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Serum Calcium, Vitamin D and C-Reactive Protein as Early Predictors for Diabetic Foot Ulcer

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Diabetic foot ulcer (DFU) has emerged as a leading cause of morbidity and mortality among diabetic patients, with the numbers of amputation and death are significantly increasing. DFU has a complex multifactorial etiology with several pathophysiological mechanisms and risk factors implicated. Finding reliable indicators to predict the prognosis of diabetic foot has become an urgent and critical need. The current study aimed to investigate the association between levels of serum calcium, vitamin D and C-reactive protein and the development of DF. One hundred and twenty diabetic patients with a confirmed diagnosis of DFU were included in the study. The ratio of cases and control subjects was 1:1. We measured serum calcium, vitamin D, CRP, HbA1C, blood urea nitrogen (BUN), creatinine, total cholesterol, low-density lipoproteins (LDL) and triglycerides in both cases and control subjects. The study found significantly low levels of serum calcium and vitamin D as well as high levels of HbA1C, CRP, serum creatinine, LDL, and TAG among cases. Serum calcium, vitamin D and CRP were significantly associated with DFU. As the treatment of DFU is challenging, these parameters might predict the development of DFU in diabetic patient and might therefore help reduce the morbidity and mortality associated with DFU.

Keywords: Calcium; Diabetic; DFU; Diabetes; Foot; Predictors; Ulcer; Vitamin D.

Diabetic foot ulcer (DFU) is a common and devastating complication of long standing, poorly controlled diabetes mellitus,[1] with a significant morbidity and mortality,[2] in addition to the psychological and financial burden on the patient and his family[3]. Globally, 19-34% of all diabetic patients will develop DFU.[4]Around 20% of patients with DFU eventually necessitate amputation.[4] Additionally, around 10% of DFU patients will die within one year after the diagnosis has been made.[5] DFU is defined as a break through the epidermis and part of the dermis in a diabetic patient that fails to heal quickly[6]. The literature suggested that DFU has a multifactorial origin.[7] The most important factors in the development of DFU are the existence of peripheral neuropathy, peripheral artery disease (PAD) and secondary bacterial infaction.[2] Other contributing factors include poor glycemic control, the presence of foot deformities and calluses, as well as trauma.[7]

However, peripheral artery disease remains the most significant contributing factor.[2] Around 66% of diabetic patients develop peripheral neuropathy.[8] According to the presence or absence of peripheral neuropathy, DFU can be classified into purely neuropathic, purely ischemic, and mixed neuroischemic.[9] Around 50% of all cases of DFU have a neuroischemic origin.[9]

Peripheral neuropathy causes loss of pain and temperature sensation, resulting in an insensitive foot.[10] In this case, even a minor trauma can precipitate foot ulceration. In addition to the sensory deficit, motor dysfunction can also precipitate the foot ulceration.[11] Autonomic neuropathy can also decrease sweating leading to skin dryness and increased predisposition to infection.[11] The end result is a progressive foot ulceration that is difficult to heal.[12]

PAD usually presents with intermittent claudication and rest pain.[13] Intermittent claudication aggravates the disability already present.[14] Risk factors for PAD include hypertension, smoking and dyslipidemia[14]. Together, these factors precipitates the ischemia and worsen the ulceration.[15] In addition to the direct effect of PAD on the ulceration process, it also contributes to the ongoing neuropathy.[15] The hypoglycemia accompanying diabetes augments to the ongoing neuropathy and ulceration via the formation of advanced glycation end-products (AGEs).[16]

Both neuropathy and peripheral artery disease precipitate secondary infection. Staphylococcus aureus accounts for 30% of all cases.[17] Chronic recurrent infections are mostly caused by impaired immune system,[17] which is aggravated by the interplay of production of AGE and reactive oxygen species secondary to hyperglycemia. The interplay of these two factors slows down the process of wound healing.[18] Several classification systems for DFU were developed, including Wagner-Meggitt classification,[19] University of Texas Classification,[20] and the SINBAD[21] (site, ischemia, neuropathy, bacterial infection, area, depth) system. The SINBAD system is straightforward and fast to use, necessitating no specialized equipment beyond clinical examination alone. The SINBAD system assesses six elements (site, ischemia, neuropathy, infection, area, and depth) with scores ranging from 0 to 1, creating a severity scale from 0 to 6. It contains all the essential information required by specialized team.[21] Furthermore, this classification has been rigorously validated for both ulcer healing and amputation prediction, demonstrating exceptional results and undeniable reliability.[21] The SINBAD system scores 0 or 1 for the site, extent and depth of the ulcer, presence or absence of ischemia, neuropathy, or infection. The total score for each category is one, and the overall score is six.[21]

Risk factors for DFU

Risk factors for DFU can be categorized as patient-related and foot-related[22]. One of the most significant patient-related factors is age.[23] Moreover, literature stated that DFU is significantly associated with males rather than females.[24] Other factors include race and ethnicity,[25] low socioeconomic class,[26] smoking,[27] high body mass index[28] (BMI) and obesity,[28] as well as poor glycemic control[29]**.** Comorbidities associated with high risk and severe outcome of DFU include cardiovascular disordersæ[30] end-stage renal disease,[31] as well as retinopathy.[32]

Literature has investigated the biochemical and radiological findings as predictors for DFU in diabetic patients. For an instance, Caruso[33] concluded that parathyroid hormone and high alkaline phosphatase levels are independent risk factors for DFU. Potential biomarkers that can be helpful in early diagnosis of DFU included procalcitonin, C-reactive protein (CRP), interleukins, TNF-á, arginine, leucine and isoleucine.[34] Moreover, a study found low lymphocyte absolute value and high platelet count to be associated with DFU.[34] Interestingly, a study by Vijaya[35] concluded that LDL cholesterol and cell-surface expression of CD63 on monocytes are predictors of DFU. Furthermore, a study by Katya[36] showed significantly higher levels of blood urea and serum creatinine among DFU patients. In his review, Fujita[37] finalized that methyl glyoxal, adiponectin, semaphorin, and nerve growth factor are biomarkers for diabetic neuropathy that can predict the development of DFU. In addition, Guttikonda[38] stated that high levels of total cholesterol, LDL, and triglycerides were associated with a higher risk of DFU. Interestingly, Xu showed that neutrophil-to-lymphocyte ratio (NLR), serum calcium and albumin levels can be reliably used as predictors for the development and prognosis

of DFU.[39] In a meta-analysis-study investigating the association between vitamin D levels and DFU, lower levels of vitamin D were significantly associated with DFU.[40]

With the global increase in the prevalence of DFU together with the associated morbidity and mortality and the doubtful effectiveness of treatment modalities for DFU, finding a biomarker that can reliably predict DFU as early as possible becomes a must. In the present study, we aimed to investigate the association between serum calcium, vitamin D, and parathyroid hormones and DFU to assess their predictive and prognostic roles in DFU.

MATERIALS AND METHODS

Study type and population

The current observational case-control study was conducted among diabetic patients attending different primary healthcare facilities in Khartoum, Sudan during the period from February-June 2023. Cases included diabetic patients with an established diagnosis of DFU. DFU was graded using the SINBAD system[21]. Scores e" 3 signify a severe ulceration. Control individuals were selected from diabetic patients without DFU.

Sample size

Sample size was calculated based on a 95% confidence interval, a 5% margin of error, an estimated 5% population proportion, assuming a normal sample distribution[41]. The population proportion was taken as 18% based on previous study in Sudan[42]. The following formula[43] was used to calculate the sample size:

$$n = (t\alpha 2 \times p \times q \times N)/((N-1) \times e2+t\alpha 2 \times p \times q)$$

Given that: n = sample size, N = population size, p = expected percentage of the variable, q = 1-p, e = accepted margin of error, tá = 1.96 for 95% confidence interval. Accordingly, A total of 120 diabetic patients with DFU were recruited for the current study. The ratio of the case to control participants was 1:1.

Data collection

Sociodemographic and medical data related to diabetes and DFU were obtained from hospital medical records and by using a validated self-administered questionnaire. The questionnaire was composed of two parts and 10 closed-ended questions. The first part included questions covering the patient's sociodemographic data (age, gender, and smoking). The second part included questions inquiring about the medical history of diabetes and DFU, as well as associated comorbidities. All patients signed informed consent before participating.

Biochemical investigations

About 10 ml of fasting venous blood was collected in an EDTA tube and centrifuged for 10 minutes at 1,000-2,000 x g for plasma and serum extractions. Serum calcium was measured using RayBio® Calcium Colorimetric Assay Kit. The normal range is 8.8 -10.4 mg/dL.[44] Additionally, vitamin D3 level was measured using Elabscience® VitD ELISA kit**.** The normal range is given as 40-60 ng/mL.[45] Regarding renal profile, BUN was measured using DetectX® BUN kit, with a normal range of 5-20 mg/dl**.**[46] For serum creatinine, it was measured using DetectX® Serum Creatinine Kit. The normal range for creatinine is 0.6-1.2 mg/dl.[46] Moreover, total cholesterol, triglyceride, and low-density lipoproteins were measured using Qucare® Multi Meter kit. The normal value for total cholesterol is less than 200 mg/dl.[47] (TAG is less than 150 mg/dl[47]) and LDL is less than 100 mg/dl.[47] Furthermore, CRP was measured using Human CRP Instant ELISA™ Kit (normal value is lower than 0.3 mg/dl[48]). Finally, HbA1C levels were measured using Getein HbA1c fast test kit with the normal value lower than 5.7%.[49] BMI was obtained from the patient's medical records.

Data presentation and analysis

Descriptive data were presented as means and standard deviations for quantitative variables and frequencies for qualitative variables. Results were statistically analysed using the SPSS (18th version). Unpaired T-test was used to compare the mean age, BMI as well as the various biochemical parameters among cases and control subjects. Chi-Square test was used to compare distribution of gender and comorbidities between cases and control subjects. Analysis of variances (NOVA) was used to compare means of serum calcium, vitamin D, and C-reactive protein among groups of patients having mild, moderate and severe DFU. P-value was considered significant when d" 0.05.

Ethical approval

All participants signed an informed consent before recruitment. Ethical approval was

obtained from the ethical committee at College of Medicine, Neelain University, Sudan and Ministry of Health, Sudan.

RESULTS

Sociodemographic findings

The current study included 120 diabetic patients with DFU. The ratio of the case to control participants was 1:1. The mean age was 53.54 years for cases and 46.71 years for control subjects (table 1). As shown in table (2), mean BMI was 29.55kg/m^2 for cases and 25.41 kg/m^2 for control subjects.

The cases included 89 males and 31 females whereas the control subjects included 56 males and 64 females (table 3).

Table 4 shows the distribution of smoking among cases and control subjects. As shown in the table, smokers represented 61.7% of cases and 45.8% of control subjects.

The frequency and percentage of DFU among patients with DFU was shown in chart 1. All cases had SINBAD scores of either 4, 5 or 6 which were considered to be severe ulceration with variable degrees (4 the less severe and 6 the most severe). As shown in the chart, approximately 43% of patients had a score of 6, 29% of patients had a score of 5, and 28% had a score of 4.

Comorbidities

The frequency of retinopathy, hypertension, cardiovascular disease (CVD), and renal diseases among cases and control subjects are shown in table 5. As shown in the table, the most common comorbidity associated with DFU was renal disease and retinopathy.

Biochemical findings

The mean values for serum calcium, vitamin D3, BUN, creatinine, total cholesterol, LDL, TAG, HbA1C and CRP were shown in table 6.

We have also compared the mean vitamin D, serum calcium and CRP levels among groups of patients having grades 4, 5, and 5 of DFU as shown in table 7.

Table 1. SINBAD classification system

Category	Explanation	Score
Ulcer site	Anterior foot	0
	Back and middle foot	1
Ulcer extent	< 1 cm2	0
	> 1 cm2	1
Ulcer deepness	Confined to skin	0
	Extends beyond skin	1
Ischemia	Normal blood flow	0
	Reduced blood flow	1
Neuropathy	Intact sensation	0
	Lost sensation	1
Bacterial infection	No	0
	Present	1
Total score		6

DISCUSSION

The global mortality of DFU is as high as 50% within five years,[50] with ischemic heart disease being the leading cause of mortality associated with DFU.[51] Morbidity associated with DFU is high too, with an approximate rate of recurrence of 65%

Table 2. Mean age and BMI among cases and control subjects

Age (years)	Cases	Controls	Analysis
Count, N:	120	120	
Sum, £x:	6425	5606	Two-tailed p value is less than 0.0001
Mean, x:	53.54	46.72	t = 10.3964
SD	2.3005145	6.8134609	df = 238
SEM	0.2100073	0.621981	Standard error of difference = 0.656
BMI	Cases	Controls	
Count, N:	120	120	The two-tailed P value is less than 0.0001
Sum, £x:	3547	3050	t = 18.8859
Mean, x:	29.55	25.41	df = 238
Variance, s2:	3.0890056	2.6820728	Standard error of difference = 0.219
SD	1.7575567	1.6377035	
SEM	0.1604422	0.1495012	

within 3-5 years[51] and around eight-fold increase in the risk of amputation than non-diabetics.[52] Additionally, around 50% of amputated patients will die within 5 years.[52] The main focus of the present study is to identify sociodemographic and biochemical findings that can predict the incidence of DFU in diabetic patients.

The study revealed that DFU was more prominent among older patients. Middle aged group in the study was not an exclusion.[53] The effect of age on the incidence of DFU has been already established.[23] The effect of age on DFU can be attributed to the long-term cumulative effects of hyperglycemia and advanced end-glycation products (AGEs) with the subsequent modulation of gene expression, production of ROS, and improper functioning of nitric oxide and growth factors.[54] Moreover, the study found that severe and advanced DFU was significantly associated with middle-aged group. Accordingly, we agreed with the conclusion that middle aged patients have more advanced ulceration and a higher rate of hospitalization than elderly patients.[55] DFU is associated with advanced age. However, Shi[56] found that the prognosis of DFU is independent of age. Moreover, elderly patients with DFU still have some probability of healing despite the poor outcome.[57]

Our study found a significant association between DFU and male gender. In this context, we agreed with the previous studies which confirmed a higher risk of DFU in males than in females.[23,58] It's important to consider that sex differences in health outcomes are often shaped by a range of factors such as access to care, screening procedures, and the commitment to following treatment plans.

Table 3. Gender distribution among cases and control subjects

Males		Cases	Controls	Row totals	Analysis
	Observed	89	56	145	Chi-Square Statistic: 18.9735
	Expected	72.5	72.5		df: 1
	Chi Square distribution	3.7552	3.7552		*p*-value: 0.000
Females	Observed	31	64	95	
	Expected	47.5	47.5		
	Chi Square distribution	5.7316	5.7316		
Column totals		120	120	240	

Table 4. Distribution of smoking among cases and control subjects

Smoking		Cases	Controls	Row totals	Analysis
	Observed	74	55	129	Chi-Square Statistic: 6.0507
	Expected	64.5	64.5		df: 1
	Chi Square distribution	1.3992	1.3992		*p*-value: 0.0139
No smoking	Observed	46	65	111	
	Expected	55.5	55.5		
	Chi Square distribution	1.6261	1.6261		
Column totals		120	120	240	

Table 5. Distribution of comorbidities among cases and control subjects

Comorbidity	Cases	Controls	df	Chi Square statistic	p-value
Retinopathy	45	28		5.6894	0.0171
Hypertension	33	24	1	1.8637	0.1722
CVD	7	2		2.886	0.0894
Renal disease	56	33		9.4471	0.0021

Moreover, women were more likely to follow guidelines related to foot care.[59] Furthermore**,** it's crucial to recognize that men with diabetes face a significantly elevated risk of developing peripheral neuropathy (PN), peripheral artery disease (PAD), and cardiovascular disease. This underscores the urgent need for increased awareness and proactive management of these associated health challenges.[60]

Our study suggested an increased risk of DFU with higher BMI. This suggestion was also made by other studies.[28,61] Excessive body fat can affect important biological processes involved in wound healing. For instance, having more fat can lead to reduced blood vessel formation and poor circulation, which can hinder the healing of ulcers. This poor circulation can also result in decreased oxygen delivery, creating an environment that promotes the growth of certain bacteria and fungi.[62]

In the present study, we concluded that smoking is associated with the development of DFU. The study was in an agreement with Conte.[27] This association can be explained based on the fact that smoking negatively impacts glycemic control and increases the production of AGEs and ROS.[63]

The most common comorbidity associated with DFU in the present study was chronic kidney disease. Chronic kidney disease is unequivocally associated with a significantly heightened risk of diabetic foot ulcers (DFU), prolonged healing times, increased recurrence rates, and increased rates of lower extremity amputation.[31] Even the minimal impairment in kidney function was linked to DFU.[64] This finding is quite explainable as peripheral artery disease and chronic kidney disease were considered as one disease from a pathophysiological point of view. In other words, The histopathophysiological processes that link oxidative stress to PAD from one side, and oxidative stress to CKD from the other side, are

Table 6. Mean values for different biochemical findings among cases and control subjects

Parameter	Cases	Controls	*p*-value
Serum calcium	6.17	8.63	0.0001
HBA1C	8.07	5.1	0.0001
Vitamin D3	35.99	55.73	0.0001
BUN	17.28	16.83	0.5424
Creatinine	2.67	1.16	0.0001
Total cholesterol	159.05	158.5	0.9373
LDL	122.2	89.15	0.0001
TAG	125.3	115.65	0.0107
CRP	6.7	0.975	0.0001

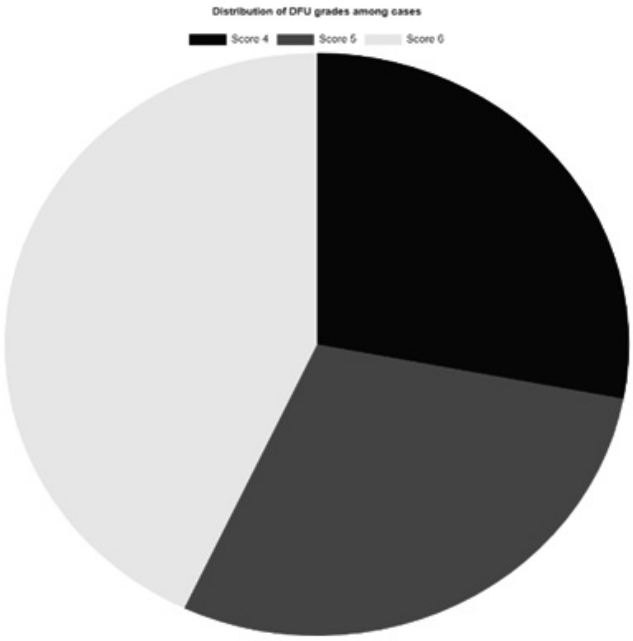

Chart 1. Distribution of DFU grades among cases

Table 7. Mean vitamin D level among different grades of DFU

Grade	n	Mean VD	F-statistic	SD	p-value
4	35	35.0606	4.6754	5.6399	0.0112
5	33	35.1818		2.0533	
6	52	33.2115		1.7415	
Grade	n	Mean calcium	F-statistic	SD	p-value
4	35	6.0857	3.1016	0.8179	0.0487
5	33	6.2424		0.7084	
6	52	6.4528		0.574	
Grade	n	Mean calcium	F-statistic	SD	p-value
4	35	5.7353	3.1241	0.7511	0.0477
5	33	5.7188		0.7719	
6	52	6.0943		0.8381	

similar.[64] Proteinuria, lower extremity edema, malnutrition and renal dialysis are key factors that directly contribute to the occurrence and exacerbation of DFU, significantly increasing the complexity of condition65. The second most common morbidity associated with DFU in our study was retinopathy. Again, our study was consistent with other studies in this regard.[22] DFU and retinopathy serve as strong indicators of advanced microvascular disease, offering compelling evidence that may help elucidate this association.[22] In fact, the oxidative stress and endothelial dysfunction responsible for retinopathy also contributes to DFU.[66] In contrast to our study, Hwang[67] did not find a significant association with retinopathy. Unlike other studies that found a strong association linking DFU to hypertension[68] and cardiovascular diseases,[30] the present did not a significant association.

The present study supported a strong association between DFU and abnormally high levels of HBA1C, suggesting an association with poor glycemic control. Accordingly, the study was consistent with previous studies in this regard.[29] The cumulative effects of hyperglycemia and the related microvascular complications are cornerstones in the firm establishment of this connection.[29]

Our study revealed a significant association between CRP and DFU. Furthermore, we reported the highest levels of CRP among the group of patients with grade 6 OF DFU. In fact, CRP was considered as the most significant inflammatory marker to be associated with DFU.[69] The value of CRP in predicting, diagnosing, as well as determining the prognosis of DFU relies on being a highly sensitive acute phase protein inflammatory marker.[69] Moreover**,** Sharma[70] considered CRP and procalcitonin to be the best markers for DFU.

With regards to lipid profile, our study concluded that high levels of LDL and TAG were significantly associated with DFU. This finding supported Guttikonda[38] who stated that high levels of total cholesterol, LDL, and triglycerides were associated with a higher risk of DFU. These lipids have a direct link to the development of atherosclerosis, which significantly impedes blood flow to the lower limbs and substantially raises the risk of developing foot ulcers.[71]

With regards to renal function profile, our study found a significant association between DFU and elevated serum creatinine, supporting Fujita[37] and other studies. According to one study,[72] nephropathy was considered to be a contributing factor in impaired microcirculation accompanying DFU. In fact, high serum creatinine signifies a chronic kidney disease, which was already proved to predict the development of DFU.[31,64]

Interestingly, the present study has significantly and successfully linked low levels of vitamin D to DFU. Moreover, we found that the lowest levels of vitamin D were significantly associated with grade greater severity of DFU. In his meta-analysis study, Dai[1] studies reported low serum vitamin D levels among patients with DFU. The exact mechanism behind the link between low vitamin D level and DFU is not fully understood. Vitamin D deficiency was linked to several chronic disorders including cardiovascular diseases, metabolic disorders, autoimmune and infectious diseases, cancer and

diabetes mellitus.[53] The active form of vitamin D, vitamin D3 or dihydroxycholecalciferol is thought to affect the expression of several genes involved in regulation of immune function and promoting insulin sensitivity and pancreatic function.[53] The vitamin inhibits the release of inflammatory mediators and proinflammatory cytokines, thereby minimizing inflammation. Additionally, it modifies both cellular and humoral immunity.[73] In his meta-analysis study, Li[40] has proposed several theories explaining the negative effect of low vitamin D levels on DFU. These theories highlighted the positive effect of vitamin D on the glycemic control , endothelial system, and immune functioning.

Several studies reported low serum calcium levels among patients with DFU. In the present study, patients with grade 6 of DFU displayed lower levels of serum calcium than groups with grade 1 or 2. In his study, Xu[39] related low serum calcium levels to DFU, amputation and death. The study considered serum calcium as an inflammatory marker that decreases as the inflammation progresses. As a micronutrient, calcium is essential for promoting effective wound healing via its powerful antioxidant and anti-inflammatory properties, coupled with its role in stabilizing collagen and regulating cell growth and differentiation.[74]

Limitations

The study has several limitations. First, the sample size was relatively small. Second, we could not assess other important molecules like other minerals and ptoteins that might have important roles in the process of wound healing . Finally, the study was conducted in local health care facilities in Khartoum City, which renders generalization to the entire population illogical.

CONCLUSION

In conclusion, the current study found a significant association between low levels of serum calcium and vitamin D with DFU. Moreover, the study reported significantly higher levels of HbA1C, CRP, serum creatinine, LDL, and TAG among patients with DFU. patients with severe DFU have the lowest levels of serum calcium and vitamin D, and the highest level of CRP.

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Conflict of interests

The author(s) do not have any conflict of interest.

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Informed Consent Statement

All participants signed an informed consent before recruitment.

Clinical Trial Registration

This research does not involve any clinical trials.

Author contribution

The authors confirm contribution to the paper as follows: Study conception and design: Nasir Abdelrafie Hamad; Data collection: Nasir Abdelrafie Hamad; Analysis and interpretation of results: Habab Merghani Yassin; Draft manuscript preparation: Lienda Bashier Eltayeb.

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