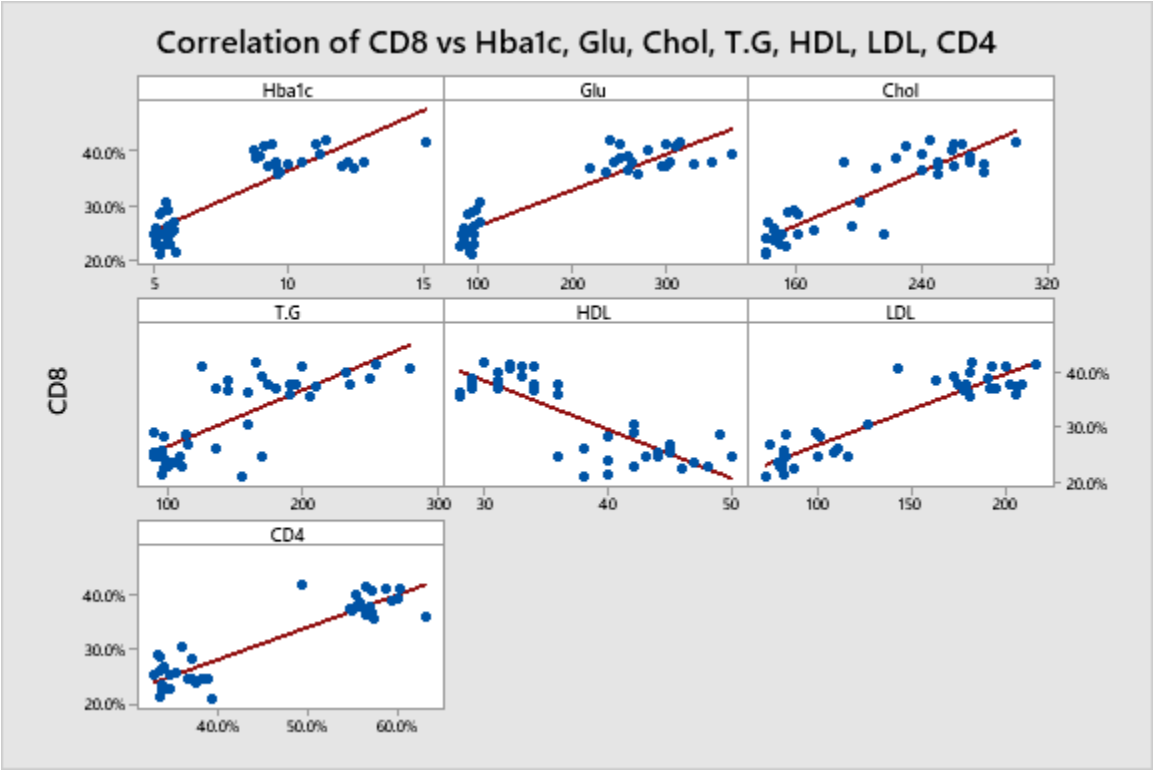

Figure 5 Correlation between CD8 levels with lipid profiles and Diabetic profiles in the diabetic group.

4. DISCUSSION

The study reveals several important findings on the relationship between diabetes and these other parameters. As a poorly controlled long-standing metabolic disorder, diabetes mellitus frequently result in cardiovascular complications. More and more studies suggest that T2DM is an immune-related chronic inflammatory disease and that a reduced number of β cells and insulin secretion disorders may occur when it affects the island (Donath, 2013). The results show that HbA1c in (diabetic group) is higher

than (control group) by 97% as shown in fig. 1 and these data were in line with Oliwia Witczak's study (Witezak and Haugen, 2014). Changes in lipid metabolism have been informed in diabetic patients. In the present study as shown in figs. 3, 4 significantly higher average serum levels of total cholesterol, triglycerides, and LDL cholesterol were noted in diabetic patients who are well-known cardiovascular risk factors compared with normal values (Krauss, 2009). Several studies have demonstrated that insulin affects the production of the liver apolipoprotein and regulates the enzymatic activity of lipoprotein lipase and cholesterol ester transport protein that causes diabetes mellitus dyslipidemia. Besides, insulin deficiency reduces the lipase activity and the production of biologically active lipoprotein lipase by several stages (Mooradian, 2009).

The results show that CD4 in (diabetic group) is higher than (control group) by 61% and CD8 in (diabetic group) is higher than (control group) by 54% as shown in fig. 5 and these data were in line with Lee's study (Lee et al., 2019). The increased number of CD4 + T cells in peripheral blood could be caused by the increased proliferation of CD4+T cells. Suggesting that poor control of glycemia may cause severe infection in T2DM patients (Schuetz et al., 2011). However, few studies have concentrated on the effect of acute changes in glucose metabolism resulting from glucose loading on the peripheral blood T cell subset. Although our study showed that the proportion of CD4+ T cells increased in (diabetic group), the reason why the proportions of CD4+ T cells changed remains unclear (Van et al., 2014).

CD8 + cells may, therefore, have an important role in the inflammatory state of the metabolic syndrome. In a metabolic syndrome scenario, some CD8 + cytokines, such as TNFα, are significantly elevated (Pilatz et al., 2017). Also, the expression of the adhesive molecules in T2DM is increased. Platelet activation (CD31, CD49b, CD62P, and CD63) has been increased in comparison to healthy individuals by patients with T2DM (Ghoshal & Bhattacharyya, 2014). In healthy subjects, acute hyperglycemia occurs, which is demonstrated by elevated surface level (P-selectin and CD40 ligand) and soluble markers (sP-selectin), leading to higher platelet activation and reactivity (Undas et al., 2008). The WBC and diabetes mellitus are linked with the result of higher inflammatory mediators. Inflammatory agents, insulin and human blood constituents are a key signal for all anomalies that lead to invasion and inflammation of other agents (Ohshita et al., 2004).

Technically speaking, various limitations professed to be noted for the current study. Our study was constrained by the moderate number of subjects with diabetes mellitus and the possibility of our investigation was restricted to estimating biochemical profiles, lipid profiles, and CD4 and CD8 levels. In the top of that, Different factors, for example, different adipocytokines, hormones, and quality articulation in tissues, and assessment of metabolic rates besides different parameters that may have influenced changed CDs were not analyzed surrendering the instrument by which coursing CD4 levels are expanded in patients with Type 2 Diabetes.

5. CONCLUSION

We investigated that a close relationship exists between type 2 diabetes mellitus and CD4 and CD8. Study shows that CD4 and CD8 levels in Type 2 Diabetic patients are higher than in healthy people.

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Ethical approval

The study was approved by the Ethical committee Number BSF-022-2019 at Faculty of Science, Beni-Suef University, Beni-Suef, Egypt.

Conflict of interest

The authors declare that there were no conflicts of interest in this study.

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A -Work concept and design
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C –Responsibility for statistical analysis
D –Writing the article
E –Critical review
F –Final approval of the article

Informed consent

Written & Oral informed consent was obtained from all individual participants included in the study. Additional informed consent was obtained from all individual participants for whom identifying information is included in this manuscript.

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Data and materials Availability

All data associated with this study are present in the paper.

Peer-review

External peer-review was done through double-blind method.

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General Note

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