Assessment of C-Reactive Protein and Macrophage Migration Inhibitory Factor in Diabetic Foot Infection

Mohamed R. Ahmed[1], Gehan A. Ibrahim[1], Hoda G. Bakr[1]
Takwa E. Meawed[2]

[1]*Internal Medicine Department, Faculty of Medicine, Zagazig University, Egypt.*
[2]*Microbiology & Immunology Department, Faculty of Medicine, Zagazig University, Egypt.*

Background and study aim: Diabetic foot ulcer is a universal health problem. Neuroischemic changes and infection are responsible for its occurrence and complications. Altered or complete loss of sensation and microvascular disease complicated by unchecked infection can precipitate tissue necrosis and gangrene. A threat for a rapid test predicting early infected foot ulcer emerges. C-reactive protein (CRP) and macrophage migration inhibitory factor (MIF) are involved in innate inflammatory response. We aimed at evaluation of the ability of C-reactive protein and macrophage migration inhibitory factor to differentiate between early infected and non infected diabetic foot ulcers and to detect risk factors of diabetic foot ulceration.

Patients and methods: 52 diabetic patients were selected, examined and classified into 3 groups : **Group (I):** Included 12 patients with non-infected diabetic foot ulcer (grade I), **group (II):** Included 30 patients with mildly infected diabetic foot ulcer (grade Π) and **group (III)**: Included 11 diabetic patients free from foot wounds used as a control group. In addition to routine laboratory investigations, serum CRP was measured using Enhanced Immuno-tubidimetric Assay. MIF level was detected by ELISA. Swabs from the diabetic foot ulcers were taken for aerobic and anaerobic cultures.

Results: Statistically significantly elevated Hb A1C%, MIF and CRP levels were detected in mild infected diabetic foot ulcer compared to studied groups (P <0.05). Dermatological changes were statistically significant risk factors for diabetic foot ulcers, accounted for 88.1% of ulcer cases. The most frequently isolated organism was *E coli*. The most common site for ulcers was the toes representing 50% of the cases.

Conclusion: CRP and MIF can differentiate early infected from non-infected diabetic foot ulcers.

INTRODUCTION

Foot ulcers are common diabetic complications. About 15% of diabetics develop foot ulcers within their life time and up to 70% of all non-traumatic amputations in the world occur in diabetics. Many of these amputations are preventable as about 85% are preceded by a foot ulcer [1]. Although most foot infections stay superficial, they can spread to muscle, joints and bone. Unchecked infection can precipitate tissue necrosis and gangrene, especially in an ischemic limb [2].

Diagnosis of infection must be based not on microbiological findings only but also on clinical criteria to avoid unnecessary antimicrobial treatment and emergence of multidrug-resistant organisms [3]. Aerobic gram-positive cocci are the predominant pathogens, *Staph aureus* and the *β-hemolytic Streptococci a*re the most commonly isolated pathogens [4].

Biochemical parameters such as sedimentation rate and leucocytosis are reputed to be of poor value for diagnosing diabetic foot infections [5]. C-reactive protein (CRP) is a highly conserved acute phase protein of innate inflammatory response synthesized by hepatocytes under cytokines stimulation originating at the site of pathology and leading to dramatic rise in CRP level within 48 h after stimulation [6,7]. An altered immune response in diabetic foot

ulcer patients is interesting as some markers of inflammation are upregulated (CRP, fibrinogen, IL-6, MIF, macrophage inhibitory protein (MIP-1α) and IP-10) while others are not (IL-8, IL-18, and macrophage chemo-attractant protein (MCP-1) [8].

Macrophage migration inhibitory factor (MIF) is a proinflammatory cytokine of the innate immune system that plays a major role in the induction of immune-inflammatory reaction. MIF may play a causal role in the etiology of type 2 diabetes and elevated MIF levels confer a higher disease risk [9].

AIM OF THE WORK
Evaluation of the ability of CRP and MIF to differentiate between early infected and non infected diabetic foot ulcers and to detect risk factors of diabetic foot ulceration.

PATIENTS AND METHODS
A prospective case control study was conducted at diabetic foot clinic of Internal Medicine and Microbiology and Immunology Departments, Faculty of Medicine-Zagazig University Hospitals during the period from Feb 2009 to Jan 2010.

Fifty-two diabetic patients were included, 42 of them were suffering from diabetic foot ulcer and 10 were control group. They were treated by oral antidiabetic drugs, insulin or combined treatment. Inclusion criteria: Cases to be included should have history of diabetes mellitus, fasting plasma glucose ≥126 mg/dl (7.0 mmol/l) or 2-h post prandial plasma glucose ≥200 mg/dl (11.1 mmol/l). They were classified into 3 groups according to the grade of diabetic foot ulcer (IDSA-IWGDF) (3) into :
Group (I): Included 12 patients with non infected diabetic foot ulcer as there were no symptoms or signs of infection (Grade I), 7 patients were males, 5 were females with age range 35- 64 years and mean of age 50.16 ± 8.5 years. Their mean duration of diabetes was 7.16 ± 3.51 years, 75% of them were using insulin and 25% were using oral anti-diabetics.

Group (II): Included 30 patients with mildly infected diabetic foot ulcer (Grade Π), 15 patients were males, others were females with age range 26-76 years and mean 52.3 ± 11.4 years. Their mean duration of diabetes was 14.6±7.18 years, 70% of them were using insulin, 16.7% were using oral anti-diabetics and 13.3% on combined therapy. Grade Π diabetic foot

ulcer was diagnosed if there was infection involving skin and subcutaneous tissue only (without involvement of deeper tissues and without systemic signs). At least two of the following signs were fulfilled:
• Local swelling or induration
• Erythema > 0.5 - 2 cm around the ulcer
• Local tenderness or pain
• Local warmth
• Purulent discharge (thick, opaque to white secretion).

Group (III): Included 10 diabetic patients free from foot wounds used as a control group. 5 patients were males and 5 were females, their age range (35-75) years and mean age was 52.7±11.1 years, 80% of them using insulin, 10% using oral anti-diabetic agents and 10% were on combined therapy.

Exclusion criteria : Any patient who had any of the following was excluded: foot wound that was more than grade 2, other causes of inflammatory response of the skin (e.g. trauma, gout, acute Charcot neuro-osteoarthropathy, fracture, thrombosis and venous stasis), treatment with antibiotics and lastly peripheral arterial disease.

Any systemic illness that might elevate the inflammatory markers as: allergic complication of infection (rheumatic fever, and erythema nodosum).

Other inflammatory diseases as (rheumatoid arthritis, chronic arthritis, systemic vasculitis, Familial Mediterranean Fever and Chron's disease), necrosis, trauma and malignancy.

Informed consent was taken. Patients included in this study were subjected to full history taking, complete clinical examination and foot examination were performed to detect any of the following:
• Signs of neuropathy that included 10 gm monofilament detection and deep tendon reflex.
• Signs of vasculopathy included pulse examination for dorsalis pedis and posterior tibial arteries, presence of edema and ankle brachial index.

• Risk factors that promote ulcer development as presence of toes deformity, bunions, Charcot foot, drop foot, equinus, prominent metatarsal heads and amputation) or dermatological factors as (corns, callus, dry skin, hair loss, nail changes and changed color of skin).

Methods:

Consent was taken from all of patients included in the study. After fasting for at least 12 h, 10 ml of blood was aseptically collected. Complete blood count was done on automated cell counter (cell Dyn 1700). Liver and kidney functions were done on automated analyzer (cobas 6000). Hemoglobin A1C was done on automated analyzer (cobas 6000). Swabs from the diabetic foot ulcers were taken for aerobic and anaerobic cultures. Tissue specimens were obtained from the debrided base and sides (Levine technique) [10].

Serum CRP was done on automated analyzer (Integra 400), CRP latex is an in vitro test for quantitative determination of CRP in human serum and plasma on Roche/Hitachi Cobas systems using particles Enhanced Immunotubidimetric Assay [11].

MIF level detection was done by using RayBio® Human MIF ELISA.

Statistical analysis:

Data entry and analysis were performed using SPSS (statistical package for social science version 10) (SPSS, Inc., Chicago, IL, USA). Data were presented as number and percentage, mean and standard deviation .The chi-square (χ2), t-test and ANOVA were used. Mann Whitney-U test and Kruskal- Wallis H test are non parametric tests equivalent of the t-test and ANOVA. P value of <0.05 was considered significant.

RESULTS

Non-significant difference was found between cases and control group regarding age, sex, type of diabetes or treatment modalities of diabetes. Ulcer size and depth was not statistically different in mildly infected than non infected ulcers. But in mildly infected ulcer patients, there was significantly longer duration of diabetes 14.6 ± 7.18 years compared to 7.16 ± 3.51, 10.7 ± 6.7, in non infected ulcer and control groups respectively $(P<0.05)$ also hypertension was significantly found in group II (33.3%), compared to 8.3 % and 0.0 %, in (group I) and control group respectively) $(P<0.05)$ Table (1).

Decreased Hb % and increased WBC were found in group II than group I but it was statistically insignificant $(p>0.05)$. A significantly elevated HB A1C% and highly statistically significant elevated CRP level were detected in (group II) compared to the studied groups $(p<0.05)$ and $(p<0.001)$ respectively. Also highly significantly elevated MIF levels were detected in (group II) compared to the studied groups $(p<0.001)$ Table (2). There was no statistical significant difference between group (III) and group (I) as regarding HB%, WBC count, A1C%, ALT, AST, serum creatinine and CRP level Table (2).

A statistical significant difference was found between ulcer cases and non ulcer (control group) as regards dermatological changes (corns, callus, dry skin, hair loss, nail changes and color of skin), 88.1% of diabetic ulcer cases had dermatological changes compared to 50 % of non ulcer cases $(P<0.05)$. While, there was no statistical significant difference between cases and control group regarding neuropathy, 76.2% of the diabetic ulcer cases compared to 70 % of non ulcer ones $(P<0.05)$. Also, no significant difference was found regarding deformity, 28.6% cases compared to 10% of non ulcer cases $(P<0.05)$, Table (3).

The most frequently isolated organism from infected diabetic foot ulcer was *E-coli* 12 (43.3%) then *Staph aureus* 9 (30.0%) followed by *Proteus mirabilis* 3 (10.0%) then *Klebsiella* 2 (6.7%), *Candida* 1 (3.3%) and lastly sterile culture was found in 2 (6.7%) Table (4).

The most common site for diabetic foot ulcer was the toes representing 50% of cases then metatarsal heads, mid foot and heel (45.2%), then dorsum of foot (4.8%) (Figure 1).

Table (1): Comparison between the studied groups as regards demographic data and clinical criteria

	Group (I)Non infected ulcer (N = 12) No (%)	Group (Π) Mild infected ulce (N = 30) No (%)	Group (ΠI) (N = 10) NO (%)	Test statistics	P
Age(years) Mean ± SD	50.1± 8.5	52.3 ± 11.4	52.7± 11.1	F= 0.21	0.81 (NS)
Sex : no (%) Male Female	7 (58.3%) 5(41.7%)	15(50%) 15(50%)	5 (50%) 5 (50%)	χ2= 0.25	0.88 (NS)
Ulcer (mean±SD) Size(cm): Depth(cm):	1.7±0.7 0.8±0.4	1.7±1.1 0.7±0.5	------	Z=0.22* Z=0.44*	0.83 (NS) 0.69 (NS)
Diabetes (Type I) Diabetes (Type ΙΙ)	1(8.3%) 11(91.7%)	7(23.3%) 23(76.7%)	2(20%) 8(80%)	χ2 1.246	0.536 (NS)
Duration of DM mean ± SD (years)	7.16± 3.51	14.6± 7.18	10.7± 6.7	F 5.99	0.048 (S)
Treatment of DM Insulin Oral combined	9 (75%) 3(25%) 0(0.0%)	21(70%) 5(16.7%) 4(13.3%)	8(80%) 1(10%) 1(10%)	χ2 2.42	0.658 (NS)
Ischemic heart diseases	0(0.0%)	4(13.3%)	0(0.0%)	χ2 3.17	0.2 (NS)
Hypertension	1(8.3%)	10(33.3%)	0(0.0%)	χ2 6.53	0.038 (S)

(*) = Kruskal – Wallis test

Table (2): Serum levels of CRP, MIF and other laboratory criteria of the studied groups.

Studied groups / Lab Data	Group (I) Non infected ulcer (N = 12)	Group (Π) Mild infected ulcer (N = 30)	Group (III) (N = 10)	P
Hb %	12.2±1.7	11.6±1.8	11.8±1.8	0.534 (NS)
WBCx10^3	7.2±1.9	9.1±3.1	7.9±2.4	0.133 (NS)
HbA1C%	7.8 ± 1.1	8.9±1.8 ab	7.7±2.1	0.05 (S)
CRP mg/dl	13.4±27.1	17.4±7.5 ab	4.6±3.3	0.0001 (HS)
MIF ng/dl	7.7±1.3	11.9±2.2 ab	4.5±0.9	0.0001 (HS)
ALT U/L	24.6±11.3	27.1±18.9	25.5±12.33	0.997 (NS)
AST U/L	26.2 ±15.2	26.4±19.1	24.9±10.6	0.778 (NS)
Creatinine mg/dl	0.8 ±0.2	1.2±1.6	0.9±0.2	0.928 (NS)

a= Significant difference between group (II) and group (I).
b=Significant difference between group (II) and group (III).
    No-significant statistical difference between group (III) and the group (I).

**Table (3):** Culture results of the diabetic foot ulcers.

Organism	Group (I) Non infected ulcer (N = 12)	(Group Π) Mild infected ulcer (N = 30)	χ2	P
E-coli	1(8.3%)	12(43.3%)		
Staph aureus	0 (0.0%)	9(30.0%)		
Proteus mirabilis	1(8.3%)	3(10.0%)	25.608	0.0001 (HS)
Klebsiella	0(0.0%)	2(6.7%)		
Candida	0(0.0%)	1(3.3%)		
Sterile Culture	10(83.3%)	2(6.7%)		

Table (4): Risk factors of ulceration in diabetic foot patients.

Risk factor	Ulcer cases (N = 42) (%)No	Control group (N = 10) No (%)	P
Neuropathy	32 (76.2%)	7 (70.0%)	0.697 (NS)
Deformity	12 (28.6%)	1 (10.0%)	0.419 (NS)
Dermatological changes	37 (88.1%)	5 (50.0%)	0.022 (S)

Table (5): MIF ELISA sensitivity, specificity and positive predictive value

	Sensitivity	Specificity	Positive Predictive Value
MIF ELISA Test	93.3%	83.3%	93.3%
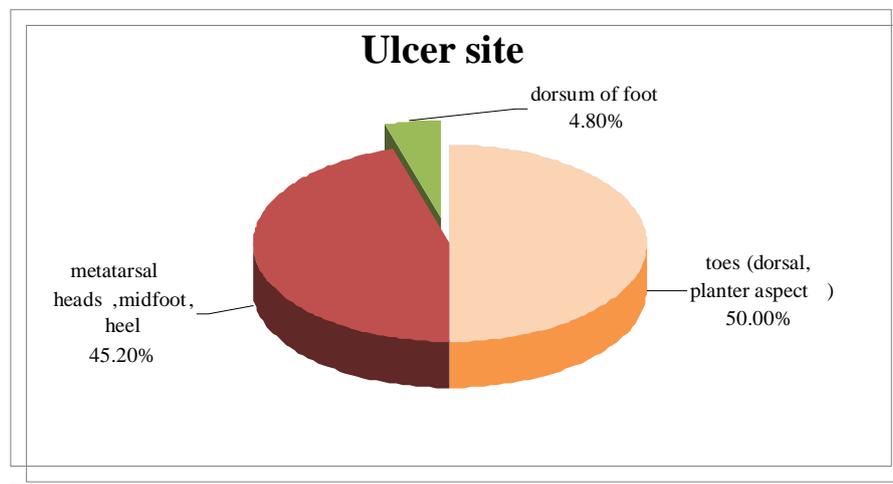
Figure (1): The ulcer site prevalence in the ulcer groups

DISCUSSION

There are 285 million people suffering from DM, corresponding to 6.4% of the world's adult population, which is estimated to rise to 438 million by 2030. Estimated Egyptian diabetics among adults aged 20-79 years is 11.4% of diabetic world population for the year 2010 and to the national population is 10.4 % [13].

C-reactive protein (CRP) takes part in the systemic inflammatory response, acute injury, infection or other stimuli, CRP binds to specific molecular configurations typically present in case of cell death and additionally found on the surface of pathogens, and therefore CRP increases rapidly after tissue injury or infections and reflects the intensity of the inflammatory process [6,14].

Serum concentration of CRP represents a very

useful non specific inflammatory biomarker and plays an important role in screening for organic diseases, monitoring the response to treatment and helps in detection of recurrent infection [10].

MIF is a proinflammatory cytokine of innate immune system, High levels of MIF were detected in chronic non healing diabetic ulcers then began to fall with successful healing [9].

In this study, there was no significant difference between studied groups regarding age and gender, the same as our results was reported by Kumar et al. [15], however, Frykberg [16] found that male sex was a risk factor for ulceration.

Data from the National Hospital Discharge Survey (NHDS) 1987–1990 in the US reported the highest percentage of hospital discharges for foot ulcers in patients aged 45–64 years and lowest in patients < 45 years. Elderly patients are

usually less mobile, have poor vision, live alone and have other medical problems [17].

Longer duration of diabetes and elevated percentage of hypertension (33.3%), were detected in mild infected ulcer patients than others. Reiber [17] showed a six-fold increased risk of foot ulcer in patients ≥20 years DM duration than patients ≤9 years. In another study, diabetic patients with foot ulcer had a longer duration of DM, 17 years versus 12 years [16].

In this study, HbA1c % was significantly increased in mild infected than non infected diabetic foot ulcers. The level of glycaemic control has been shown to play a role, with a 26% increased risk of peripheral vascular disease for every 1% increase in HbA1c [18]. Moss et al [19] found that increasing HbA1c % was associated with subsequent foot ulcer in their cohort study. Boyko et al. [20] found that, severe hyperglycaemia was associated with a higher risk for diabetic foot ulcer. There was a great evidence that leukocyte functions such as migration, phagocytosis, intracellular killing and chemotaxis were impaired in the presence of uncontrolled diabetes [21].

In the present study, there was a high significantly elevated CRP level in mild infected diabetic foot ulcer compared to non infected ulcer and control groups. Lee et al. [22] sharing us the same result and reported that CRP was more useful method in predicting and diagnosing infection than WBC, ESR in diabetic foot ulcer patients. Upchurch et al. [23] supported our results, they reported increased CRP and fibrinogen levels in diabetic patients with a foot ulcer compared with diabetic patients without foot ulcer.

Lin et al. [24] added that, reduced CRP levels (<50 mg/L), indicates a low infection severity and may serve as a major predictor of successful percutaneous transluminal angioplasty outcome in diabetic patients with infected foot ulcers.

Jeandrot et al.[25] studied value of CRP, procalcitonin and other usual biological inflammatory markers in differentiating early infected from uninfected diabetic foot ulcers. They found that CRP as a single marker had the highest sensitivity and specificity and the use of a high-sensitivity CRP assay brought no additional accuracy of diagnosis.

CRP values have been shown to significantly increase in response to local infection, while procalcitonin increases more in systemic infection [26].

Regarding MIF level, a high significantly elevated MIF level was detected in our mild infected diabetic foot ulcers compared to non-infected ulcer and control groups. Both sensitivity of MIF ELISA test and precision (positive predictive value) were relatively high (93.3%) indicating that MIF ELISA test can be used as a screening test for mass population to differentiate between early-infected and non-infected ulcer. It doesn't need high technical skills, but further tests should confirm diagnosis. Its specificity was (83.3%) indicating that MIF ELISA test is not highly able to identify negative results (Table 4).

Only few researchers studied MIF level changes in diabetic foot ulcer patients. However, our results regarding elevated levels of CRP and MIF agreed with the results reported by Weigelt and his colleagues [8] that Patients with an acute foot ulcer had significantly higher levels of CRP, fibrinogen, interleukin (IL-6), MIF, macrophage inflammatory protein-1α, and interferon-γ–inducible protein-10

Other authors discussed MIF level in diabetic patients with complications including ulcers, reported that elevated levels of MIF or its cell surface receptor (CD74) were found in patients with diabetic complications including diabetic nephropathy [27], diabetic retinopathy [28], and diabetic foot syndrome.

In two different cohort studies done by Herder et al [9,27], there was a stronger association between MIF with impaired glucose tolerance (IGT) and type 2 diabetes much more than the associations of CRP and IL-6 with IGT and type 2 diabetes.

A consistent triangular relationship between MIF genotypes, serum levels and incident type 2 diabetes was found especially in women indicating that MIF may play a causal role in the etiology of type 2 diabetes and elevated MIF levels confer a higher disease risk [29].

Regarding risk factors for the development of diabetic foot ulcer, the statistically-significant factor (P<0.05) was dermatological changes (88.1%) versus (50%). Deformity and neuropathy were risk factors, but the difference was insignificant.

Chronic, repeated pressure and recurrent trauma from biomechanical changes can lead to

hyperkeratosis. Callus tissue is tough and increasing pressure leading to increasing the incidence of plantar ulcerations [30,31]. The same finding was reported by El-Nahas et al. [32] in their large-scale study that included 1200 Egyptian diabetic patients.

One of the commonest combinations causing ulceration is peripheral neuropathy, foot deformity and inappropriate footwear. Patients with deformed feet bones are at risk of skin damage and infection [33,34,35].

Kumar et al. [15] found that over 40% of type 2 DM patients had significant neuropathy. Bowering [36] reported that 60% of diabetic foot ulcers are the result of underlying neuropathy. Loss of sensation is one of the strongest risk factors for ulceration [37]. This was supported by Abbot et al. [38] in a cohort study included 6613 diabetic patients found that, neuropathy predictors as abnormal ankle reflex and 10 gm monofilament insensitivity predict new foot ulcers.

In this study *E .coli* was the most frequently isolated organism from ulcer cases (43.3%) followed by *S. aureus* (30.0%), then *Proteus* (10.0%), however, the picture was different in the study done by Gadepalli et al. [39] as *S. aureus* represented (13.7%), *Proteus* was isolated from (12.6%), followed by *E.coli* (12.0%) of from diabetic foot ulcer isolates. Richard et al. [40] reported *S. aureus* was the most frequent pathogen (36.5% of all isolates).

In this study, the most common site for the ulceration was the toes, representing 50% of the cases. The study done by Reiber et al. [41] supported our results, they reported that, lesion sites were in toes in 52%, while, metatarsal heads, mid foot & heel in 37%, dorsum of foot in 11%. Gefen [42] found nearby results as up about 60% of all diabetic ulcers, typically involve sites exposed to high pressure such as near the metatarsal heads and toes.

We can conclude that, CRP and MIF can differentiate early infected from non infected diabetic foot ulcers to avoid unnecessary antimicrobial treatment leading to emergence of multidrug-resistant pathogens. The statistically significant risk factor for development of diabetic foot ulcers was dermatological changes. However, Deformity and neuropathy were other non-significant risk factors. Toes were the most common site exposed to diabetic foot ulceration.

Recommendations :
The role played by other immune-mediators should be investigated in diabetic patients with ulcers, to determine the outcome of this problem and try to minimize the adverse effects by modification of these mediators.

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