JSRMBS Journal of Scientific Research in Medical and Biological Sciences
ISSN 2709-0159(print) and ISSN 2709-1511 (online)
Volume 5, Issue 3 Article 14
DOI: https://doi.org/10.47631/jsrmbs.v5i3.796

EVALUATION OF SOME IMMUNOLOGICAL PARAMETERS ASSOCIATED WITH DIABETIC FOOT INFECTION IN KERBALA GOVERNORATE

Sarah Mohammed Mohsin[1], Ahmed Jawad Al-Shekerchi[2]

[1]University of Kerbala, Collage of Pharmacy, Kerbala, Iraq.

[2]University of Kerbala, Collage of Sciences. Kerbala, Iraq

ARTICLE INFO

Recieved: 11 June 2024
Revised: 20 July 2024
Accepted: 27 Augt 2024

Keywords:
Diabetic foot infection, TLR2, IL-17A, CRP

Corresponding Author:

Sarah Mohammed Mohsin

Email:
sarah.mohsin@s.uokerbala.edu.iq
ahmed.jawad@s.uokerbala.edu.iq

ABSTRACT

Diabetes mellitus is a long-term illness that gets worse with time. Diabetic foot infection (DFI) is one of its numerous side effects that can cause major alterations in a number of immunological markers. In Kerbala, Iraq, this study examined a number of immunological markers in patients with type 2 diabetes mellitus (T2DM) with or without diabetic foot ulcers, along with in healthy volunteers. The markers included Toll-like Receptor-2 (TLR-2), Interleukin-17A (IL-17A), and C-reactive protein (CRP). The study, which included 120 patients with diabetic foot of females and males and ages ranging from 35 to 75 years, was carried out at the Imam Al-Hassan Center for Endocrinology and Diabetes in the city of Karbala Iraq. Each participant had seven milliliters of blood extracted using a disposable syringe; the serum was separated in a cooled centrifuge by centrifugation at 4000 xg. Before analysis, the serum samples were kept at -20°C. As compared to the T2DM and control groups, the average concentration of TLR-2 in DFI patients had been substantially greater (7.36±1.85 ng/ml) ($p < 0.001$). Comparably, compared to the T2DM and control groups, the average concentration of IL-17A in DFI patients was considerably higher (123.7±33.52 ng/L) ($p < 0.001$). Furthermore, DFI patients had a mean CRP level that was considerably higher than that of the T2DM as well as control groups (92.9±78.26 mg/L; $p < 0.001$). The study concluded that, in comparison to the T2DM and control groups, DFI patients had notably higher levels of the immunologic biomarkers TLR-2, IL-17A, and CRP. These results point to a possible involvement of these indicators in the development of type 2 diabetes.

INTRODUCTION

As one of the top 10 causes of death worldwide, diabetes is a serious global health concern, based according to International Diabetes Federation's Diabetes Atlas [1]. A major increase in the overall incidence of diabetes is anticipated, according to a new study. 9.3% of the world's population (463 million) is predicted to develop diabetes by 2030; by 2045, this number is predicted to rise to 10.2% (578 million) [2]. One dangerous side effect of diabetes is diabetic foot ulcers, which frequently result in amputation. Diabetes patients account for more than half of all amputations, which usually result from infected foot ulcers [3]. These ulcers are brought on by poor circulation (vascular disease), which is frequently linked to diabetes, and nerve damage (peripheral

neuropathy). About half of diabetic foot ulcers get infected if treatment is not received, and 20% of these instances result in bone infections (osteomyelitis). Twenty percent of ulcer patients require lower limb amputation due to diabetic foot, which is also one of the primary causes of non-traumatic amputation of lower limbs [4]. With a roughly 40% 5-year survival rate, diabetic foot ulcer patients also have high death rates. The death rate rises sharply to almost 60% for patients who have had an amputation [4].

Researchers are looking into the immune system's possible involvement in diabetic foot ulcers and type 2 diabetes. Toll-like receptors (TLRs), in particular, seem to be important components of the innate immune system. According to studies, certain TLRs may cause the immune system to react to signs of bodily injury [5]. TLRs may play a role in the onset of type 2 diabetes since the disease is characterized by the accumulation of these damaging signals [5]. Furthermore, studies have indicated the adaptive immune system's role, particularly that of Th17 cells, which generate the chemical IL-17A and may be involved in the pathophysiology of type 2 diabetes [6]. Moreover, raised C-reactive protein (CRP) levels may indicate a higher chance of type 2 diabetes [7].

The Goal of Our Study

The aim of this study was to measure the levels of several important immune system markers in three different participant groups: people who have diabetic foot ulcers, diabetic individuals without foot ulcers, and healthy control participants without diabetes. This was done because it appears that the immune system plays a critical role in both type 2 diabetes and diabetic foot ulcers. Toll-Like Receptor 2 (TLR-2), Interleukin-17A (IL-17A), and C-Reactive Protein (CRP) levels in these three groups were specifically measured as part of the study. The researchers aimed to obtain additional understanding regarding the immune system's possible role in the onset and advancement of diabetic foot ulcers by examining the concentrations of these critical immune system components in each of the three study groups.

METHODOLOGY

Study design and Samples collection:

The design of this study was case control. A total of 120 blood samples were drawn from three distinct participant groups: forty individuals with type 2 diabetes mellitus without foot ulcers, Forty individuals with diabetic foot ulcers, and forty healthy people without diabetes. Patients with type 2 diabetes mellitus and diabetic foot ulcers who were over 35, of both sexes, and who had received a previous diagnosis from a clinical medical professional within the Imam Al-Hasan Center for Endocrinology and Diabetes between October 2022 and January 2023 were eligible to join the diabetic foot ulcer group.

Before being centrifuged in a cooled centrifuge, the peripheral blood samples were gathered and kept in gel tubes. After the serum was separated, it was kept in a deep freezer until it was time to assess three important markers of the immune system: C-Reactive Protein (CRP), Interleukin-17A (IL-17A), and Toll-Like Receptor 2 (TLR-2).

People with type 1 diabetes mellitus, pregnant women, patients with foot ulcers who were not diabetics, and patients under the age of 35 were not included in the study.

Determination of immunological parameters in serum:

Determination of Toll-like receotor-2 (TLR-2):

An ELISA kit for human Toll-like Receptor-2 was used to measure the level of Toll-Like Receptor 2 (TLR-2). Enzyme-Linked Immunosorbent Assay (ELISA) kit produced in China by Bioassay

Technology Laboratory. Antibodies specific to human TLR-2 were pre-coated on the ELISA plate used in this experiment. After that, the TLR-2 in the sample was able to attach itself to the antibodies on the plate. A human TLR-2 antibody that had been biotinylated was then added, and it too bound to the TLR-2 present in the sample. Streptavidin-HRP, that attaches to the biotinylated TLR-2 antibody, was then added. After that, any unattached Streptavidin-HRP was removed with an ELISA washer.

Subsequently, a substrate solution had to be added, and its color changed in direct proportion to the quantity of human TLR-2 found in the specimen. The process was then stopped by adding an acidic stop solution, while the optical density (OD) at 450 nm was recorded. The researchers were able to measure the amounts of TLR-2 in the blood samples taken from each study group using an ELISA-based technique.

Standard solution preparation:

The standard solution was reconstituted with an initial concentration of 48 ng/ml, following the guidelines included with the ELISA kit. In order to achieve this, 120 microliters of the standard diluent and 120 microliters of the standard solution were combined, yielding a standard stock solution with a 24 ng/ml concentration.

The method was then repeated in serial dilutions to get standard solutions at concentrations of 1.5 ng/ml, 1.5 ng/ml, 6 ng/ml, and 3 ng/ml. The zero standard was the standard diluent alone, with a concentration of 0 ng/ml.

After that, a microplate reader calibrated to 450 nm was used to directly measure the optical density (OD) of each of these standard solutions. Within ten minutes after applying the stop solution to the mixture, this measurement was made.

A TLR-2 standard curve was created utilizing these standard solutions, which had known TLR-2 concentrations. As seen in Figure 1, this standard curve included a range of 0.000 ng/L to 24.000 ng/ml. Using the measured OD values at 450 nm, the researchers were able to calculate the TLR-2 concentrations in the samples that were not known thanks to the use of this standard curve.

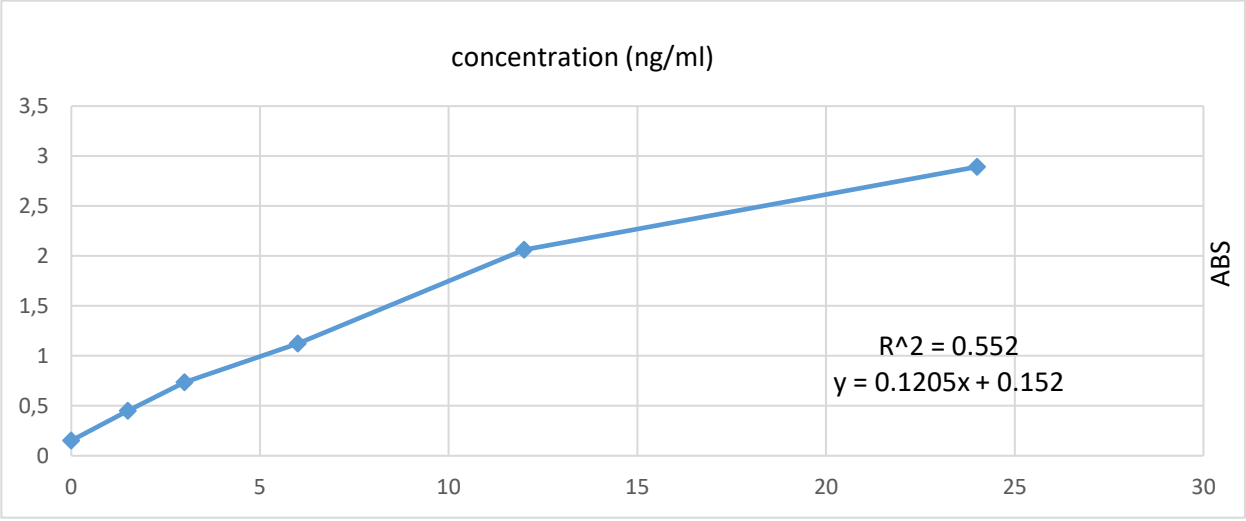

Fig. No.1: Standard curve of TLR-2

Determination of Interleukin-17A (IL-17A):

This was performed by using of Human Interleukin-17A ELISA kit as described in TLR-2 determination.

Standard solution preparation:

The standard solution was reconstituted with an initial concentration of 1280 ng/L, following the guidelines included with the ELISA kit. In order to do this, 120 microliters of the standard diluent and 120 microliters of the standard solution were combined, yielding a standard stock solution with a 640 ng/L concentration.

The same process was then used in serial dilutions to create standard solutions at concentrations of 40, 80, 160, and 320 ng/L. The zero standard was the standard diluent alone, with a concentration of 0 ng/L.

Next, using a microplate reader set to 450 nm, the optical density (OD) of these standard solutions was directly measured. After applying the stop solution to the samples, the samples were measured within ten minutes.

An IL-17A standard curve was created using these standard solutions, which had known IL-17A concentrations. Figure 2 illustrates the range of this standard curve, which was 0.000 ng/L to 640.000 ng/L.

By using this standard curve, the researchers were able to infer the IL-17A concentrations from the obtained OD values at 450 nm in the unknown samples.

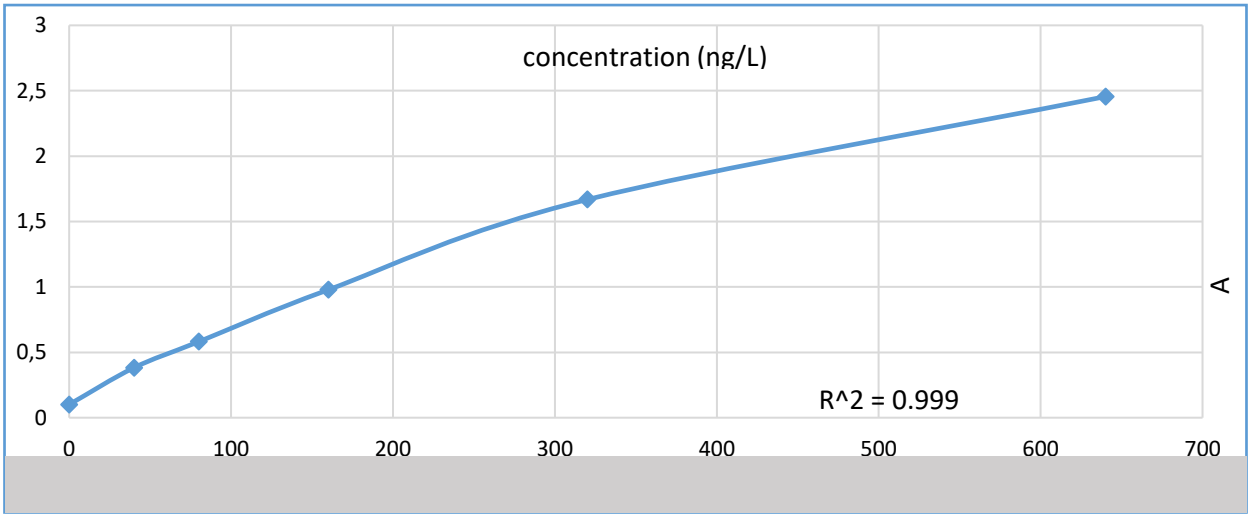

Fig. No.2: Standard curve of IL17-A

Determination of C-reactive protein (CRP):

This parameter was determined according to turbidimetry method by using of CRP kit prepared by Shenzhen Mindary Bio-Medical Electronics Co., China.

Reaction principle:

An immunoagglutination reaction served as the basis for determining the concentration of C-Reactive Protein (CRP). The kit's anti-human CRP antibodies interacted with the sample's CRP molecules to produce an immunocomplex. The amount of CRP present in the sample was exactly correlated with the turbidity or light absorbance of this immunocomplex. Stated differently, an increase in CRP leads to an increase in immunocomplex formation and an increase in observed absorbance.

The following stages were engaged in the assay procedure: • The kit components were combined and incubated at 37°C for 3 minutes. Next, the absorbance of the blank was measured. The blank and sample solutions were then combined completely at 37°C with 50 µL of Reagent 2.

• Five minutes later, the absorbance was tested once more.

• The analyzer then automatically determined each sample's CRP concentration following the necessary calibration.

By measuring the immunocomplex formation photometrically, the researchers were able to measure the amounts of CRP in blood samples taken from each of the study groups.

Ethical consideration:

The University of Karbala's College of Science Ethical Committee gave its approval for this research. Before any samples were collected, all subjects who were engaged in this research project were informed about it and gave their verbal agreement.

Statistical Analysis:

The main objective was to find any significant differences between the several groups under investigation.
If a stringent statistical test was passed, the differences were deemed statistically significant. The researchers used a method known as Receiver Operating Characteristic (ROC) analysis to ascertain the best approach to identify crucial circumstances.

ROC analysis is a potent statistical technique that aids in assessing the performance and diagnostic accuracy of various tests or prediction models. The researchers could discover the best cut-off values or thresholds for recognizing significant clinical or research-related results by employing ROC analysis.

Based on the data gathered, the investigators were able to determine the most efficient methods for identifying key moments and make trustworthy findings regarding the variances among the study groups thanks to the analytical technique they employed.

RESULTS AND DISCUSSION

A case-control study was how the current investigation was planned. The analysis comprised 120 persons in total:

1. The "DFI" group consisted of 40 patients who had both type 2 diabetes mellitus (T2DM) and diabetic foot ulcers.

2. T2DM patients without foot ulcers made up 40 of the subjects.

3. Forty people appeared to be healthy controls.

The samples were taken at the Imam Al-Hassan Center for Endocrinology and Diabetes over the course of four months, from October 2022 to January 2023.

The findings demonstrated that, in comparison to the healthy control group, patients with diabetic foot infections (DFI) had noticeably greater levels of Toll-like receptor 2 (TLR-2). The DFI patients had a mean TLR-2 level of 7.36 ± 1.85 ng/ml, as indicated in Table 1. This was substantially greater than the values seen in the T2DM and control group ($p < 0.001$). These results imply that in people with type 2 diabetes mellitus, the onset of diabetic foot infections may be linked to elevated TLR-2 expression.

Tab. No. 1: Mean difference of some biomarkers among the Three Studied Groups

Biomarker	DFI N=40 (mean±SD)	DM N=40 (mean±SD)	Control N=40 (mean±SD)	*P*-value
TLR-2 (ng/ml)	7.36±1.85*	6.46±2.09	4.74±0.92	<0.001
IL-17A (ng/L)	123.7±33.52	107.4±32.10*	91.78±13.58	<0.001
CRP (mg/L)	92.9±78.26*	9.16±3.00	7.47±2.79	<0.001
ANOVA was *: significant at p ≤ 0.05, Post hoc (LSD) N: number of cases; SD: standard deviation; *: significant				

The study's findings regarding the relationship between age and TLR-2 levels showed that, in the diabetic foot infection (DFI) group, mean TLR-2 levels rose with age, especially in the 55–64 age range. The DFI cohort's mean TLR-2 levels were greater in the older age groups than in the younger age groups, as Table 2 illustrates. This implies that the age of the individuals may have an impact on TLR-2 expression, as older DFI patients show noticeably higher TLR-2 levels than younger patients.

These results suggest that age should be taken into account when assessing TLR-2's involvement in the etiology of diabetic foot infections. Age-related alterations in the immune system and the emergence of problems connected to diabetic foot disease may be explained by the elevated TLR-2 levels seen in the older age groups of DFI patients.

Tab. No. 2 : Age effects on the Toll Like Receptor-2 levels according to the three studied groups

Groups	(35 – 44) Years (mean±SD)	(45 – 54) Years (mean±SD)	(55 – 64) Years (mean±SD)	(65 – 74) Years (mean±SD)	(≥75) Years (mean±SD)
	Concentration (ng/ml)				
DFI	6.20±0.55	7.61±0.55*	8.68±0.55	7.21±0.60	7.12±0.60*
DM	6.01±0.55*	7.27±0.55	6.58±0.60	5.98±0.60*	6.49±0.55
Control	4.55±0.55	4.16±0.55	4.27±0.55	5.35±0.55	5.38±0.55
ANOVA was *: significant at p ≤ 0.05, Post hoc (LSD)					

Determination of Interleukine-17A (IL-17A) levels:

Interleukin-17A (IL-17A) levels were considerably greater in patients with diabetic foot infections (DFI) than in the healthy control group, according to the study's findings. Table 1 indicates that the DFI group had a mean level of IL-17A of 123.7 ± 33.52 ng/L, significantly greater than the values found in the control and diabetic mellitus (DM) groups ($p < 0.001$). These results suggest that, in comparison to the healthy persons, the DFI patients exhibited a significantly higher range of IL-17A concentrations.

Moreover, the examination of IL-17A levels in correlation with age demonstrated that these levels significantly increased with age, especially in the 45–54 and 55–64 age groups, as indicated by Table 3.

These findings imply that age plays a significant role in determining the levels of this proinflammatory cytokine in people with diabetic foot infections and that elevated expression of IL-17A may be linked to the onset and progression of diabetic foot problems.

.

Tab. No. 3 : Age effects on the IL-17A levels according to the three studied groups

Groups	35 - 44 Years (mean±SD)	45 - 54 Years (mean±SD)	55 - 64 Years (mean±SD)	65 - 74 Years (mean±SD)	≥75 Years (mean±SD)
			Concentration (ng/L)		
DFI	119.57±10.98	144.00±12.03*	140.87±10.98	119.61±10.98*	94.42±12.03
DM	107.04±10.98	105.85±12.03	111.78±10.98*	119.30±12.03	93.01±10.95
Control	100.15±10.98	95.34±10.98	87.41±10.98	88.92±10.98	87.08±8.92

ANOVA was *: significant at $p \leq 0.05$, Post hoc (LSD)

Determination of C-Reactive Protein (CRP) levels

When comparing the mean C-Reactive Protein (CRP) levels of patients with diabetic foot infections (DFI) to those with diabetes mellitus (DM) and healthy control groups, a substantial rise was observed.

The DFI group's mean CRP level was 92.9 ± 78.26 mg/L, as indicated in Table 1. This was substantially higher than the CRP levels seen in the DM and control groups ($p < 0.001$). According to these results, the CRP concentrations of the DFI patients were significantly higher than those of the other research groups.

Moreover, an examination of CRP levels in various age groups showed that CRP rose with advancing years. Table 6 shows that the CRP levels were higher in the older individuals, especially in the 55–64 age range, and gradually decreased.

These findings imply that the onset and advancement of diabetic foot problems are linked to elevated CRP production. Furthermore, it seems that age has a significant role in determining the levels of this inflammatory marker, as middle-aged DFI patients had the highest CRP values.

Tab. No. 4: Age effects on the CRP levels according to the three studied groups

Groups	(35 – 44) Years (mean±SD)	(45 – 54) Years (mean±SD)	(55 – 64) Years (mean±SD)	(65 – 74) Years (mean±SD)	(≥75) Years (mean±SD)
	Concentration (mg/L)				
DFI	85.97±18.40*	112.30±18.40*	129.37±18.40*	76.12±20.16*	60.76±20.16*
DM	8.47±18.40	9.72±18.40	11.24±20.16	8.82±20.16	7.55±18.40
Control	6.63±18.40	8.53±18.40	8.23±18.40	8.12±18.40	5.88±18.40
ANOVA was *: significant at $p \leq 0.05$, Post hoc (LSD)					

Odd ratio:

The relationship between the biomarkers (C-Reactive Protein (CRP), Interleukin-17A (IL-17A), and Toll-like Receptor 2 (TLR-2)) and the onset of diabetic foot infection (DFI) and diabetes mellitus (DM) was examined using a multinomial logistic regression analysis. The biomarkers (CRP, IL-17A, and TLR-2) demonstrated a highly significant correlation with instances of diabetic foot infection (DFI), according to the regression analysis's findings. It has been determined that certain biomarkers increase the likelihood of developing diabetic foot problems.

More specifically, a higher CRP level was associated with a considerably increased risk of DFI, as indicated by the odds ratio (OR) for CRP of 55.058 (95% CI: 3.000-56.021). Comparably, the OR for IL-17A was 1.035 (95% CI: 1.012-1.059), indicating that for every unit rise in IL-17A levels, there was a 3.5% greater risk of DFI.

Additionally, the OR for TLR-2 was 4.347 (95% CI: 2.257-8.371), indicating that greater TLR-2 levels are associated with a risk of DFI that is more than four times higher. These results demonstrate the potential value of CRP, IL-17A, and TLR-2 biomarkers as

significant risk factors and predictive markers for the onset of diabetic foot infections in individuals with diabetes mellitus.

ROC analysis of TLR-2:

Examining TLR-2 as a Diagnostic Tool:

Using Receiver Operating Characteristic (ROC) curve analysis, the study assessed Toll-like Receptor 2 (TLR-2)'s potential as a diagnostic marker for diabetes mellitus. The results are shown in Table 5.

The ROC curve analysis's results were encouraging, showing that diabetes patients could be distinguished from healthy controls with reasonable accuracy. At a TLR-2 level of 5.87 ng/ml, the analysis revealed a sensitivity of 81.4% and a specificity of 97.6%. With a p-value of less than 0.001, Youden's J statistic (as seen in Figure 3) further reinforced the statistical significance of this conclusion.

According to these findings, TLR-2 may prove to be a useful biomarker for diabetes mellitus diagnosis. TLR-2 is an invaluable tool for identifying and making a diagnosis of diabetes due to its high sensitivity and specificity, which were noted in the ROC curve analysis. TLR-2 can efficiently discriminate between those who have diabetes and those who do not. TLR-2's clinical relevance in the administration of diabetes is highlighted by its statistical value and Youden's J statistic, which offer extra confidence in the marker's function as a diagnostic.

TLR-2 in Diabetic Foot Infections (DFI) and Diabetes Mellitus (DM):

The usefulness of Toll-like Receptor 2 (TLR-2) in the diagnosis of diabetes mellitus (DM) and diabetic foot infections (DFI) independently was also investigated in this investigation. In comparison to the whole diabetic group, the results showed that TLR-2 levels were considerably more accurate and precise to identify those with diabetic foot infections (DFI). At a TLR-2 level of 6.347 ng/ml, the ROC curve analysis revealed a sensitivity of 80% and a specificity of 70% for the diagnosis of DFI. As seen in Tables 6, Figures 4, and 5, this result was statistically significant with a p-value of less than 0.001.

These findings imply that TLR-2 has a great deal of promise as a marker for diagnosis for DM and DFI patients' differentiation. TLR-2 can be a useful tool in the early detection and diagnosis of diabetic foot problems, which are a major source of morbidity and mortality in people with diabetes, as shown by the enhanced sensitivity and specificity seen for the DFI group. The therapeutic usefulness of TLR-2 as a diagnostic marker for diabetic foot infections is further strengthened by the statistical significance of these results, which offer important new information for the treatment and avoidance of these serious diabetes sequelae.

Tab. No. 5: ROC screening sensitivity and specificity of TRL-2 in DM (both DFI and DM) patients compared to control

ROC analysis	TRL-2
AUC	92%
Sensitivity	81.4%
Specificity	97.6%
P value	<0.001[S]
Cut off	5.87
Youden index	0.781
CI% (Lower- upper)	(0.860-0.973)
PPV	92.68%
NPV	88%
Accuracy	70%

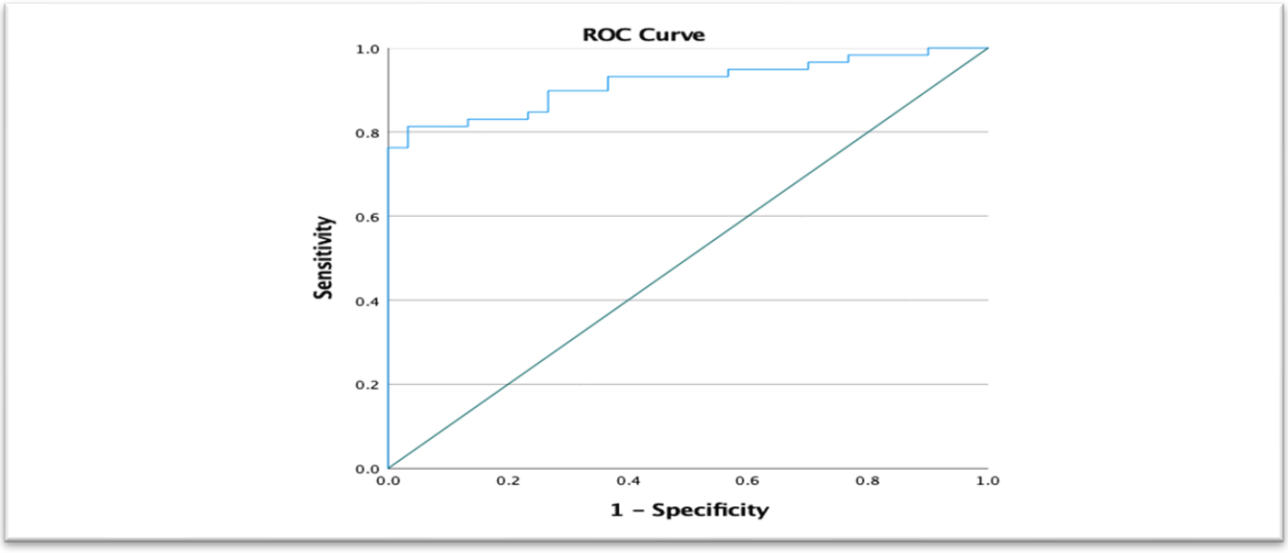

Fig. No. 3: ROC curve of TLR-2 levels in Patient and Control, The area under the ROC curve: 92%

Tab No. 6: ROC display sensitivity and specificity of TRL-2

Test Result Variable(s)	DFI	DM
AUP	74.6%	67.3%
Sensitivity %	80%	79.3%
Specificity %	70%	65%
Youden index	0.461	0.36
Cut-off points	6.46	5.87
CI (95%)	(0.643-0.849)	(0.557-0.790)
P value	<0.001[S]	0.008[S]

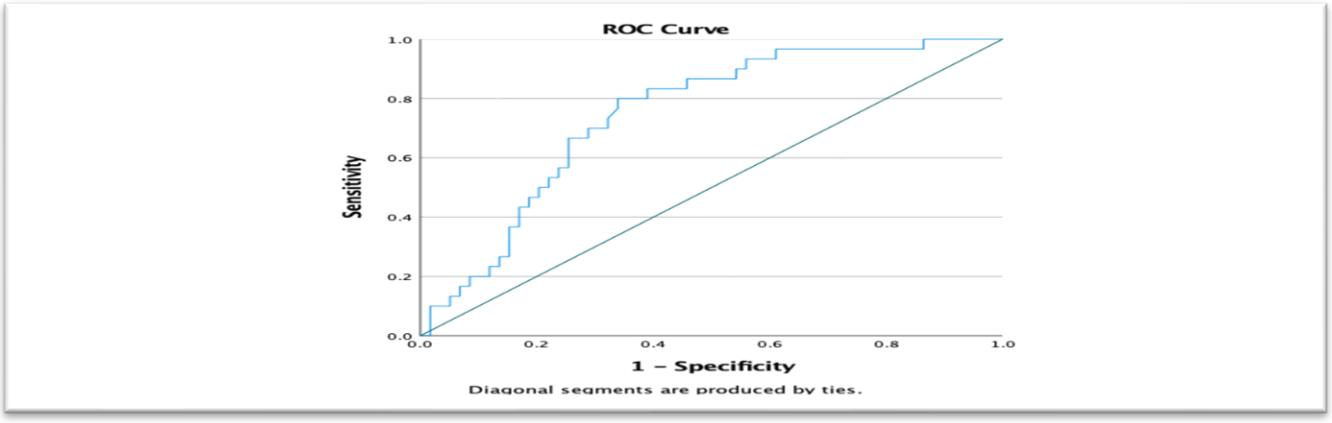

Fig. No. 4: ROC curve of Toll-like levels in Patients for DFI, The area under the ROC curve: 74.6%,

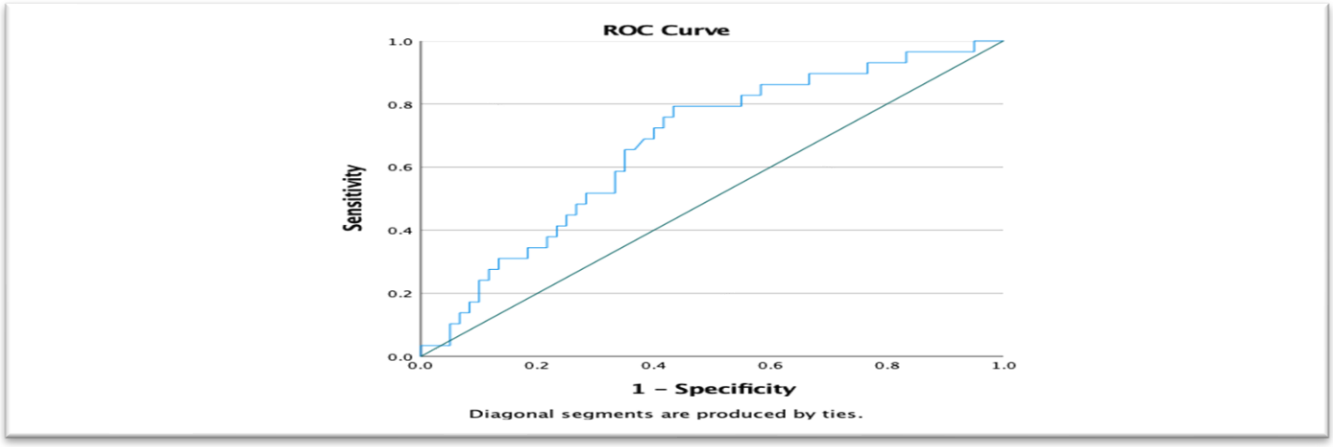

Fig. No. 5: ROC curve of Toll-like levels in Patient for DM, The area under ROC curve: 67.3%

Discussion:

Numerous research have examined the expression levels of Toll-like Receptor 2 (TLR2) in relation to diabetes and diabetic foot infections. According to a 2014 study conducted in the USA by Dasu and Martin, diabetic patients' wounds had considerably higher TLR1, 2, 4, and 6 mRNA expressions than non-diabetic wounds (8). Comparing those with well-controlled diabetes and problems to non-diabetic volunteers, an Irish study conducted in 2017 by Gupta et al. discovered that those with higher TLR2, 4, and 5 levels had diabetes-related issues (9). In an Egyptian investigation, Wifi et al. (2017) discovered no statistically significant variation in TLR2 expression among the three categories they looked at (10).

On the other hand, in their US-based investigation, Mohammad et al. (2006) observed elevated TLR2 expression in the bone marrow-derived macrophages of non-obese diabetic mice (11). Furthermore, a different study conducted in the UK demonstrated increased TLR2 expression in the adipose tissue of individuals diagnosed with type 2 diabetes (T2DM) (12). It is known that TLR-2 functions as a signaling receptor for a variety of microbial products, such as those produced by mycoplasma and Gram-positive bacteria (13). According to a 2009 study by Ajuwon et al., peptidoglycan produced from Staphylococcus aureus caused adipocyte cell lines to express TLR2 more highly (14). The discovery that TLR2-deficient mouse strains are more prone to infections by Gram-positive bacteria like Staphylococcus aureus indicates that TLR2 has an anti-infectious effect (15).

Regarding the impact of aging on TLR expression and signaling in peripheral leukocytes, the data is wildly contradictory. research like the Australian work of Simpson et al. (2013) (16) have shown rising TLR-2 function and expression; conversely, research from the USA (17) have shown decreases in TLR-2. Furthermore, some American investigations have reported no aging-related changes in TLR-2 (18). Moreover, elderly mice with decreased TLR function were studied by Renshaw et al. (2002) in Atlanta (19). Although TLR miRNA analysis has been shown in previous papers to show varying expression patterns with age, the functional significance of these modifications in the transcriptional regulation of TLR adaptor molecules remain to be determined (20).

According to a study by Van Duin and collaborators done in the United States, older persons respond less favorably to TLR1/2-specific activation than younger participants do, producing less TNF-α and IL-6 as a result (21).

In terms of IL-17A, the present findings concurred with those of a local study by Kadhim (2021), which showed that patients with diabetic foot ulcers (DFUs) had significantly higher levels of IL-17A than did the control group (22).

Comparing all diabetic groups to the healthy control group, AL-Sahi and colleagues also found a very substantial difference ($p<0.01$) in IL-17A levels, with the DFU group having the highest amount of IL-17 (23).

The present findings are also consistent with the research conducted in India by Parhi et al. (2019), who reported that IL-17 levels were greater in diabetes patients than in healthy controls, and that the levels of IL-17 were considerably higher in patients with problems than in those without (24). Additionally, Zareian and Mirzaii Dizgah's (2014) study from Iran found that patients with type 2 diabetes mellitus (T2DM) had significantly higher serum concentrations of IL-17 than the controls ($p=0.002$) (25).

Furthermore, compared to the control group, the diabetic foot group and the diabetic group had higher levels of HbA1c, white blood cells, IL-17, and IL-18 in a study conducted in Turkey by Kaleli et al. (2019) (26).

As a pro-inflammatory cytokine, IL-17A has two functions: it stimulates the immune system's early defenses against infections but also plays a part in autoimmune diseases and harmful inflammatory states. As IL-17 is a pro-inflammatory cytokine, the high levels in DFI patients may be caused by the inflammation associated with ulceration, compromised skin barrier, and different kinds of infectious bacteria. These findings support the hypothesis that the higher levels of IL-17 found in the diabetic foot infection (DFI) and diabetes mellitus (DM) groups. One possible explanation is that metalloproteinase stimulation, hypertension, and vascular dysfunction may be amplified when IL-17A interacts with its receptor. An additional mechanism involves the activation of the JAK/STAT system, which may result in the dysregulation of molecules associated to gluconeogenesis, beta-cell and liver cell death, and hepatic insulin resistance (27). Given that the DFI and DM groups had higher levels of IL-17 than the control group, it is possible that IL-17 is a marker for diabetes diagnosis and is linked to the course of the diabetic condition. Additionally, the DFI group's significantly greater level of IL-17 suggests that the infection can be diagnosed as a pro-inflammatory marker for diabetic foot (26).

Age-associated traits emerge as a result of intrinsic changes in stem cells as well as tissue-specific alterations in the microenvironment during the aging process. The aging skin environment seems to be in a low-level but permanent state of chronic inflammation, which is similar to that seen in severe skin illnesses (28). IL-17-mediated signaling is significantly connected with the onset of chronic inflammatory and autoimmune diseases.

Numerous research projects have looked into the possible relationship between age-related disorders and the persistence of robust inflammatory responses. Since inflammation is a known amplification factor, the persistence of severe inflammatory responses with aging, in the absence of immune system counterbalancing and therapeutic responses, may greatly worsen disease presentation (29)

According to earlier research, an aging immune system may accelerate the development of a number of diseases by causing chronic inflammation, particularly through the activation of interleukin signaling (30). Research has indicated that IL-17 is dysregulated as mice age, and that older mice have a higher percentage of IL-17-producing cells than younger mice (31). Diabetes and other autoimmune and chronic inflammatory disorders have been linked to IL-17A overexpression (32). According to a recent study, one of the key cytokines influencing the development of diabetic complications is IL-17A (33).

The current findings on C-reactive protein (CRP) were in line with a local study conducted by Kadhim (2021), which found that the diabetic foot infection (DFI) group had statistically significant higher levels of CRP than the diabetes mellitus (DM) and healthy people (22). Our findings are also consistent with a local study conducted by Muhanedalnajer (34), which confirmed that CRP levels were 2.68 ± 1.7 in healthy individuals, 8.95 ± 4.61 in diabetic patients, and $103.11 = 68.35$ in DFI patients. Furthermore, our findings are corroborated by a Chinese study

conducted by Xu and his colleagues, which found that DFI patients had higher CRP levels than DM and healthy people (35).

Acute-phase protein CRP is thought to be the main inflammatory mediator the liver produces after an acute infection or inflammation. Infection and damage can cause its concentration to rise up to a thousand times. Furthermore, the onset of cardiovascular disease and type 2 diabetic mellitus (T2DM) can be predicted by elevated CRP levels (36). CRP has been shown to be a significant risk factor for diabetes and is linked to both the severity of glycemic control and the several complications that might arise from the disease (37).
The age group comparison results are consistent with local Shaalan research that found increased CRP levels in the 50-to-over-60 age group compared to younger DFI patients (38). Increased inflammatory marker levels are frequently linked to age-related illnesses (39).

The mechanism behind age-related issues also involves low-grade inflammation (40). Immunosenescence is the term for the general reduction in immune system function that occurs with aging. The three theories that underpin immunosenescence are immune dysregulation (disturbance in the regulation of multiple immune system components), immune insufficiency (decreased effectiveness of the immune system), and autoimmune (reduced capacity to distinguish between invaders and normal tissues). Aging is linked to higher levels of pro-inflammatory markers such CRP, according to numerous studies (41). Over 70% of older populations suffer from several chronic disorders, making up a large number of those over 65 who suffering from various diseases (42).

Because wounds are a perfect place for bacteria to colonize and multiply because they provide a great medium for bacterial development, the higher CRP levels in diabetic foot patients correspond with the fact that the majority of lesions are infected (43). According to earlier research, type II diabetes might be an inherent immune system illness (44).

The findings of Arbibe et al. (2000), which demonstrated the significance of TLR-2 in the pathophysiology of DM and associated consequences, were consistent with the results of the ROC analysis for TLR-2. According to the suggested mechanism, Rac1, a crucial mediator of oxidative stress in monocytes, is necessary for TLR2-mediated NF-kB activation in monocytes. Thus, TLR2 may be important for oxidative stress in diabetic wounds because it activates Rac1, which in turn activates NF-kB and pro-inflammatory cytokines. The TRL-2 level was demonstrated to have good sensitivity and specificity towards DFI patients (45), supporting this notion.

CONCLUSION

The observed differences in the levels of these immunological markers between DFI, DM, and control groups highlight their potential utility as diagnostic or prognostic biomarkers for T2DM and its complications. Further investigation into the mechanistic links between these markers and the pathogenesis of T2DM could provide valuable insights into the disease's underlying pathways and inform the development of targeted therapeutic interventions.

REFERENCES

1. Boyko, E. J., Magliano, D. J., Karuranga, S., Piemonte, L., Riley, P., Saeedi, P., ... & Sun, H. (2021). IDF Diabetes Atlas, 10th edition. Brussels, Belgium: International Diabetes Federation.
2. Saeedi, P., Petersohn, I., Salpea, P., Malanda, B., Karuranga, S., Unwin, N., Colagiuri, S., Guariguata, L., Motala, A. A., Ogurtsova, K., Shaw, J. E., Bright, D., & Williams, R. (2019). Global and regional diabetes prevalence estimates for 2019 and projections for 2030 and 2045: Results from the International Diabetes Federation Diabetes Atlas, 9th edition.

Diabetes Research and Clinical Practice, *157*. https://doi.org/10.1016/j.diabres.2019.107843

3. Boulton, A. J. M., Armstrong, D. G., School, K., Kirsner, R. S., Blank, H., Attinger, C. E., Lavery, L. A., Mills, J. L., & Steinberg, J. S. (2018). *2018 CONTRIBUTING AUTHORS Diabetic Foot Complications*.
4. Mponponsuo, K., Sibbald, R. G., & Somayaji, R. (2021). A Comprehensive Review of the Pathogenesis, Diagnosis, and Management of Diabetic Foot Infections. In *Advances in skin & wound care* (Vol. 34, Issue 11, pp. 574–581). NLM (Medline). https://doi.org/10.1097/01.ASW.0000791876.10485.d4
5. Sepehri, Z., Kiani, Z., Nasiri, A. A., & Kohan, F. (2016). Toll-like receptor 2 and type 2 diabetes. In *Cellular and Molecular Biology Letters* (Vol. 21, Issue 1). BioMed Central Ltd. https://doi.org/10.1186/s11658-016-0002-4
6. Xia, C., Rao, X., & Zhong, J. (2017). Role of T Lymphocytes in Type 2 Diabetes and Diabetes-Associated Inflammation. In Journal of Diabetes Research (Vol. 2017). Hindawi Publishing Corporation. https://doi.org/10.1155/2017/6494795
7. Stanimirovic, J., Radovanovic, J., Banjac, K., Obradovic, M., Essack, M., Zafirovic, S., Gluvic, Z., Gojobori, T., & Isenovic, E. R. (2022). Role of C-Reactive Protein in Diabetic Inflammation. In Mediators of Inflammation (Vol. 2022). Hindawi Limited. https://doi.org/10.1155/2022/3706508
8. Dasu MR, Martin SJ. (2014). Toll-like receptor expression and signaling in human diabetic wounds. World J Diabetes. Apr 15;5(2):219-23. https://doi.org/10.4239/wjd.v5.i2.219 PMID: 24748934; PMCID: PMC3990321.
9. Gupta, S., Maratha, A., Siednienko, J., Natarajan, A., Gajanayake, T., Hoashi, S., & Miggin, S. (2017). Analysis of inflammatory cytokine and TLR expression levels in Type 2 Diabetes with complications. Scientific Reports, 7(1). https://doi.org/10.1038/s41598-017-07230-8
10. Wifi, M. N. A., Assem, M., Elsherif, R. H., El-Azab, H. A. F., & Saif, A. (2017). Toll-like receptors-2 and -9 (TLR2 and TLR9) gene polymorphism in patients with type 2 diabetes and diabetic foot. *Medicine (United States)*, *96*(17). https://doi.org/10.1097/MD.0000000000006760
11. Mohammad, M. K., Morran, M., Slotterbeck, B., Leaman, D. W., Sun, Y., von Grafenstein, H., Hong, S. C., & McInerney, M. F. (2006). Dysregulated Toll-like receptor expression and signaling in bone marrow-derived macrophages at the onset of diabetes in the non-obese diabetic mouse. *International Immunology*, *18*(7), 1101–1113. https://doi.org/10.1093/intimm/dxl045
12. Creely, S. J., Mcternan, P. G., Kusminski, C. M., Fisher, F. M., Da Silva, N. F., Khanolkar, M., Evans, M., Harte, A. L., & Kumar, S. (2007). Lipopolysaccharide activates an innate immune system response in human adipose tissue in obesity and type 2 diabetes. *Am J Physiol Endocrinol Metab*, *292*(7), 740–747. https://doi.org/10.1152/ajpendo.00302.2006.-Type
13. Flo TH, Halaas O, Torp S, Ryan L, Lien E, Dybdahl B, Sundan A, Espevik T. (2001). Differential expression of Toll-like receptor 2 in human cells. J Leukoc Biol. Mar;69(3):474-81. PMID: 11261796.
14. Ajuwon, K. M., Banz, W., & Winters, T. A. (2009). Stimulation with Peptidoglycan induces interleukin 6 and TLR2 expression and a concomitant downregulation of expression of adiponectin receptors 1 and 2 in 3T3-L1 adipocytes. *Journal of Inflammation*, *6*. https://doi.org/10.1186/1476-9255-6-8
15. Kuo, I. H., Carpenter-Mendini, A., Yoshida, T., McGirt, L. Y., Ivanov, A. I., Barnes, K. C., Gallo, R. L., Borkowski, A. W., Yamasaki, K., Leung, D. Y., Georas, S. N., De Benedetto, A., & Beck, L. A. (2013). Activation of epidermal toll-like receptor 2 enhances tight junction

function: Implications for atopic dermatitis and skin barrier repair. *Journal of Investigative Dermatology*, *133*(4), 988–998. https://doi.org/10.1038/jid.2012.437

16. Simpson, J. L., McDonald, V. M., Baines, K. J., Oreo, K. M., Wang, F., Hansbro, P. M., & Gibson, P. G. (2013). Influence of age, past smoking, and disease severity on tlr2, neutrophilic inflammation, and MMP-9 Levels in COPD. *Mediators of Inflammation*, *2013*. https://doi.org/10.1155/2013/462934
17. Panda, A., Qian, F., Mohanty, S., van Duin, D., Newman, F. K., Zhang, L., Chen, S., Towle, V., Belshe, R. B., Fikrig, E., Allore, H. G., Montgomery, R. R., & Shaw, A. C. (2010). Age-Associated Decrease in TLR Function in Primary Human Dendritic Cells Predicts Influenza Vaccine Response. *The Journal of Immunology*, *184*(5), 2518–2527. https://doi.org/10.4049/jimmunol.0901022
18. Nyugen J, Agrawal S, Gollapudi S, Gupta S (2010) Impaired functions of periph-eral blood monocyte subpopulations in aged humans.J. Clin. Immunol.30,806–813
19. Renshaw M, Rockwell J, Engleman C, Gewirtz A, Katz J, Sambhara S. Cutting edge: impaired Toll-like receptor expression and function in aging. (2002) . J Immunol. Nov 1;169(9):4697-701. https://doi.org/10.4049/jimmunol.169.9.4697 PMID: 12391175.
20. Dunston, C. R., & Griffiths, H. R. (2010). The effect of ageing on macrophage Toll-like receptor-mediated responses in the fight against pathogens. In *Clinical and Experimental Immunology* (Vol. 161, Issue 3, pp. 407–416). Blackwell Publishing Ltd. https://doi.org/10.1111/j.1365-2249.2010.04213.x
21. Van Duin, D., Allore, H. G., Mohanty, S., Ginter, S., Newman, F. K., Belshe, R. B., Medzhitov, R., & Shaw, A. C. (2007). Prevaccine determination of the expression of costimulatoiy B7 molecules in activated monocytes predicts influenza vaccine responses in young and older adults. *Journal of Infectious Diseases*, *195*(11), 1590–1597. https://doi.org/10.1086/516788
22. Kadhim, F., H.; (2021). Bacteriological and Immunological study of Diabetic Patients with and without Foot Ulcer in Kerbala Province as a Partial Fulfillment of the Requirements for the Degree of master's in clinical laboratories
23. AL-Sahi, M. M., Jabbar AL-Hasnawi, S. M., & Mohammed Ali, M. (2023). EVALUATION OF IMMUNOLOGICAL LEVELS OF IL-37, IL-38 AND IL-17A IN IRAQI PATIENTS WITH DIABETIC FOOT ULCERS. https://doi.org/10.17605/OSF.IO/4WN8J
24. Parhi, A., Das, S., Mahapatra, S., Pradhan, N., Behera, M., Patnaik, B., & Rattan, R. (2019). The Level and Role of Interleukin-17 in Patients of Type 2 Diabetes Mellitus with and without Complications. *Journal of Diabetes Mellitus*, *09*(04), 176–185. https://doi.org/10.4236/jdm.2019.94017
25. Zareian, P., & Mirzaii Dizgah, I. (2014). Serum Interleukin 17 in Type 2 Diabetes Mellitus. *Journal of Archives in Military Medicine*, *2*(4). https://doi.org/10.5812/jamm.24689
26. Kaleli, S., Varim, C., Nalbant, A., Yazar, H., & Akdoğan, M. (2019). Interleukins As a Marker of Inflammation in Diabetic Foot Syndrome and Type 2 Diabetes Mellitus. *Bezmialem Science*, *7*(1), 1–7. https://doi.org/10.14235/bas.galenos.2017.2077
27. Yousefidaredor, H., Zare-Bidaki, M., Hakimi, H., Assar, S., Bagheri, V., & Arababadi, M. K. (2014). IL-17A plays an important role in induction of type 2 diabetes and its complications. *Asian Pacific Journal of Tropical Disease*, *4*(5), 412–415. https://doi.org/10.1016/S2222-1808(14)60598-3
28. Koester J, Miroshnikova YA, Ghatak S, Chacón-Martínez CA, Morgner J, Li X, Atanassov I, Altmüller J, Birk DE, Koch M, Bloch W, Bartusel M, Niessen CM, Rada-Iglesias A, Wickström SA.(2021). Niche stiffening compromises hair follicle stem cell potential during ageing by reducing bivalent promoter accessibility. Nat Cell Biol. 2021 Jul;23(7):771-781. https://doi.org/10.1038/s41556-021-00705-x Epub 2021 Jul 8. PMID: 34239060.

29. Sfanos, K.S. and De Marzo, A.M., (2012). Prostate cancer and inflammation: the evidence. *Histopathology*, *60*(1), pp.199-215. https://doi.org/10.1111%2Fj.1365-2559.2011.04033.x

30. De Angulo, A., Faris, R., Cavazos, D., Jolly, C., Daniel, B. and DeGraffenried, L., (2013). Age-related alterations in T-lymphocytes modulate key pathways in prostate tumorigenesis. *The Prostate*, *73*(8), pp.855-864. https://doi.org/10.1002/pros.22631

31. Schmitt, V., Rink, L. and Uciechowski, P., (2013). The Th17/Treg balance is disturbed during aging. *Experimental gerontology*, *48*(12), pp.1379-1386. https://doi.org/10.1016/j.exger.2013.09.003

32. Kumar P, Subramaniyam G. (2015). Molecular underpinnings of Th17 immune-regulation and their implications in autoimmune diabetes. Cytokine.;71(2):366–376. https://doi.org/10.1016/j.cyto.2014.10.010

33. Qiu, A. W., Cao, X., Zhang, W. W., & Liu, Q. H. (2021). IL-17A is involved in diabetic inflammatory pathogenesis by its receptor IL-17RA. Experimental Biology and Medicine, 246(1), 57–65. https://doi.org/10.1177/1535370220956943

34. Muhanedalnajer, H., Abdul, S., Al-Shammaree, W., & Salman, I. N. (2020). Determination Of Advanced Oxidative Protein Products Levels And Its Correlation With Inflammation In Diabetic Foot Patients. *Biochem. Cell. Arch*, *20*(2), 0–000. www.connectjournals.com/bca

35. Xu, M., Li, Y., Tang, Y., Zhao, X., Xie, D., & Chen, M. (2022). Increased Expression of miR-155 in Peripheral Blood and Wound Margin Tissue of Type 2 Diabetes Mellitus Patients Associated with Diabetic Foot Ulcer. *Diabetes, Metabolic Syndrome and Obesity*, *15*, 3415–3428. https://doi.org/10.2147/DMSO.S376292

36. Festa, A., D'agostino Jr, R., Howard, G., Mykkanen, L., Tracy, R. P. & Haffner, S. M. (2000). Chronic subclinical inflammation as part of the insulin resistance syndrome: the Insulin Resistance Atherosclerosis Study (IRAS). Circulation, 102, 42-47.

37. Mohan, V., Deepa, R., Velmurugan, K. & Premalatha, G. (2005). Association of C-reactive protein with body fat, diabetes and coronary artery disease in Asian Indians: The Chennai Urban Rural Epidemiology Study (CURES-6). Diabetic medicine, 22, 863-870

38. Shaalan, K. (2016). Relation of C-Reactive Protein with Age and BMI in Diabetic Foot Patients. Diyala Journal for pure sciences. Vol: 12 No:3, July 2016 ISSN: 2222-8373

39. Singh T, Newman AB. (2011). Inflammatory markers in population studies of aging. Ageing Res Rev. Jul;10(3):319-29. https://doi.org/10.1016/j.arr.2010.11.002 Epub 2010 Dec 8. PMID: 21145432; PMCID: PMC3098911.

40. Trollor, J.N., Smith, E., Agars, E., Kuan, S.A., Baune, B.T., Campbell, L., Samaras, K., Crawford, J., Lux, O., Kochan, N.A. and Brodaty, H., (2012). The association between systemic inflammation and cognitive performance in the elderly: the Sydney Memory and Ageing Study. *Age*, *34*, pp.1295-1308.

41. Varadhan R, Yao W, Matteini A, Beamer BA, Xue QL, Yang H, Manwani B, Reiner A, Jenny N, Parekh N, Fallin MD, Newman A, Bandeen-Roche K, Tracy R, Ferrucci L, Walston J. (2014). Simple biologically informed inflammatory index of two serum cytokines predicts 10 year all-cause mortality in older adults. J Gerontol A Biol Sci Med Sci. 2014 Feb;69(2):165-73. https://doi.org/10.1093/gerona/glt023. Epub 2013 May 20. PMID: 23689826; PMCID: PMC4038244.

42. Bähler, C., Huber, C.A., Brüngger, B. and Reich, O., (2015). Multimorbidity, health care utilization and costs in an elderly community-dwelling population: a claims data based observational study. *BMC health services research*, *15*, pp.1-.

43. Shler GR., Al- Barzinji RM., Al-Dabbagh AA. (2012).: Serum C-Reactive Protein Level in Diabetic Foot Patients and Their Relation with Bacterial Isolates. Diyala Journal of Medicine; 3(1) :106

44. Mutluoglu M, Uzun G, Ipcioglu OM, Sildiroglu O, Ozcan O, Turhan V, et al., (2011). Can procalcitonin predict bone infection in diabetic persons with infected foot
45. Arbibe L, Mira JP, Teusch N, Kline L, Guha M, Mackman N, Godowski PJ, Ulevitch RJ, Knaus UG. (2000). Toll-like receptor 2-mediated NF-kappa B activation requires a Rac1-dependent pathway. Nat Immunol. 2000 Dec;1(6):533-40. https://doi.org/10.1038/82797 PMID: 11101877.