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Monocytes and Fibrinogen as Biomarkers in Type 2 Diabetes Mellitus

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ABSTRACT

Background: In the diagnosis of diabetic foot, in addition to the inflammatory biomarkers that have been widely interested and used, for example procalcitonin, C-reactive protein (CRP), ESR, leukocyte count, neutrophil count, Fibrinogen and Monocytes are considered as biomarkers in this study. possible.

Methods: A prospective study was designed to examine the utility of fibrinogen and monocytes in estimating disease severity in patients with DFU (Diabetic Foot Ulcer). The severity of DFU was assessed using the Wagner criteria distinguishing between patients with diabetic foot without ulcer (WDFU) and with non-infected diabetic foot ulcer (NIDFU) or with infected ulcer (IDFU). In this study the AA also wanted to correlate HbA1c to the concentration of Fibrinogen and the appearance of DFU, as well as the level of lymphocytes and monocyte precursors of macrophages in the evolution of ulcerated and non-ulcerated diabetic feet.

Results: Mean blood fibrinogen values were significantly higher in patients with DFU grade ≧2 compared to those with DFU grade ≤1 (424.4±138.8 mg/dL versus 395.3±130.0 mg/dL; p=0.091). Fibrinogen values were correlated with CRP levels, neutrophils, ESR and leukocyte count. Monocytes presented a significant difference between non-diabetic patients with ulcer and without ulcer (0.41 *vs* 0.29 k/□L; p=0.000) and between the diabetic without ulcer (WDFU) and non-diabetic without ulcer (NDWU) groups (0.39 *vs* 0.29 k/□L; p=0.000). The Procalcitonin (PCT) value was <0.5 ng/dl, therefore it had no diagnostic significance. Only 1% of the values found were higher than 0.5 with an average of 1.04 ng/dl (range: 0.52-2.5).

Conclusions: Neither monocytes nor HbA1c can be considered biomarkers for the risk of ulcer formation in the diabetic foot, but only as biomarkers of type 2 Diabetes Mellitus. Differently, fibrinogenemia, its pre/post intervention ratio, the □□ angle and the k value of thromboelastography (TEG), have a clinical significance on the risk of onset and development of ulcerated diabetic foot.

The cut-off for ulcer formation for both the pre/post intervention ratio of fibrinogenemia and monocythemia is 1.10, with a sensitivity of 84.1% and a specificity of 24.5% for fibrinogen and 93.8% and 14.8% for monocytes.

ARTICLE DETAILS

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INTRODUCTION

Since Type 2 Diabetes Mellitus (T2DM) is associated to long term complications, of both microvascular and microvascular type, early detection of these complications is desirable. Given the last century demographical changes, brought by diabetes global epidemics, the term CLI (critical

limb ischemia) has become more and more prominent. Hence, biomarkers are crucial in early clinical diagnosis, in prevention and progression/prediction of the disease.

DFU (diabetic foot ulcer) healing process is usually divided in at least 4 overlapping phases: coagulation, inflammation, migration-proliferation (including matrix deposit) and remodeling of the scar tissue.

T2DM patients have an increased incidence of atherosclerosis, increased basal membrane thickness, arteriosclerosis and endothelial hyperplasia. These risk factors reduce blood flow to tissues, leading to ischemia of the surrounding tissues, which leads in turn to DFU. Chronic wounds are stuck in inflammatory phase and are not able to heal (Wang et al. 2021)[1]. Given their plasticity, macrophages and peripheral blood mononuclear cells (PBMNC) have a key role in tissue repair processes of trophic lesions, through the transition from inflammatory phase to proliferative phase, which is made possible through the polarization of M1 inflammatory macrophages in M2 regenerative phenotype. Diabetes has a distinct impact on macrophages function, as it compromises monocytes recruiting to wound sites, reducing phagocytosis and impairing the transition of inflammatory macrophages to anti-inflammatory ones (M1 to M2); hence, it slows wound healing and scarring (Rehak et al. 2022)[2].

Inflammatory events, hence, impair both wound healing and polymorph nucleated neutrophils proliferation, increasing tissue damage, until chronic wounds are formed. Acute phase reagents, like C-reactive protein (CRP), white blood cells (WBC), neutrophil and platelet count are commonly used as predictors for amputation in patients with DFU, but they only exhibit modest accuracy as disease severity indexes (Li e coll. 2016)[3]. Another simple and accurate marker of inflammation is the acute phase protein, fibrinogen, which is usually assumed to be elevated in patients with diabetic foot disease. Even if it has been demonstrated that DFU patients have higher fibrinogen levels compared to patients without ulcers, according to the authors it was necessary to conduct more accurate tests to establish if fibrinogen levels (Fib) can be useful to predict the evolution of "diabetic foot" disease, using also other biomarkers (VES; PCR; WBC; N.G; etc.) as confirmation.

Fibrinogen constitutes 10% of the *α-granular* protein, and is the predominant adhesive protein secreted by platelets. Recent data indicate that platelet fibrinogen is not synthesized by megakaryocytes, as previously thought, but is acquired exclusively from plasma by endocytosis. As fibrinogen is present in the *α*-granules of platelets at a higher concentration than other proteins acquired exclusively from plasma (e.g., albumin), endocytosis of fibrinogen seems to be receptor-mediated. Since the primary fibrinogen receptor on cells of the platelet-megakaryocyte lineage is the $\alpha_{IIb}\beta_3$ receptor, and since Glanzmann thrombosthenics lacking this receptor are also deficient in *α*-granular platelet fibrinogen, it was hypothesized that fibrinogen was endocytosed through this receptor.

Fibrinogen is also an important determinant of blood viscosity and platelet aggregation and may play a role in endothelial damage, low osmolar fibrin clot formation, thrombosis, blood flow abnormalities, and platelet hyperactivity. These studies suggest that fibrinogen is significantly associated with vascular injury and thrombosis. Fib is closely related to DF. The relationship between angle α, k value of kaolin thromboelastography, fibrinogen and diabetic foot is an index of the risk of onset and progression of diabetic foot (Li et al. 2016).

A rather large clinical study (Mills J.L. et al. 2014)[4] found that PAD (peripheral vascular disease) prevalence in patients with T2DM is 23.5%, and that diabetic patients with combined PAD are more likely to develop ulceration and limb gangrene, significantly increasing the risk of amputation and CLI. CLI, as per current definition, is associated with decreased quality of life, increased risk of amputation, and increased mortality. Therefore, research of indicators predicting the onset and progression risk of diabetic foot, as well as early intervention can help improve the quality of life for patients with DM.

Glycated hemoglobin (HbA1c) represents the average of blood glucose levels over time, while blood glucose represents a snapshot at the time of sampling. HbA1c is an index of average glucose levels in the 2-3 months before the test was performed. If daily glucose levels are kept stable (both with normal and elevated levels), HbA1c and blood glucose are correlated. It is important to keep in mind the delay associated with HbA1c. Good glycemic control achieved in the previous 2-3 weeks may not affect the HbA1c result, which instead reflects levels across a larger number of weeks.

Markers of infection in the diabetic foot are: White Blood Cells (WBC) ≥15.0 x 109/L (p=0.014)(42.1%); ESR≥100 mm/h (p=0.001)(41.5%); CRP≥15.0mg/dl (p=0.0017)(46.2%); these might be predictive factors of limb loss (Aziz et al.2011)[5].

Previous studies have shown that elevated CRP (C-reactive protein) levels are associated with diabetes (Wang et al. 2021). Patients with acute DFU had significantly increased CRP and fibrinogen compared to patients with WDFU (diabetic foot without ulcer). Increased mortality is usually heralded by a preoperative/postoperative CRP ratio ≤ 1.5 ($p<0.001$), combined with an age of 73 years or older ($p<0.001$), so elevated serum CRP levels are the main predictors of mortality. In Wang's study, analysis of blood samples showed that CRP, as a single biomarker, was the most effective indicator for distinguishing grade 1 and grade 2 ulcers. In contrast, WBC and neutrophil counts had no predictive effect.

Ultimately, according to Wang, only the two biomarkers CRP and PCT play an important role in distinguishing IDFU and NIDFU, ensuring the rational use of antibiotics.

Korkmaz et al. (2018)[6] conducted a study enrolling 38 IDFU patients, 38 NIDFU patients, and 43 WDFU patients; the results showed that the WBC, CRP, ESR, and fibrinogen levels of IDFU patients were significantly higher than those of NIDFU patients ($p < 0.01$). During the identification process of IDFU and NIDFU, serum CRP had the highest AUC_{ROC} value (0.998; $p<0.001$), while PCT showed to have no significant effect. Based on the evidence of increasing current data, serum IL-6 and fibrinogen are considered two potential inflammatory biomarkers for the differentiation of IDFU. Different studies, including ours, have also indicated that the predictive effect of PCT (procalcitonin) is less effective in patients with mild DFU infection, but more effective in patients with severe form of IDFU.

Glossary:

Diabetics with Ulcer (*DFU*); Diabetics without Ulcer (*WDFU*); Diabetics with infected ulcer (*IDFU*); Non-Diabetic with Ulcer (*NIDFU*); Non-Diabetic without Ulcer (*NDWU*);

MATERIALS AND METHODS

4 groups of patients were enrolled in this study: 40 patients without diabetic foot ulcer (WDFU), 26 patients with diabetic foot ulcer (NIDFU and IDFU), 26 non-diabetic patients without ulcer (NDWU) and 8 non-diabetic patients with ulcer (NDFU). It was not necessary to ask for authorization from the Local Ethics Committee responsible for the experimentation since no drugs or chemical substances were administered to the patients, but the investigation only used the analysis of routine hematological data at the time of clinical admission. However, all patients were asked to sign the Informed Consent for the research.

Exclusion criteria were patients suffering of type 1 diabetes, gestational diabetes and secondary diabetes, patients with acute complications of combined diabetes (such as diabetic ketoacidosis, non-ketotic hyperosmolar state), patients with various other acute and chronic infections, trauma and surgery, patients with combined cardiac, hepatic and renal failure, arterial and venous embolism and cerebrovascular events, patients with rheumatic immune diseases, hematological diseases and tumors, patients treated with drugs impacting coagulation function (such as exogenous fibrinogen, hormones, anti-platelet drugs, anticoagulants, etc.).

Patients scoring a Wagner grade <1 were defined as mild DF (without ulcer), while patients scoring a Wagner grade ≥1 were defined as severe DF (with ulcer) (Table I).

Table I. Classification according to Wagner

Grade 0	Peri-ulcerative lesion, post-ulcerative scar, presence of bone deformities
Grade 1	Superficial ulcer without involvement of the subcutaneous tissue
Grade 2	Penetration through the subcutaneous tissue; there may be exposure of bones, tendons, ligaments, or joint capsules
Grade 3	Osteitis, abscesses or osteomyelitis
Grade 4	Gangrene of a finger
Grade 5	Gangrene requiring amputation of the foot

The severity rating was determined by observation of the lesion and the scoring of a series of factors, including medical, anatomical and patient-related factors, through the PUSH system (Version 3.0: 15/09/98, © National Pressure Ulcer Advisory Panel) which allowed the calculation of a Score at patient's admission, following photographical imaging of the lesion and analysis according to the system developed by our group (Calcderm)(Crisci et al., 2014)[7], at discharge (72 h) and 45 days after.

Figure 1. The National Pressure Ulcer Advisory Panel developed the Pressure Ulcer Scale for Healing (PUSH) tool to track healing in pressure ulcers II through IV. The PUSH tool consists of three parameters: length by width, amount of exudate (none, light, moderate, and heavy), and tissue type (necrotic tissue, slime, granular tissue, epithelial, and closed tissue). Each parameter is scored, and the sum of the three produces a total wound status score.

(Version 3.0: 9/15/98, © National Pressure Ulcer Advisory Panel)

Patient Name:_____ Patient ID#:_____

Ulcer Location: _____ Date:_____

Directions:

Observe and measure the pressure ulcer. Categorize the ulcer with respect to surface area, exudate, and type of wound tissue. Record a subscore for each of these ulcer characteristics. Add the subscores to obtain the total score. A comparison of total scores measured over time provides an indication of the improvement or deterioration in pressure ulcer healing.

Length × Width	0 0 cm^2	1 <0.3 cm^2	2 0.3–0.6 cm^2	3 0.7–1.0 cm^2	4 1.1–2.0 cm^2	5 2.1–3.0 cm^2	Subscore
		6 3.1–4.0 cm^2	7 4.1–8.0 cm^2	8 8.1–12.0 cm^2	9 12.1–24.0 cm^2	10 >24 cm^2	
Exudate Amount	0 None	1 Light	2 Moderate	3 Heavy			Subscore
Tissue Type	0 Closed	1 Epithelial Tissue	2 Granulation Tissue	3 Slough	4 Necrotic Tissue		Subscore
							Total Score

Length × width: Measure the greatest length (head to toe) and the greatest width (side to side) using a centimeter ruler. Multiply these two measurements (length × width) to obtain an estimate of surface area in cm^2. Caveat: Do not guess! Always use a centimeter ruler and always use the same method each time the ulcer is measured.

Exudate amount: Estimate the amount of exudate (drainage) present after removal of the dressing and before applying any topical agent to the ulcer. Estimate the exudate (drainage) as none, light, moderate, or heavy.

Tissue type: This refers to the types of tissue that are present in the wound (ulcer) bed. Score as a 4 if there is any necrotic tissue present. Score as a 3 if there is any amount of slough present and necrotic tissue is absent. Score as a 2 if the wound is clean and contains granulation tissue. A superficial wound that is reepithelializing is scored as a 1. When the wound is closed, score as a 0.

4 - Necrotic tissue (eschar): black, brown, or tan tissue that adheres firmly to the wound bed or ulcer edges and may be either firmer or softer than surrounding skin.

3 - Slough: yellow or white tissue that adheres to the ulcer bed in strings or thick clumps, or is mucinous.

2 - Granulation tissue: pink or beefy red tissue with a shiny, moist, granular appearance.

1 - Epithelial tissue: for superficial ulcers, new pink or shiny tissue (skin) that grows in from the edges or as islands on the ulcer surface.

0 - Closed/resurfaced: the wound is completely covered with epithelium (new skin).

Blood samples were drawn after 10–12 h of overnight fasting; fasting blood glucose, glycosylated hemoglobin (HbA_{1c}), fibrinogen levels, and peripheral monocyte levels without mobilization were analyzed (Rehak et al. 2022) . PCT, CRP, ESR, WBC, N.G and lymphocyte levels were also studied. Blood samples were also collected from each patient to study a complete blood count (CBC) using 5.4 mg EDTA K3E tubes (VacuMed). Samples were processed with a HECO 5 hematology analyzer (Seac Radim Company). All tests were studied in the Biochemistry Laboratory of our facility and performed in a blinded manner. Fibrinogen was measured by immunoturbidimetric test by ACL 3000 (Beckman Instrumentation Laboratory), monocytes, lymphocytes, like WBC and N.G. were determined with a HECO 5 hematology analyzer (Seac Radim Company). In this prospective study the authors also wanted to evaluate whether the ratio between pre- and post-operative fibrinogen and between pre- and post-operative monocytes may have a relationship with the appearance of ulcers in DFU patients. This investigation also analyzes the value of lymphocytes in the peripheral blood of diabetic patients with and without ulcers and with and without infection. In our study we also wanted to correlate HbA_{1c} to the concentration of fibrinogen and the appearance of DFU.

Inflammatory markers (ESR, CRP, WBC, percentage of NG), fibrinogen, lymphocytes and monocytes (PBMNC), were evaluated in all patients at the time of admission and at the time of discharge, that is after 3 days. Procalcitonin values were considered positive if they were found to be higher than 0.5 ng/ml. The patients were re-evaluated clinically and through hematological tests after 45 days, to highlight the evolution of the ulcerative lesion and to find whether there is a correlation between the evolution of the diabetic and non-diabetic ulcer and the pre- and post-hospitalization fibrinogen blood levels. HbA_{1c} was evaluated using Pictus 400 Diatron (MI ZRT, Budapest, Hungary).

The kaolin thromboelastographic examination (TEG) was performed with a dedicated machine (Thromboelastography Analyzer CFMS LEPU-8800 Lepu Medical Technology Company, Beijing, China).

STATISTICAL ANALYSIS

Due to the small sample size, data evaluation was expressed in means±SD and medians. *All normally distributed data were analyzed using t-student test* and ANOVA test to evaluate differences in mean±SD. Data found to be non-normally distributed were analyzed using the Mann-Whitney *U*-test for independent subgroups. Correlation and regression analysis was performed between fibrinogen and other inflammation markers through the Spearman test. A *p* value lower than 0.05 was considered statistically significant, $p<0.005$ is highly statistically significant and therefore the null hypothesis is rejected. The Bland-Altman test and the ROC curve were also performed on the fibrinogenemia and monocythemia values for DFU and WDFU patients to evaluate the degree of congruence between them (%) and their sensitivity and specificity as indicators of ulcerative pathology. Data were analyzed using version 6.0 of the Santon-Glantz 2007 Statistics for Biomedical Disciplines package. Receiver operating

characteristic (ROC) curve changes were plotted using MedCalc software (22.019 32 bit).

The questions and objectives in this study are:

1- Evaluate a possible correlation between blood fibrinogen and the presence of diabetic foot disease.

Do patients with diabetic foot ulcers have higher fibrinogen values than those without ulcers and patients without diabetes?

2-Establish if there is a correlation between Glycosylated Hemoglobin (HbA_{1c}) and DFU Markers (PCT, PCR, ESR, WBC, Fibrinogen, Monocytes, Lymphocytes).

Do the HbA_{1c} values change in relation to the presence of a diabetic foot ulcer?

3-Compare the levels of fibrinogen and monocytes pre- and post-intervention and correlate them with the evolution of the diabetic and non-diabetic ulcer.

Is there a cut-off beyond which it is possible to predict the appearance of an ulcer?

RESULTS

The mean age of the patients subjected to evaluation was 65.07±14.24 years, S.E.: 2.12; Median 66 years.

All patients in our study, even those presenting diabetic foot infection with ulcer (IDFU), had a Procalcitonin (PCT) value <0.5 ng/dl, and only 1% of the values found were higher than 0.5 with an average of 1.04 ng/dl (range: 0.52-2.5), hence it had no diagnostic significance, probably because our patients, even with infected ulcers, were not at risk of sepsis and apyretic (Valencia L., 2023)[11]. The average initial score, evaluated with the PUSH method, in patients with diabetic foot ulcer (NDFU) was 12.62 ±1.68 (range: 10-15), while the final score for the same patients was 9.33 ±2.08 (range: 7 -11); the initial one for patients with diabetic foot ulcer (DFU) was 10.06±3.52 (range: 4-16), and the final score was 7.08±4.4 (range: 0-16).

The mean fibrinogen values in non-diabetic patients with ulcer and in non-diabetic control group without ulcer were 407.90±188.2 mg/dL and 274.7±82.0 mg/dL respectively (p<0.005*) (Fig. 2). Inflammatory markers, like white blood cells and neutrophils, were elevated in patients with non-diabetic ulcer compared to non-diabetic controls without ulcer (WBC 8.64 *vs* 5.56 109/L: p=0.005*; Neutrophils 5.33 vs 4.46%; p=0.172). No significant differences were observed in platelet levels between the study group and the control group. Spearman correlation analysis for non-diabetic patients with ulcers indicated a significant correlation of Fibrinogen with ESR (r=0.721; p=0.001), with neutrophils (r=0.739; p=0.012) and a moderately positive correlation with lymphocytes (r=0.559; p=0.073) and with PCR (r=0.522; p=0.011) (Tab.II).

In this study, a significantly higher level of fibrinogen was observed in the blood of women compared to men. We also reported a progressively increased fibrinogen level with age for both sexes. Even in diabetic patients, the same differences in fibrinogen levels were observed between the two genders, again with increased values in women.

Fibrinogen levels are directly associated with fibrin clot properties (structure, time to form and resistance to fibrinolysis), and therefore they may be a key parameter in determining the characteristics of membranes derived from self compressed platelet-rich fibrin (PRF) clots (Aldana et al., 2022)[12].

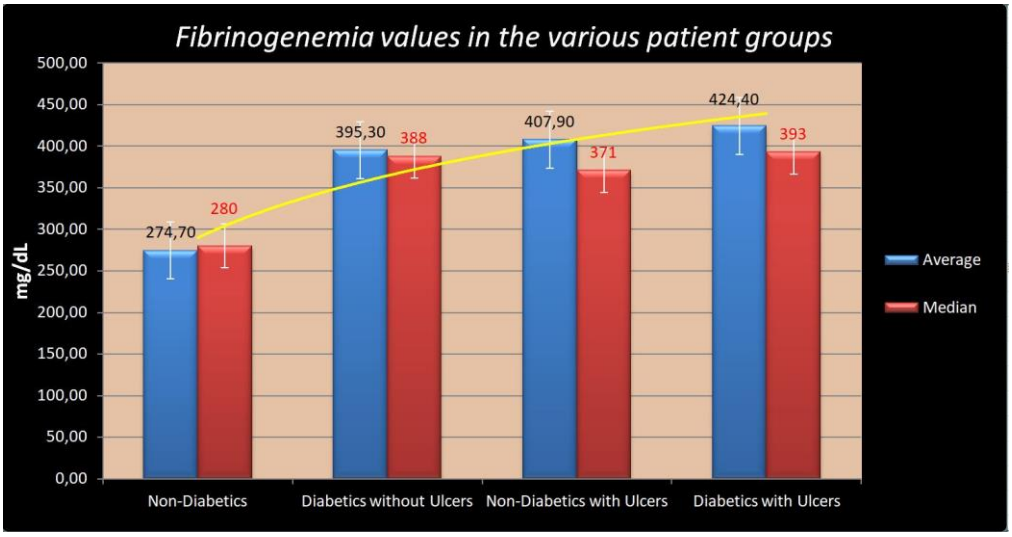

Figure 2. Total fibrinogenemia values by group

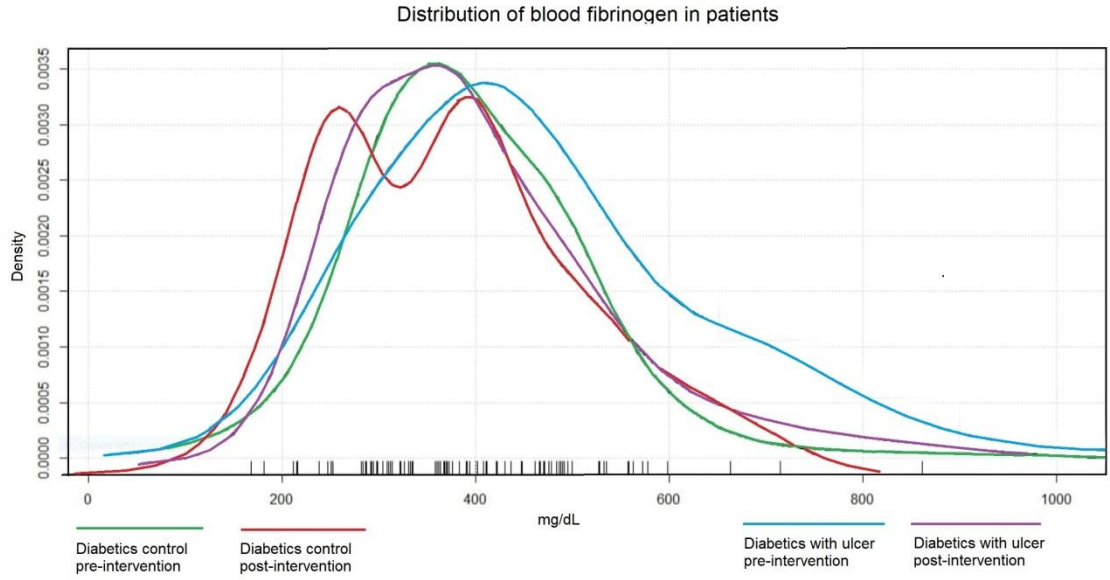

Figure 3. Pre- and post-intervention fibrinogenemia density for DFU and WDFU diabetic patient groups

The mean fibrinogen values in diabetic patients without ulcer (WDFU) were 395.30±130.0 mg/dL. The inflammatory markers, including white blood cells and neutrophils, weren't different and elevated in non-diabetic patients compared to diabetic controls without ulcer (WBC: p=0.642; Neutrophils: p=0.915). Again, no significant differences were observed in platelet levels between the study group and the control group. Spearman correlation analysis for control diabetic patients indicated only a moderately positive correlation of Fibrinogen with ESR (r=0.439, p=0.001) and with CRP (r=0.445, p=0.001)(Tab. III).

Table II. Comparison between the values examined in male and female non-diabetic patients without ulcer and with ulcer, with significance and Spearman correlation test.

TYPE	NDWU Total					NDFU Total									
	Average	D.S	E.S.	Median	N°	Average	D.S	E.S.	Median	N°	t-Stud	M.W.	ANOVA	correlation	
Fibr. mg/dL	274.7	82.0	12.5	280	43	407.9	188.2	40.1	371	23	0.000*	0.000*	0.000*	r	p
PCR mg/dl	6.77	5.91	0.9	5	43	10.7	8.98	1.9	7.26	23	0.036**	0.037**	0.033**	0.522	0.011
WBC 10^9/L	6.56	2.3	0.35	5.9	43	8.64	3.5	0.75	8	23	0.005*	0.005*	0.010**	0.410	0.053
Gr.Neu.%	4.46	1.48	0.26	4.1	33	5.33	2.56	0.81	4.2	11	0.172	0.172	0.448	0.739	0.012
VES mm/h	18.3	15.2	2.3	13	42	48.8	27.6	5.9	48.8	23	0.000*	0.000*	0.000*	0.721	0.001
HbA1c mmol/mol	4.28	1.8	0.48	4.2	14	4.38	1.33	0.66	4.4	5	0.911	0.911	0.817	0.100	>0.05
Monocytes K/uL	0.29	0.1	0.02	0.3	43	0.41	0.18	0.039	0.4	23	0.000*	0.000*	0.010**	0.231	0.285
Linfocytes K/uL	1.64	0.93	0.16	1.4	33	2.91	1.22	0.38	2.2	11	0.000*	0.000*	0.000*	0.559	0.073

**p<0.005 highly significant difference **p<0.05 significant difference*

Table III. Comparison between the values examined in male and female non-diabetic patients without ulcers and diabetic patients without ulcers, with significance and Spearman correlation test.

TYPE	NDWU Total					WDFU Total									
	Average	D.S	E.S.	Median	N°	Average	D.S	E.S.	Median	N°	t-Stud	M.W.	ANOVA	correlation	
Fibr. mg/dL	274.7	82.0	12.5	280	43	395.3	130	11.07	388	138	0.000*	0.000*	0.000*	r	p
PCR mg/dl	6.77	5.91	0.9	5	43	8.17	7.85	0.67	5	138	0.283	0.283	0.475	0.445	0.001
WBC 10^9/L	6.56	2.3	0.35	5.9	43	6.71	1.68	0.14	6.7	138	0.642	0.642	0.152	0.209	0.014
Gr.Neu.%	4.46	1.48	0.26	4.1	33	4.49	1.31	0.15	4.4	80	0.915	0.915	0.717	0.215	0.056
VES mm/h	18.3	15.2	2.3	13	42	34.7	23.99	2.04	30	138	0.000*	0.000*	0.000*	0.439	0.001
HbA1c mmol/mol	4.28	1.8	0.48	4.2	14	6.57	2.26	0.42	5.8	29	0.002*	0.002*	0.006**	-0.001	1.000
Monocytes K/uL	0.29	0.1	0.02	0.3	43	0.39	0.18	0.01	0.3	136	0.000*	0.000*	0.000*	0.054	0.527
Linfocytes K/uL	1.64	0.93	0.16	1.4	33	2.05	1.72	0.19	1.8	80	0.279	0.279	0.013**	0.195	0.083

**p<0.005 highly significant difference **p<0.05 significant difference*

Table IV. Comparison between the values examined in male and female diabetic patients with ulcer and diabetic patients without ulcer, with significance and Spearman correlation test.

TYPE	DFU Total					WDFU Total									
	Average	D.S	E.S.	Median	N°	Average	D.S	E.S.	Median	N°	t-Stud	M.W.	ANOVA	correlation	
Fibr. mg/dL	424.4	138.8	13.3	393	109	395.3	130	11.07	388	138	0.091	0.091	0.476	r	p
PCR mg/dl	10.6	11.5	1.09	5	111	8.17	7.85	0.67	5	138	0.049**	0.049**	0.183	0.413	0.001
WBC 10^9/L	6.96	1.97	0.19	6.7	111	6.71	1.68	0.14	6.7	138	0.281	0.281	0.336	0.340	0.001
Gr.Neu.%	4.99	1.83	0.20	4.7	85	4.49	1.31	0.15	4.4	80	0.046**	0.046**	0.133	0.490	0.001
VES mm/h	33.8	23.2	2.20	30	111	34.7	23.99	2.04	30	138	0.766	0.766	0.191	0.621	0.001
HbA1c mmol/mol	7.2	3.7	0.90	5.86	17	6.57	2.26	0.42	5.8	29	0.476	0.476	0.820	-0.350	0.180
Monocytes K/uL	0.38	0.17	0.016	0.4	111	0.39	0.18	0.01	0.3	136	0.657	0.657	0.965	0.223	0.019
Linfocytes K/uL	1.79	0.78	0.085	1.6	84	2.05	1.72	0.19	1.8	80	0.211	0.211	0.173	-0.252	0.022

**p<0.005 highly significant difference **p<0.05 significant difference*

Table V. Comparison between the values examined in male and female diabetic patients with ulcer and non-diabetic patients with ulcer, with significance and Spearman correlation test.

TYPE	DFU Total					NDFU Total							
	Average	D.S	E.S.	Median	N°	Average	D.S	E.S.	Median	N°	t-Stud ANOVA	M.W.	
Fibr. mg/dL	424.4	138.8	13.3	393	109	407.9	188.2	40.1	371	23	0.629	0.629	0.413
PCR mg/dl	10.6	11.5	1.09	5	111	10.7	8.98	1.9	7.26	23	0.969	0.969	0.547
WBC 10^9/L	6.96	1.97	0.19	6.7	111	8.64	3.5	0.75	8	23	0.002*	0.002*	0.037**
Gr.Neu.%	4.99	1.83	0.20	4.7	85	5.33	2.56	0.81	4.2	11	0.582	0.582	0.945
VES mm/h	33.8	23.2	2.20	30	111	48.8	27.6	5.9	48.8	23	0.007**	0.007**	0.016**
HbA1c mmol/mol	7.2	3.7	0.90	5.86	17	4.38	1.33	0.66	4.4	5	0.115	0.115	0.050
Monocytes K/uL	0.38	0.17	0.016	0.4	111	0.41	0.18	0.039	0.4	23	0.447	0.447	0.448
Linfocytes K/uL	1.79	0.78	0.085	1.6	84	2.91	1.22	0.38	2.2	11	0.000*	0.000*	0.001*

**p<0.005 highly significant difference **p<0.05 significant difference*

In patients with DFU and in diabetic controls without ulcer (WDFU) the mean fibrinogen values were 424.4±138.8 mg/dL and 395.3±130.0 mg/dL, respectively (p>0.05). WBC and N.G. were not significantly different in patients with DFU compared to controls (WBC: p=0.281; Test M.W.=0.336; Neutrophils: p=0.046**; Test M.W.=0.133). No significant differences were observed in platelet, monocytes and lymphocytes levels between the DFU study group and the WDFU control group (Tab. IV).

Spearman correlation analysis indicated a significant correlation of fibrinogen with ESR (r=0.621, p=0.001), but only a moderately positive correlation with CRP (r=0.413, p=0.001) and with Neutrophilic Granulocytes (r= 0.490, p<0.001) and a poorly positive correlation with WBC (r=0.340, p<0.001). Furthermore, the correlation between fibrinogen and monocyte concentration is weakly positive (r=0.223, p=0.019) while that with lymphocytes is weakly negative (r=-0.252, p=0.022)(Akoglu, 2018)[13] (Tab. IV).

In patients with NDFU and DFU, the mean fibrinogen values were 407.90±188.2 mg/dL and 424.4±138.8 mg/dL respectively (p<0.629).

WBC inflammatory markers were significantly elevated in non-diabetic patients with ulcer compared to DFU (WBC: 8.64 vs 6.96 109/L; p=0.002*; Test M.W.=0.037**). Neutrophils were not different in patients with NDFU compared to DFU (p=0.582; Test M.W.=0.945). While the ESR (48.8 vs 33.8 mm/h; t-Student p=0.007*; Test M.W.=0.016**) and lymphocytes (2.91 vs 1.79 k/µL; t-Student p=0.000*; Test M.W.=0.001*) were significantly different in non-NDFU subjects compared to DFU subjects, no significant differences were observed in levels of platelets and monocytes, as well as between the study group with DFU and the non-diabetic control group with ulcer (Tab. V).

The ROC curve analysis in the work of Li et al. (2016) suggested that the optimal fibrinogen cut-off point for complete amputation among the 152 patients they examined with DFU was 513 mg/dL, with sensitivity, specificity, PPV (Positive Predictive Value) and NPV (Negative Predictive Value) of 80.9%, 82.6%, 78.6% and 89.0%, respectively.

We did not find significant changes in HbA_{1c} (glycated hemoglobin) levels in the various comparisons, except between the diabetic and non-diabetic control groups (4.28 vs 6.57 mmol/mol p=0.002) (Tab. III).

Concerning monocytes blood levels, we found a significant difference between NDFU and NDWU patients (0.41 *vs* 0.29 k/µl; p=0.000*) and between the diabetic without ulcer (WDFU) and non-diabetic without ulcer (NDWU) groups. (0.39 *vs* 0.29 k/µL; p=0.000*) (Tab. II and III). However, no difference in monocytes blood levels between the diabetic patients with ulcers group (DFU) and diabetics without ulcers (WDFU) group was found (Tab. IV), as well as between diabetic and non-diabetic patients without ulcers (Tab. V).

At the thromboelastographic examination, diabetic subjects with DF showed increased levels of α angle and reduced levels of k value compared to patients in the control group without DF (p<0.01) (Ramanujam et al., 2023)[14].

According to the study by Zang, 2022[15], fibrinogen levels and the α angle were positively correlated with the diabetic foot classification (r=0.635, p<0.01; r=0.616, p<0.01), while k value was negatively correlated with DF (r=-0.589, p<0.01). Accordingly, Fib levels were positively correlated with the α angle and negatively correlated with the k value (r=0.553, p<0.01, r=-0.526, p<0.01). The optimal cut-off for

fibrinogen was 4.12 g/L, with a sensitivity of 85.7% and a specificity of 93.5% (Tab. VI).

The average values of *k* and angle *α* of kaolin thromboelastography (TEG, CFMS LEPU-8800) found in our study were: 1.5 mm (range 1.3-2.0); 68.85 deg (range 62.38-72.70) respectively.

Table VI. Fibrinogenemia and thromboelastography values reported by Zhang et al. 2022 compared to the values obtained in our study (in red).

Group	Non-diabetic control group	Diabetic group without diabetic foot	Diabetic group with diabetic foot and ulcer	Non-diabetic group with ulcer	Diabetic group with mild diabetic foot	Diabetic group with severe diabetic foot
Fibrinogen (v.n.:150-400 g/L)	2.82±0.57 2.75±0.82	3.04±0.52 3.95±1.30	4.94±2.01 (p<0.01) 4.24±1.39	4.08±1.88	3.26±0.96	5.82±1.86 (p<0.01)
Angle □□ (v.n.: 55-78 deg)	58.8±5.1	61.4±7.7 (p<0.05)	69.5±7.3 (p<0.01)		63.7±5.5	72.5±6.4 (p<0.01)
Value *k* (v.n.: 1-3 min)	2.1±0.4	1.9±0.5 (p<0.01)	1.3±0.4 (p<0.01)		1.6±0.3	1.2±0.4 (p<0.01)

The ratio between the Fib blood level values found in diabetic patients control group and in diabetic patients with ulcer pre- and post-intervention was 1.09 (p=0.015)(Spermann's r^2=0.75) *vs* 1.11 (p=0.011)(Spermann's r^2= 0.71). The cut-off for the risk of forming an ulcer is 1.10 (p=0.087) with a sensitivity of 84.06% and a specificity of 24.47%. The Odds Ratio (probability of the event occurring) is equal to 1.007; 95% I.C. 0.816-1.244; $\chi 2$ =0.000; p=0.989(Fig.3).

The relationship between the fibrinogen values found in control diabetic patients and in diabetic patients with ulcer has an AUC_{ROC} value (0.610; p=0.014), with a specificity of 59.57% and a sensitivity of 59.42% with a cut-off > 408 mg/L (Fig.4).

The relationship between the Monocythemia values found in control diabetic patients and in diabetic patients with Ulcer has an AUC_{ROC} value (0.533; p=0.466) with a specificity of 83.51% and a sensitivity of 28.38% with a cut-off ≤ 0.2 k/μL (Fig. 4).

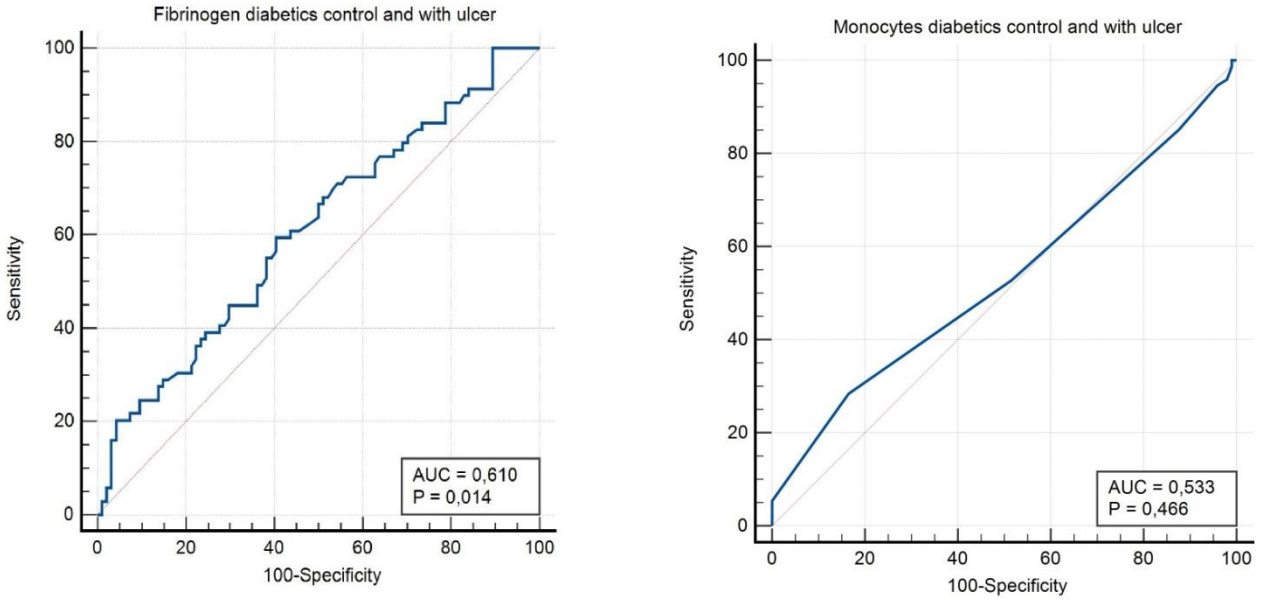

Figure 4. ROC curve for Fibrinogen and Monocytes in DFU and WDFU

The ratio between the monocyte blood values found in control diabetic patients and in diabetic patients with ulcer pre- and post intervention was 1.18 (p=0.18) (Spermann's r^2=0.37) *vs* 1.04 (p=0.658) (Spermann's r^2=0.46). The cut-off for the risk of forming an ulcer is 1.10 (p=0.087) with a sensitivity of 93.81% and a specificity of 14.86%. The Odds Ratio =1.095; 95% I.C. 0.894-1.342; $\chi 2$ =0.687; p=0.407.

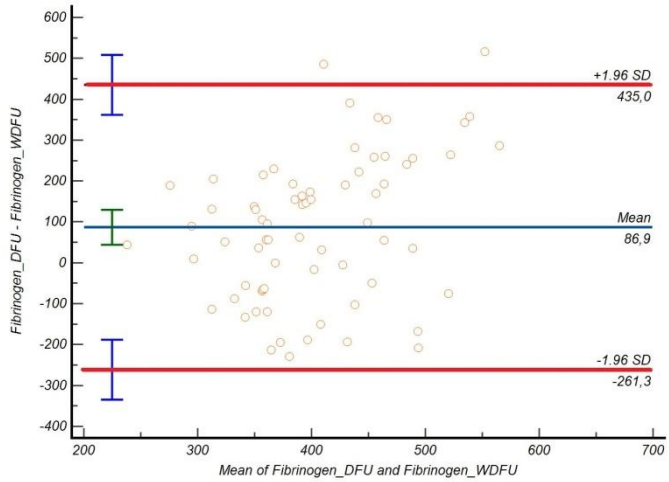

Figure 5. Bland-Altman test for Fibrinogen in DFU and WDFU

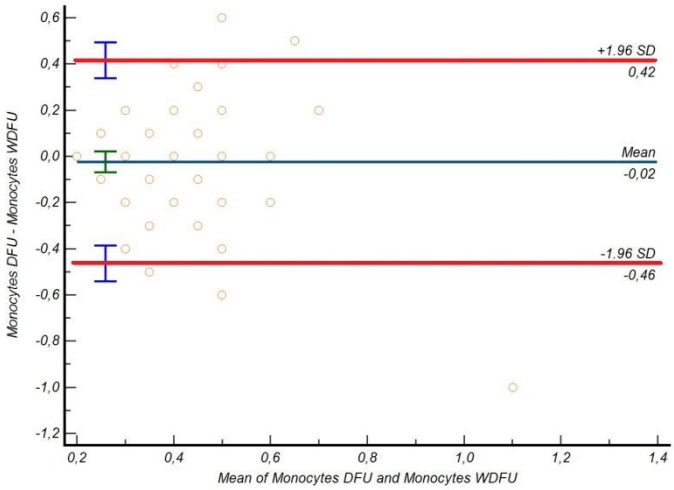

Figure 6. Bland-Altman Test for Monocytes in DFU and WDFU

The Bland-Altman test for Fibrinogen and Monocytes values was also performed to evaluate the congruity of the values obtained in DFU and WDFU patients (Figs. 5 and 6) and it was found that the fibrinogen values in DFU subjects are 93% adequate, in WDFU patients they are 95% adequate, while those relating to monocytes are 97% adequate in DFU subjects and 98% in WDFU ones.

DISCUSSION

The increasing incidence of diabetes and diabetic foot ulcers (DFU), as well as an increased incidence of peripheral artery disease (PAD) in patients with diabetes requires a reconsideration of the concept of CLI. In many current studies, the prevalence of diabetes in reports of patients undergoing limb re-vascularization is between 50% and 80%. DFUs can be broadly classified into three groups: purely neuropathic, purely ischemic, and neuroischemic (mixed type). Based on these studies, the prevalence of neuro-ischemic ulcers has steadily increased from approximately 20% to 25% in the 1990s to over 50% of patients today. Therefore, neuroischemic type is now the most common etiology of DFUs in most Western countries. The current estimated prevalence rates of neuropathic, ischemic, and neuroischemic ulcers in patients with diabetes are 35%, 15%, and 50%, respectively.

In this study, in agreement with other previous studies (Li et al. 2016, Zhang et al. 2022) patients with DF had higher α angle and Fib levels on TEG compared to diabetic patients without DF, while k value levels in patients with DF were significantly lower than those of diabetic patients without DF. However, it would be necessary to evaluate whether α angle, Fib levels and k values are modified in the initial phase of the diabetic foot.

Abnormal platelet hyper-reactivity, coagulation status, and fibrinolytic function are prevalent in patients with diabetic foot and they worsen with disease progression. Metabolic abnormalities increase fibrinogen levels, causing an increased incidence of blood clotting in patients with diabetic foot, which in turn predisposes to thrombosis and causes microvascular and lower extremity macro-angiopathy.

The results of our study also revealed that people with diabetes and DFU have elevated fibrinogen blood levels compared to people with diabetes but without DFU.

Elevated fibrinogen levels have been found to provide high sensitivity, specificity, and predictive values in patients with DFU, suggesting superiority of fibrinogen over CRP. The predictive superiority of fibrinogen found may be attributed to its more stable nature compared to PCR.

It is therefore essential to find effective markers to assess the severity of the disease, as well as to personalize therapy. Fibrinogen, an inflammatory marker, as one of the main blood coagulation proteins, represents an important determinant of blood viscosity and platelet aggregation, and also represents a risk factor for vascular events. In fact Kunutsor et al. (2016)[16] suggest that fibrinogen is positively, linearly and independently associated with the risk of sudden cardiac death.

Data suggests that patients with diabetic foot disease have higher fibrinogen levels compared to those without ulcers, hence elevated fibrinogen levels may represents a significant clue to estimate the severity of DFU disease for physicians.

Our study used fibrinogen to predict diabetic foot incidence and assess its severity, and its optimal cut-off point for determining the risk of an ulceration was found to be 4.08 g/L; therefore Fib may be involved in the onset and development of diabetic foot as an important factor, causing a state of increased blood coagulation in patients with DF, in turn predisposing to the formation of clots and causing macro- and micro- angiopathy of the lower limbs. Meanwhile, this study further confirmed that Fib is positively correlated with diabetic foot classification, suggesting the need for timely clinical control of Fib levels to reduce or delay the onset and ulcerative progression of diabetic foot, and therefore asserting once again that the addition of drugs reducing blood clotting can have a beneficial effect on the prevention and delay of the onset and progression of diabetic foot.

CONCLUSIONS

Recent therapeutic strategies for DFU can be divided into two categories: non-cellular therapies (growth factors) and autologous cell therapies (Casado-Diaz, 2022; Caravaggio et al. 2021)[17, 18]. Monocytes and macrophages, present in all body tissues, represent a cell population with different functions and phenotypes, and can therefore regulate local homeostasis in various ways. These leukocytes can produce cytokines, chemokines, growth factors, inflammatory mediators and can also phagocytose damaged tissues, "non-self" substances and microorganisms. Regardless of the specific cause, whenever inflammation persists in injured tissues, regenerative processes are hindered or even inhibited.

Neither monocytes nor HbA_{1c} can be considered biomarkers for the risk of ulcer formation in the diabetic foot, but only as biomarkers of type 2 diabetes mellitus. Differently, fibrinogen levels, their pre/post intervention ratio, the α angle and the *k* value of kaolin thromboelastography (TEG), have a clinical significance on the risk of onset and development of ulcerated diabetic foot.

These studies suggest that TEG could be more sensitive than the Fib-test for an early diagnosis of diabetic foot and therefore for an early treatment; hence, our group aims to carry out a specific scientific trial. Nonetheless, this study still has several limitations and further studies are needed to establish if active interventions may help in reducing adverse prognostic events. Furthermore, the sample size of this study was reduced and not large enough, hence confirmation by a comprehensive study with a larger sample is needed.

However, unified quantitative standards for diabetic foot incidence and progression haven't been found yet.

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