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Correlation of Cathepsin D and hsCRP Plasma Levels with Diabetic Foot Ulcer in Egyptian Subjects: Control Study

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Abstract: Background: *The global escalating rates of diabetes mellitus make diabetic foot ulcer (DFU) a major public-health problem. Patients with DFU frequently showed increased plasma levels of cathepsin D (CatD) contributing to poor wound healing.* ***Objective:*** *To investigate the association between plasma CatD and high-sensitivity C-reactive protein (hsCRP) with DFU and its various grades.* ***Subjects:*** *This study comprised 148 type 2 diabetics (74 subjects with foot ulcer and 74 subjects without foot ulcer as controls).* ***Results:*** *Subjects with DFU compared with those without DFU showed higher mean plasma levels of CatD (32.1±30.4 vs 15.4±34.8 RFC/ml), and hsCRP (70.4±25.3 vs 59.5±31.6 mg/ml). A positive correlation was found between either of CatD (P<0.001) and hsCRP (P<0.038) with the grade of ulcer.* ***Conclusions:*** *Type 2 diabetic subjects with various grades of DFU showed higher CatD and hsCRP levels compared with diabetics without foot ulcer, independent of concomitant infections.*

1. Introduction

The worldwide incidence of diabetes is increasing rapidly and it has been estimated that by the year 2030, there will be 8.6 million adults with diabetes in Egypt, making it the country with the tenth largest population of diabetics in the world [1]. Among diabetic vascular complications, foot ulcers represents the first cause of hospitalization in diabetics and a significant cause of health care costs [2], and despite considerable international efforts, foot ulcers continue to be responsible for a high number of lower limb amputations that are associated with decrease in quality of life and increased risk of mortality [3]. Up to 70% of all non-traumatic amputations in the world occur in diabetics. However, many of these amputations are preventable as about 85% are preceded by a foot ulcer [4].

Pathogenic events able to cause DFU are multifactorial. Among the commonest causes of this pathogenic pathway it's possible to consider peripheral neuropathy, foot deformity, abnormal foot pressures, abnormal joint mobility, trauma, peripheral artery disease [5]. It is characterized by a pronounced inflammatory reaction, decreased collagen content and biosynthesis, and accelerated proteolytic degradation which are crucial in wound non-healing [6].

CatD is an aspartic endopeptidase that plays an important role in cell growth, proteolytic degradation, cell invasion and apoptosis [7], and has been associated with several human pathologies [8]. Interestingly, patients with DFU had increased plasma levels of CatD contributing to poor wound healing. Increased proteolytic degradation of collagen in wounds is reversed by external application of a CatD inhibitor, suggesting that this is the main enzyme responsible for collagen decomposition [6].

Low-grade immune activation also represents an important risk factor not only for the development of Type 2 diabetes but also for several macrovascular complications (myocardial infarction and stroke) and microvascular complications (neuropathy and nephropathy) [9]. Because pro- and anti-inflammatory processes are crucial in the different phases of wound healing, it is conceivable that disturbances of the immune system interfere with tissue homeostasis and wound healing after the manifestation of ulcers [6]. C-reactive protein (CRP), an acute phase reactant, binds to specific molecular configurations typically present in case of cell death and additionally found on the surface of pathogens, and therefore increases rapidly after tissue injury or infections and reflects the intensity of the inflammatory process [10]. Immunoassays for CRP with greater sensitivity (hence called hsCRP) than those previously in routine use, have revealed increased CRP values, even within the range previously considered normal [11]. Diabetic subjects with various grades of DFU showed a higher hsCRP plasma levels in comparison with diabetics without

foot ulcer, independent of the concomitant infections [12].

2. Subjects & methods

This prospective case control study was performed in the National Institute of Diabetes & Endocrinology (NIDE) in Egypt between September 2014 and October 2015. We recruited 74 diabetic patients (Group I) with foot ulceration, which is clinically defined as a full-thickness defect that required ≥ 14 days to heal [13], (36 females and 38 males, mean age 52.7± 8.8 years) at the Diabetic Foot Surgery outpatient clinic, and 74 diabetic patients (Group II) with no foot ulceration considered as control group (48 females and 26 males, mean age 50.9 ± 10.5 years) at the Internal Medicine outpatient clinic. All subjects who participated in this study were already previously diagnosed as type 2 diabetes mellitus cases. Patient recruitment was according to American Diabetes Association (ADA) criteria [14]. Written consent was obtained from every subject before taking samples after explaining investigations done for them. The study was approved by the General Organization of Teaching Hospitals and Institutes research ethics committee.

All patients were subjected to the following: Clinical assessment: 1- Full medical history. 2- Physical local examination by inspection of the foot and palpation of peripheral pulses. 3- Ulcer size was determined by multiplying the longest and the widest diameters and expressed in centimeters square. The wound was graded according to Wagner Grading System (WGS) which assesses ulcer depth along with presence of gangrene and loss of perfusion using six grades (0-5): grade 0 (pre-or post-ulcerative lesion), grade 1 (partial/full thickness ulcer), grade 2 (probing to tendon or capsule), grade 3 (deep with osteitis), grade 4 (partial foot gangrene), and grade 5 (whole foot gangrene) [15]. Patients with peripheral vascular diseases because of non-diabetic causes, patients with traumatic foot ulcers, and patients with joint diseases, in addition, to patients with inflammatory or infectious diseases, autoimmune and rheumatic diseases, cancer, hematological diseases, and those who were under treatment with anti-inflammatory drugs, pregnant and lactating females and type 1 diabetes mellitus patients were excluded. We also excluded patients with recent venous thromboembolism.

3. Sample collection and laboratory analysis

Fifteen ml of venous blood were withdrawn from each patient in dry sterile vacutainers after overnight fasting. First part of collected blood was taken on EDTA tubes for determination of HbA1c level by using D-10 HPLC ion exchange chromatography (USA, supplied by Bio-Rad, Afak el Mostakbal Company, Cairo, Egypt). Second part collected on serum gel tubes from which serum was separated by centrifugation after clotting. It was tested for: Fasting Blood Glucose and Lipid Profile (Total cholesterol, Triglycerides, HDL & LDL) levels by using ARCHI TECT 8000 chemistry analyzer (USA, supplied by Abbott, Al Kamal company Cairo, Egypt). Third part of blood was taken on another EDTA tubes, for the measurement of CatD and hsCRP levels, was centrifuged for 15min at 3000 rpm, and the plasma fraction was separated, divided into two aliquots and stored at −80°C until analysis. Plasma levels of soluble CatD and hsCRP were measured using Enzyme Linked Immunosorbant Assay (ELISA) technique according to manufacturer's instructions.

4. Statistical analysis

Data were analyzed using the IBM program SPSS Statistics. Categorical data were represented as frequencies (%). The differences in frequencies of categorical parameters were analyzed by X2 (chi-square) test. Data of continuous parameters were represented as mean ± SD, and analyzed using student's t-test or the Mann-Whitney U test. Correlations were done using Pearson's correlation coefficient test (r) or Spearman's coefficient. Risk assessment was done using odds ratio and risk ratio. The level of statistical significance was defined at $P<0.05$.

5. Results

The study comprised 148 Egyptian type 2 diabetic patients. They were divided into two groups. Case Group I: 74 type 2 diabetics with foot ulcer comprising 36 females and 38 males with mean age 52.7± 8.8 years. Control Group II: 74 type 2 diabetics without foot ulcer comprising 48 females and 26 males with mean age 50.9 ± 10.5 years. 87.8% of subjects in group I versus 77.0% of subjects in group II were treated with insulin and 12.2% versus 23.0% with oral anti-diabetics.

Baseline characteristics of Case Group I subjects in comparison with Control Group II subjects are

given in table (1). By comparing mean ± SD levels we found that there were highly significant statistical differences between them as regard CatD, hsCRP, Fasting blood glucose, Cholesterol, HDL, LDL, creatinine and urea, (P<0.001), and significant statistical differences between them as regard HbA1c and the duration of diabetes, (P<0.05), while there were insignificant statistical differences as regard Triglycerides and age.

Table 1. Baseline characteristic in cases & controls.

Group/ Parameter	Control Group I N=74 Mean ± SD	Control Group II N=74 Mean ± SD	P
FBS (mg/dL)	300.8 ± 152.9	230.1 ± 106.2	0.001**
HbA1c (%)	10.2 ± 2.1	9.4 ± 2.2	0.021*
Chol (mg/dL)	192.4 ± 46.5	217.5 ± 47.5	0.001**
TG (mg/dL)	162.5 ± 74	162.2 ± 76.9	0.982
HDL (mg/dL)	39.6 ± 9.8	44.8 ± 8.4	0.001**
LDL (mg/dL)	119.1 ± 33.7	137.8 ± 39.3	0.002**
Creat (mg/dL)	1.0 ± 0.3	0.8 ± 0.2	0.000**
Urea (mg/dL)	38 ± 15.1	30.6 ± 8.6	0.000**
Age (years)	52.7 ± 8.8	50.9 ± 10.5	0.274
Duration (y)	13.9 ± 6.5	11 ± 7.5	0.013*
Cathepsin	32.1 ± 30.4	15.4 ± 34.8	0.002**
hsCRP (mg/mL)	70.4 ± 25.3	59.5 ± 31.6	0.022*

**Highly Significant (P<0.001), *Significant (P<0.05)

Student's t-test used; Mann-Whitney-U test used in non-parametric samples

Factors predicting foot ulcer in diabetic patients are given in table(2). On univariate analysis, the factors which showed a positive association in predicting the foot ulcer were HbA1c (>7.0%) (OR 8.400, RR 1.925), HDL-C (<40 mg/dl) (OR 2.937, RR 1.752), and duration of diabetes (> 10 yrs) (OR 2.161, RR 1.429), and in Chi- square test, HbA1c (P = 0.001), HDL-C (P = 0.002), and duration of diabetes (P = 0.033).

Table 2. Risk estimation for developing foot ulcer in diabetic patients.

Parameters	OR	95% CI UL	95% CI LL	RR	95% CI UL	95% CI LL	P
HbA1c (>7%)	8.4	1.84	38.43	1.925	1.48	2.50	0.001**
HDL (<40 mg/dL)	2.937	1.49	5.78	1.752	1.20	2.56	0.002**
LDL (>100 mg/dL)	0.630	0.29	1.37	0.782	0.51	1.21	0.241
Cholesterol (>150 mg/dL)	0.598	0.19	1.92	0.753	0.37	1.53	0.384
Triglycerides (>200 mg/dL)	0.933	0.45	1.94	0.966	0.67	1.38	0.852
Duration DM (>10 y)	2.161	1.06	4.42	1.429	1.05	1.95	0.033*
Gender (M/F)	1.313	0.69	2.51	1.146	0.83	1.59	0.410

OR=odds ratio; RR=Risk ratio; M=Male; F=Female;
CI=confidence interval; UL=upper limit; LL=lower limit
**Highly Significant (P<0.001), *Significant (P<0.05)

On multivariate analysis, CatD (P = 0.003) (P = 0.001), and hsCRP (P = 0.031) (P = 0.001), showed a positive association in predicting the foot ulcer by multiple linear regression (table 3).

Table 3. Multiple linear regression analysis and foot ulcer in type 2 diabetic patients.

Parameter	Multiple linear regression Coefficient	P	Anova F	P
Cathepsin	0.004	0.003	7.353	0.001**
hsCRP	0.003	0.031		

**Highly Significant (P<0.001)

The following variables were considered for the model: plasma CatD, plasma hsCRP. Only the variables that had a P value <0.05 were considered in the final fitted model. HS = Highly significant. S = significant.

Correlation study of the various parameters with CatD and with hsCRP in the case group I (Pearson) are given in table (4). There was a highly significant positive correlation, between CatD (r=0.648; p=0.001), and a significant positive correlation between hsCRP and ulcer grade (r=0.242; p=0.038), respectively (Table 5).

Table 4. Correlation study of the various parameters with CatD and with hsCRP in Type 2 diabetics with diabetic foot ulcer

Group/ Parameter	Patient Group N=74 Mean ± SD	Cathepsin r	Cathepsin P	hsCRP r	hsCRP P
Ulcer Grade	2.07 ± 0.84	0.648	0.001	0.242*	0.038
FBS (mg/dL)	300.8 ± 152.9	0.060	0.613	0.030	0.801
HbA1c (%)	10.2 ± 2.1	0.027	0.820	-0.018	0.880
Chol (mg/dL)	192.4 ± 46.5	-0.056	0.633	-0.216	0.065
TG (mg/dL)	162.5 ± 74	0.104	0.376	-0.004	0.973
HDL (mg/dL)	39.6 ± 9.8	-0.033	0.780	-0.066	0.578
LDL (mg/dL)	119.1 ± 33.7	-0.104	0.378	-0.181	0.123
Creat (mg/dL)	1.0 ± 0.3	-0.133	0.259	0.127	0.28
Urea (mg/dL)	38 ± 15.1	-0.157	0.181	0.043	0.713
Age (years)	52.7 ± 8.8	0.083	0.484	0.001	0.999
Duration (y)	13.9 ± 6.5	-0.036	0.758	-0.163	0.164

r=Pearson's correlation coefficient; Spearman's correlation was used for non-parametric samples
*Significant (P<0.05)

Table 5: Correlation study of CatD, hsCRP and ulcer grade in Type 2 diabetics with diabetic foot ulcer.

Parameters	r	P
Ulcer Grade vs Cathepsin	0.648	0.001**
Ulcer Grade vs hsCRP	0.242	0.038*
Cathepsin vs hsCRP	0.067	0.568

r=Pearson's correlation coefficient; Spearman's correlation used with ordinal or non-parametric samples
**Highly Significant (P<0.001), *Significant (P<0.05)

6. 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 prevalence of 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 % [1]. Fifteen percent of diabetic patients develop foot ulcers during their lifetime and a significant number of individuals with this diabetic complication require a lower extremity amputation [16]. However, foot amputations may be preventable with prompt recognition and therapy [17]. Diabetic foot, is characterized by a pronounced inflammatory reaction, decreased collagen content and biosynthesis and accelerated degradation which are crucial in wound healing [6]. hsCRP levels have been demonstrated to be elevated in diabetic foot ulceration [18], and as a single marker, hsCRP was the most informative for differentiating grade 1 from grade 2 ulcers (sensitivity 0.727, specificity 1.000, positive predictive value 1.000, negative predictive value 0.793) [19]. CatD contributes to intracellular protein degradation and its level increases in inflammatory events [20]. It was suggested that CatD was the main enzyme responsible for collagen decomposition, and interestingly, patients with ulcerated diabetic foot had increased plasma levels of CatD, which can contribute to poor wound healing [6]. From the aforementioned, we can assume that, there is an association between CRP and CatD and the development of foot ulcer in diabetic patients with its various grades, and in the present study, we evaluated this association. We showed that plasma CatD levels were significantly higher in patients with foot ulcer than in those without foot ulcer, suggesting a potential role of CatD in the development of diabetic foot ulcer similar to the studies of [6-21]. Also, the study, showed in case group I highly significant positive correlation between CatD levels and ulcer grade in agreement with the study of [6], which means that we found a relation between the concentration of CatD and the incidence of chronic complication in the form of diabetic foot ulcers and the various grades of ulcer. As regards hsCRP, the present study reported increased levels in diabetic patients with a foot ulcer compared with diabetic patients without foot ulcer. Upchurch et al. [18] supported our results. Another finding consistent with the results of the present study was that positive correlations were evident between WGS and hsCRP [22]. In our study, diabetes duration, HbA1c, and HDL increased risk of diabetic foot ulcers significantly, however, it showed no significant correlation with sex may be because of a larger female population in the study. In other studies, HbA1c, and duration of diabetes, in agreement with ours, while age and male gender in contrast to ours increased the risk of DFU [23-25]. In the current study, we found that the mean duration of diabetes was significantly higher in DFU patients compared with non-DFU patients and those with >10 years of DM duration showed increases in the prevalence of DFU compared with patients with <10 years of DM duration. This is in agreement with other studies that showed that long duration of diabetes was the main factor causing DFUs [26-30]. This is most likely because of other risk factors such as peripheral neuropathy and peripheral vascular disease developing with time [31]. In our study, it was found that poor glycemic control was a significant risk factor for DFU. These findings were confirmed by other studies [26,30,32], that showed that poor glycemic control was the main factor causing diabetic foot problems. Also, the study [33] indicated that the patients with foot ulcers exhibit a specific and nonrandom up regulation of HbA1c % in diabetic foot compared with patients without ulcer, which were mainly associated with severity of ulceration (different grades of ulcer). There was no significant difference between the two groups studied in serum triglycerides in agreement with the study [34].

7. Conclusion

Our study demonstrated that type 2 diabetic subjects, with DFU showed, in comparison with those without DFU, higher hsCRP and CatD plasma levels and their levels were associated with ulcer grade. It is safe and simple to measure hsCRP and CatD plasma levels, and explains that a strict and prospective inflammation marker and a protease soluble lysosomal aspartic endopeptidase evaluation could be useful in practical prevention and management of foot complications in diabetics. Elucidating the mechanisms underlying the involvement of CatD in the pathogenesis of DFU, and how they can be modulated to develop new preventive and therapeutic strategies, should therefore take center stage. Future studies, although challenging, will bring more light into the field of diabetes-related complications, and most likely will result in effective therapies against DFU especially if these studies will be done on a large scale.

8. References

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