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DETECTION OF IL_6 AND IL_34 LEVELS IN DIABETIC FOOT PATIENTS IN IRAQ

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ABSTRACT
Diabetes foot ulcers are vascular complications of diabetes mellitus that have been widely reported to be very morbidity and mortality related. Interleukins are a subgroup of cytokines, a varied class of biological messenger molecules that influence cellular activity. Interleukin-6 (IL-6), a key cytokine modulator of the acute-phase response, increases the development of acute-phase proteins in the liver and has activities that support diabetes. In addition, the recently identified pro-inflammatory cytokine interleukin (IL)-34 serves as a ligand for the colony-stimulating factor-1 receptor (CSF-1R). Also, IL-34 plays a role in the development of numerous chronic inflammatory and autoimmune disorders. In this study, we aimed to examine the levels of interleukin (IL)-6 and (IL)-34 in diabetic patients with diabetic foot and in healthy controls, from January to July 2022, admitted to Al-Hussein Teaching Hospital in Samawa/ Iraq, Medical city/Baghdad Hospital and private clinics in Baghdad/ Iraq. The study was performed on two groups. They were patients with diabetic foot that included 140 patients, their ages range (29-87) and healthy individuals (control) that included 70 patients, their ages range (18-57). Serum IL-6 and IL-34 levels were measured by sandwich ELISA. Diabetes biochemical parameter (random blood sugar) also analyzed. Random blood sugar for patients was 226.99 mg/dL. The results revealed that diabetic foot ulcers factors were: 93(66.4%) are male and the rest 47(33.6%) are females, high 79(56.40%) are within (31-60) year, 50 (35.7%) of patients with DFUs were current smoker, 55 (39.3%) were overweight, 131(93.6%) were type 2 DM, 62(44.3%) were of duration between (15-30) years, both drugs using were 55 (39.3%) were factors statically associated with diabetic foot ulcer (P value < 0.05). The results also showed a significant increase in the levels of (IL-6 and IL-34) in diabetic foot patients compared to the control plasma group (P≤ 0.05).

Keywords: Diabetic foot ulcer; Interleukin (IL) _6; (IL) _34 and Sandwich ELISA.

1. Introduction
Diabetic Foot Ulcer (DFU) is defined as an ulcerated foot in a diabetic patient that is related with neuropathy and/or peripheral vascular disease of the lower limb. Neuropathy, ischemia, and infection are the typical triads of DFU. Infection and poor wound healing are more likely in people with diabetes because their metabolic processes are impaired. It occurs as a result of a number of factors, including reduced cell and growth factor responses, reduced peripheral blood flow, and reduced local angiogenesis. Damage to peripheral nerves, peripheral vascular disease, ulcerations, deformities, and gangrene all have an impact on the foot (Syafril, 2018). The ulcer can be

superficial, defined as a "full thickness lesion of the skin that does not penetrate any structure deeper than the dermis," or deep, defined as a "full thickness lesion of the skin that penetrates below the dermis to subcutaneous structures involving fascia, muscle, tendon, or bone (Monteiro-Soares *et al*., 2020).

Diabetic mellitus (DM) is a group of metabolic condition identified by hyperglycemia (Mohammed and Salloom, 2021). DM is one of the most significant health risks of the 21st century, and it is the fifth major cause of mortality in developed countries (Khalil, 2016). Patients with DM have a much weaker immune system than healthy persons. As a result, a diabetic patient's foot infection is a life-threatening and debilitating condition. Hyperglycemia induces an increase in pro-inflammatory cytokines, as well as impairments in polymorphonuclear cell functions such as chemotaxis, adhesion, phagocytosis, and intracellular killing. Reduced leukocyte activity, an unsuitable inflammatory response, and cellular immunity disruption (Atlas, 2015);(Al-Attaby and Al-Lami, 2019). In patients with poorly controlled diabetes, leukocyte phagocytosis was dramatically reduced, and improvement in microbiocidal rates was strongly connected with correction of hyperglycemia. Increased metalloproteinases and decreased chemotaxis of growth factors and cytokines obstruct normal wound healing by prolonging the inflammatory state (Al-Musawi *et al*., 2021);(Stojkov *et al*., 2022).

Cytokines are small, biologically highly active proteins that regulate the growth, function, and differentiation of cells and help steer the immune response and inflammation (Iqbal, 2017);(Baqer *et al*., 2022). Insulin resistance has been linked to increased production of proinflammatory cytokines like TNF-α and IL-6 and decreased production of anti-inflammatory mediators like IL-4 and IL-10. Despite some differences over specific cytokine levels, T2DM is now considered a chronic inflammatory illness (Nussrat and Ad'hiah, 2023), whereas T1DM is a T-helper (Th)-1 autoimmune disease (Melekoglu *et al*., 2019). Macrophages and T cells release IL-6, a cytokine that plays a vital role in host defense, IL-6 has both pro-and anti-inflammatory properties (Tahir *et al*., 2022). A large source of IL-6 is adipose tissue, particularly visceral fat. IL-6 promotes the release of acute-phase proteins from the liver, as well as the formation of neutrophils in the bone marrow and B lymphocyte proliferation. As a result, this cytokine contributes significantly to the inflammatory response. Increased levels of IL-6 are linked to diabetes insulin resistance and β-cell inflammation (Abood *et al*., 2014);(Akbari and Hassan-Zadeh, 2018). IL-34 has been linked to a variety of autoimmune and inflammatory illnesses, and its potential as a new therapeutic target has been discovered. IL-34 plays a role in a variety of autoimmune and inflammatory disorders. Furthermore, IL-34 can be found in adipose tissues, and its concentration is significantly higher in obese people and is strongly associated to insulin resistance (Li *et al*., 2018).

2. Materials and methods

2.1 Setting

This was a cross-sectional study conducted at Al-Hussein Teaching Hospital in Samawa/ Iraq and Baghdad Medical city/Baghdad Hospital and private clinics in Baghdad/ Iraq during several months in 2022. The study was approved by the ethics committees of both hospitals.

2.2 Sample collection

The current study was included 140 samples. age (29-87 years) of both sexes suffering diabetic foot ulcer. In addition to this patient group, healthy individuals (control) that included 70 patients. 5 ml of blood was collected from (210) samples of study groups: (140) diabetic with foot ulcer and (70) samples for control group (healthy persons) and transferred into a gel tube for serum separation and kept frozen at -70 C for the immunological investigation to determine to serum IL-6 and IL-34 levels by the sandwich ELISA Kit according to (Elabscience, USA).

2.3 Data collection and classification

The following demographic and health data were collected: gender, age, education, and smoking, type of diabetes, DM duration, sugar value, diabetic foot grade, ulcer duration, type and treatment: whether oral anti-diabetic, insulin, both or no receive any treatment. The type of DM was decided on the standard World Health Organization criteria for diagnosis of diabetes type (Malta *et al*., 2019). Laboratory investigation included random blood glucose.

2.4 Interleukins Assays

Blood specimens for serum IL-6 and IL-34 levels were centrifuged at 4000 rpm for 10 minutes after storage for 30–60 minutes. Serum samples obtained were stored at −70° C in a deep freezer until interleukins assays were performed. Serum IL-6 and IL-34 measurements were performed by using to Human IL-6 and IL-34 ELISA (Elabscience, USA) kit. Results were reported as pg/mL.

3. Statistical analysis

A one-way ANOVA test, an independent t-test, and an LSD test were performed in this study's completely randomized design (CRD) to statistically investigate the variations in means of planned parameters (IL-6 and IL-34) at a level of significance of 5%. Additionally, the frequency data was statistically evaluated using Chi-Square at the 5% level of significance. In order to compare the correlations between the planned parameters, Pearson's correlation coefficient was used. Excel and the Statistical Package for the Social Sciences (SPSS) version 22 were used to complete all of these tasks (2010). The data were then presented as Mean±SD (McDonald, 2014).

Ethical Approval

This study involving human participants was reviewed and the patients/participants were provided their written informed agreement to participate in this paper.

4. Results

4.1 Demographic data and clinical characteristics of patients

A cross-sectional study involved 140 patients with diabetic foot ulcers was conducted at Al-Hussein Teaching Hospital (Samawa/ Iraq), private clinics and Medical city/Baghdad Hospital (Baghdad/ Iraq) from January to July, 2022. A total of 70 healthy controls were used. We evaluated the demographic data including age, gender, education, smoking, BMI, type of DM, DM duration, DFU grade, duration of DFU, and drug using. Random blood sugar for patients was 226.99 mg/dL.

The distribution of diabetic foot ulcers patients in the current study according to age groups as the following: 2(1.40%) are ≤30 year, 79(56.40%) are within 31-60 year, and 59(42.10%) are ≥61 year. The age groups of 31-60 and ≥61 are greater than ≤30 year, Table (4-1). Also, there is

statistically significant difference (P value < 0.05) which indicates that the incidence rate of DFUs increased with the age, table (1).

Table (1): Frequency of diabetic foot ulcers patients according to age groups

Age Groups(years)	No.	%	*P value*
≤30	2	1.40	<0.0001*
31-60	79	56.40	
≥61	59	42.10	
Total	140	100	

* represents a significant difference at p<0.05.

This result of the high incidence rate with the old ages agreed with the study that described about 68% of them aged above 50 years (Fawzy *et al*., 2019). Older age was associated with higher frequency of diabetic foot occurrence it is likely to be associated with a poorer outcome probably because of a slower immune response to infection and the presence of other comorbidities that delay healing such as an impaired vascular blood flow (Musa *et al*., 2018).

Guo *et al*., reported the positive relationship between age and the risk of DFU recurrence (Guo *et al*., 2022). Our results differed with Chinese study that found no significant difference was found in age (Huang *et al*., 2019). The varying age identity criteria used by the involved studies may be the cause of the variable outcomes. As people become older, the wound healing process is impaired, due to many factors such as peripheral arterial disease, decreased defense mechanisms, and impaired immunity (Marzoq *et al*., 2019).

According to the gender, the patients in the present study distributed as follow: 93(66.4%) are male and the rest 47(33.6%) are females. This result suggests that incidence of DFUs is significantly (P value <0.05) associated with the gender factor, table (2).

Table(2): Frequency of diabetic foot ulcers patients according to gender groups

Gender	No.	%	*P value*
Female	47	33.6	<0.0001*
Male	93	66.4	
Total	140	100	

* represents a significant difference at p<0.05.

There is a Turkish study agreed with our study by showing the diabetic foot frequency is significantly different by the gender (Korkmaz *et al*., 2018). Also, the majority patients were 42 male (56.0%) from 75 participants (Kagwa *et al*., 2018). But, our result disagreed with Saudi Arabia study which showed that the occurrence that 70% of the DFUs patients were females (Fawzy *et al*., 2019). Two studies shown that gender was independent risk factors of DFU development, but the author did not give any explanation for this negative result, possibly because of the small sample size (Khalifa, 2018);(Yazdanpanah *et al*., 2018).

According to this as well of other previous studies, moreover, DF syndrome is a strongly genderoriented complication being much more prevalent among males (Crawford *et al*., 2015);(Seghieri *et al*., 2019). They argued that females could have a lower risk relative to males

partly because of less severe neuropathy, increased joint movement, and lower foot pressure (Navarro-Flores and Cauli, 2020).

The present study showed that 50(35.7%) of patients with DFUs were current smoker, whereas the rest 90(64.3%) were not as showed in table (3). The result revealed that there is significant relationship (P value < 0.05) between smoking behaviors and DFUs. This is like a study that found a statistically significant relationship between smoking and DFU (Eltilib, 2021).

Table(3): Frequency of diabetic foot ulcers patients according to smoking groups

Smoking	No.	%	*P value*
Yes	50	35.7	0.001*
No	90	64.3	
Total	140	100	

* represents a significant difference at p<0.05.

Studies by Mariam *et al*., (2017) and Zhang *et al*., (2017) also agreed with our study by showing the diabetic foot is significantly different by smoking. The our result inconsistent with Brazil study that report smoking was not associated with diabetic foot that lead to lower limb amputation or death in this diabetic population (Costa *et al*., 2017).

Previous studies have identified smoking as a risk factor for diabetic foot ulcers because daily tissue hypoxia may cause vascular and neuropathic disorders in the lower extremities of diabetic patients (Obaid *et al*., 2015);(Zhang *et al*., 2017). Its pathogenesis is reduced capacity to transport oxygen in the blood due to harmful by-products of cigarette smoking and results in tissue hypoxia and arteriospasm. This damage leads to compensatory erythrocytosis that increases blood viscosity and decreases tissue perfusion. Decreased tissue perfusion and oxygenation inhibit healing of diabetic ulcer, which can increase the risk of lower extremity amputation LEA (Lee *et al*., 2022). Regarding to the BMI, the distribution of DFUs patients came as the following: 2 (1.4%) were under weight, 41(29.3%) were normal, 55(39.3%) were overweight, and 42 (30.0%) were obesity. The finding of this study showed that overweight diabetic group greater than normal and underweight. Further, Obese diabetic patients were more likely to develop diabetic foot ulcer as compared to diabetic patients with normal body mass index, table (4)

Table(4): Frequency of diabetic foot ulcers patients according to BMI groups

BMI	No.	%	*P value*
Under Weight	2	1.4	<0.0001*
Normal	41	29.3	
Overweight	55	39.3	
Obesity	42	30.0	
Total	140	100	

* represents a significant difference at p<0.05.

This statistically significant difference (P value <0.05) which indicates that the occurrence rate of diabetic foot increased with BMI consistent with the study conducted in Ethiopia (Mariam *et al*., 2017). The possible reason could be due to the presence of higher foot pressure in those heavily

weighed and with higher body mass index (BMI) diabetic patients as well obesity and overweight might decrease intensively the normal blood circulation pattern at the lower extremities; as a result, this might lead them to develop diabetic foot ulcer.

Additionally, a study demonstrated that the association between BMI and diabetic foot, and patients with higher BMI higher increase risk of developing diabetic foot ulceration (Sohn *et al*., 2011).But, our findings disagreed with a study that discussed the association between BMI and DFU. The result showed that there is no statistically significant association between BMI and DFUs (Guo *et al*., 2022).

Out of the total population, the present study showed that only 9(6.4%) patients presented with type 1 DM and the rest 131(93.6%) were type 2 DM. Statistically there is significant relationship between DFUs and type of DM, table (5).

Table(5): Frequency of diabetic foot ulcers patients according to type of DM groups

Type of DM	No.	%	*P value*
Type one	9	6.4	<0.0001*
Type two	131	93.6	
Total	140	100	

* represents a significant difference at p<0.05.

Similar results were which indicated type 2 DM diabetes mellitus was significantly associated with the occurrence of diabetic foot ulcer (Mariam *et al*., 2017);(Zhang *et al*., 2017). Unlike our result, Hussein *et al*., (2022) found that type of diabetes and development of foot ulcers was not associated.

However, the principal mechanisms have not been elucidated. The possible explanation could be in type 2 diabetic patients; there are related complications of the disease, such as mechanical changes in the conformation of the bony construction of the foot, peripheral neuropathy, and atherosclerotic peripheral arterial disease; as a result, the patient may have less tissue epithelisation, consumption of oxygen, nutrient transportation, and cell detoxification resulting in ulceration in the extremities (Mariam *et al*., 2017). Also, there was limited evidence about diabetic foot ulcer epidemiology in type 1 diabetes.

We were using Wagner's classification (Pitocco *et al*., 2019) in determine DF grades and done by physicians. In present study, diabetic foot grades of ulcers as following: 35 (25.0%) were grade 1, 32 (22.9%) were grade 2, 25 (17.9%) were grade 3, 28 (20.0%) were grade 4 and 20 (14.3%) were grade 5. This result suggests that incidence of diabetic foot is not significantly (P value > 0.05) associated with the DF grades, table (6).

Table(6): Frequency of diabetic foot ulcers patients according to DF grade groups

Diabetic foot grade	No.	%	*P value*
Grade 1	35	25.0	0.295
Grade 2	32	22.9	
Grade 3	25	17.9	

Grade 4	28	20.0	
Grade 5	20	14.3	
Total	140	100	

* represents a significant difference at p<0.05.

Similar study conducted by Musa *et al*., (2018) in which gradation made no statically difference to DF ulcers. Unlike our result, a study reported that found relationship between the depth of the ulcer and severity of DFU measured with the Wagner's grades (Jalilian *et al*., 2020).

According to drug using, the present study showed that 40 (28.6%) of patients were insulin using, 43 (30.7%) were oral antibiotic using, 55 (39.3%) were both drugs using, and 2 (1.4%) were no drug using. Statistically, there is significant relationship (P value < 0.05) between DFUs and drug using, table (7).

Table(7): Frequency of diabetic foot ulcers patients according to drug using groups

Drug using	No.	%	*P value*
Insulin	40	28.6	
Oral antibiotic	43	30.7	
Both	55	39.3	<0.0001*
No drug	2	1.4	
Total	140	100	

* represents a significant difference at p<0.05.

Our result shown high percentage 55 (39.3%) of patients were using insulin combined with other oral antibiotic drugs. This significantly association between DFUs and drug using agreed with an Iranian study that stated a significant relationship between medication use and severity of DFU. Also, same a study reported a relation between both oral medication and insulin injections with severity of DFU (Madmoli *et al*., 2019). Turkish study disagreed with our result by report that no significant relationship between insulin treatment, antibiotic use and DFU (Sen *et al*., 2019). Although there is no clear reason for this relation, consider that insulin injection is more related to severity of DFU and can be due to the inflammation reaction in the body (Jalilian *et al*., 2020);(Welty *et al*., 2016).

4.2 An Immunological evaluations of plasma concentrations of selected interleukins:

Five ml of blood was collected from 210 samples of study groups: 140 samples for diabetic patients with foot ulcer and 70 samples for control group (healthy persons) and transferred into a gel tube for serum separation and kept frozen at -80 C for the immunological investigation to determine level interleukins plasma concentrations in human by Sandwich-ELISA methods using Human IL-34 and IL-6 (Elabscience, USA). The concentrations are expressed in pg/ml for Human IL-34 and IL-6. IL-6 and IL-34 amounts were studied in each study groups (control and patient) and shown the statistical differences among study groups, and comparison between each two groups, as shown in table (8).

Table (8): Comparison of study parameters (IL- 6 and IL- 34) among studied groups (control and patient).

Parameters	Patient n=140	Control n=70	*P value*
IL- 6 (pg/ml)	24.6±18.5	11.10±1.41	<0.0001*
IL- 34 (pg/ml)	556.47±74.44	444.74±165.2	<0.0001*

The results showed that amounts of IL-6 and IL-34 among study groups (control and patient) plasma is significantly different (P value <0.05), Table (4-2). The average levels of IL-6 and IL-34 were found to be higher in subjects with diabetic foot (24.6±18.5 pg/ml and 556.47±74.44 pg/ml, respectively) plasma levels compared to controls (11.10±1.41 pg/ml and 444.74±165.2 pg/ml, respectively) plasma levels, in agreement with Al-Salih and Ali (2021) who showed that the statistically significant increase in the levels of (IL-6) in patient groups compared to the control group due to inflammation (P≤0.05). Also, a similar study shown that patients with diabetes and diabetic foot ulcerations at various stages had higher levels of IL-6 levels of plasma compared to diabetic patients without foot ulcerations (Zubair *et al*., 2012);(Zorena *et al*., 2020).

Interleukin (IL)-6, is an important proinflammatory cytokine that plays a potential pathological role in diabetic foot disorder. Proinflammatory cytokines are involved in the pathogenesis of Diabetic foot disorder. Interleukin-6 (IL-6) plays an important role in the inflammatory and autoimmune processes (Lee *et al*., 2019). IL-6 levels are closely related to insulin resistance in type 2 diabetes patients and its complications (Chandrika, 2022). And it was proven through the study that (IL-6) reduces insulin sensitivity in the body and thus the concentration of (IL-6) independently predicts the future risks of developing type 2 diabetes and its complications (Al-Salih and Ali 2021).

Like our result, Zorena *et al*., (2016) also indicated that serum IL-34 levels were shown to be high in patients with T2DM compared to controls, and operating characteristic curve analysis showed that IL-34 has more discriminatory power for the risk of diabetic complications. Also, serum IL-34 was significantly and positively correlated with insulin resistance-related metabolic parameters (Chang *et al*., 2014).

Interleukin-34 improved fat accumulation and inhibited the stimulatory effects of insulin on glucose transport (Chang *et al*., 2014). IL-34 can stimulate monocyte and macrophage differentiation by secreting pro-inflammatory cytokines, such as IL-6 and TNF-α (Anegon, 2017). IL-34 participates in diverse autoimmune and inflammatory diseases, and its role as a novel therapeutic target has been identified. Moreover, IL-34 can be detected in adipose tissues, and the concentration of IL-34 is markedly upregulated in obesity patients and highly related to insulin resistance (Piao *et al*., 2019).

In conclusion, our study found that IL-6 and IL-34 levels are elevated in diabetic patients with foot complications as compared to diabetics without foot ulcers. This study strongly supports the hypothesis that higher plasma IL-6 and IL-34 play an important role in the pathogenesis of foot ulceration. Dependent factors were sex, gender, education, smoking, and type of DM, DM duration, DFU period and drug using. Whereas independent factor was BMI.

5. Conclusion

Excel and the Statistical Package for the Social Sciences (SPSS) version 22 were used to complete all of these tasks (2010), and data were then presented as Mean±SD. The following key points give a summary of our conclusion:

- The distribution of diabetic foot ulcers patients in the current study shown the age groups of 31-60 and ≥61 are greater than ≤30 year. There is a statistically significant difference (P value < 0.05) which indicates that the incidence rate of DFUs increased with age.
- Patients in the present study were distributed as follows: 93 (66.4%) are male, and the rest, 47 (33.6%) are female. This result suggests that the incidence of DFUs is significantly (P value <0.05) associated with the gender factor.
- The present study showed that 50 (35.7%) of patients with DFUs were current smokers, whereas the rest 90 (64.3%) were not.
- The distribution of DFUs patients according to BMI of this study revealed that overweight diabetic group greater than normal and underweight.
- The present study showed that only 9(6.4%) patients presented with type 1 DM and the rest 131(93.6%) were type 2 DM. statistically, there is a significant relationship between DFUs and types of DM.
- According to drug use, the present study showed that 40 (28.6%) of patients were insulin users, 43 (30.7%) were oral antibiotic users, 55 (39.3%) were both drug users, and 2 (1.4%) were not drug users. Statistically, there is a significant relationship (P value <0.05) between DFUs and drug use.
- Our study found that levels of IL-6 and IL-34 were higher in diabetes patients with foot problems than in diabetics without foot ulcers. This data significantly supports the concept that increased plasma IL-6 and IL-34 levels play a role in the development of foot ulceration. Dependent factors were sex, gender, education, and smoking, type of DM, DM duration, DFU period, and drug use. Where the independent factor was BMI.

Recommendations

We recommend a large number of patients and further evaluations of other cytokines in diabetic foot ulcer patients, such as IL-12, IL-17, and IL-18. Using liquid and tissue biopsy for the immunological evaluations.

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Conflict of Interest Statement

We have no conflicts of interest to disclose.

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