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ORIGINAL RESEARCH

25-(OH)D Deficiency and the Risk of Minor Amputation in Patients with Diabetes Foot Ulcers: A Chinese Cohort Study

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Background: Diabetic foot amputation represents the severe complication of patients with diabetic foot ulcers (DFU), with worsened clinical outcomes concomitant with 25-hydroxyvitamin D [25-(OH)D] deficiency. This study aimed to investigate the value of 25-(OH)D deficiency and its association with minor lower limb amputation in Chinese patients with DFU in type 2 diabetes mellitus (T2DM).

Methods: Ninety-one T2DM patients with DFU undergoing minor amputation (surgical group), 94 T2DM patients without DFU (diabetic control group), and 40 healthy controls were enrolled in the study finally. Serum 25-(OH)D levels were measured by stable isotope dilution-high performance liquid chromatography-tandem mass spectrometry. Patients in the surgical group underwent pathogen detection.

Results: The overall incidence of 25-(OH)D deficiency was 73.0%. Amputation group exhibited significantly higher prevalence versus control group (85.7% vs 60.6%, $P<0.001$). The levels of serum 25-(OH)D in the minor amputation group were significantly lower than the T2DM group [10.71 (8.2,16.7) vs 18.8 (15.0, 23.9) ng/mL, $P<0.001$]. Compared with the control group, amputation group showed lower 25-(OH)D, calcium, albumin and higher WBC, platelet, inflammatory markers (all $P<0.001$). Minor amputation subgroups showed that 25-(OH)D deficiency associated with elevated WBC ($P=0.002$), PLT ($P=0.011$), D-dimer ($P=0.041$), HbA1c ($P=0.033$), SII ($P=0.023$). While low expression of 25-(OH)D group had higher WBC ($P=0.016$), D-dimer ($P=0.004$), SIRI ($P=0.042$), lower albumin ($P=0.009$) vs high expression group. ROC analysis confirmed 25-(OH)D as superior amputation predictor (AUC=0.798, 95% CI 0.734–0.861) compared to inflammatory markers. Multivariate regression identified deficiency as independent amputation risk factor. Polymicrobial infections (26.4%) correlated with lower 25-(OH)D ($P=0.003$), with Gram-negative (52.4%) and Gram-positive bacteria (47.6%) among bacterial infections.

Conclusion: 25-(OH)D deficiency occurs in 85.7% of patients with minor amputation in DFU, and the levels of 25-(OH)D decreased. 25-(OH)D deficiency is a vital risk factor for minor amputation and measures should be taken to prevent 25-(OH)D deficiency in T2DM patients with DFU.

Keywords: 25-(OH)D deficiency, diabetic foot ulcers, minor amputation, type 2 diabetes mellitus

Introduction

Diabetic foot ulcer (DFU), the most common chronic complication of diabetes, typically arises from distal neuropathy or lower limb vascular disease, resulting in skin ulcers and deep tissue destruction in the feet and ankles.[1] Surgical debridement, weight-bearing pressure reduction, improvement of lower limb ischemia, anti-infective treatment, and early referral to multidisciplinary treatment are vital treatment measures for DFU.[2] Alarmingly, approximately 20% of DFU patients will ultimately undergo lower limb amputation.[3] Moreover, 85% of diabetes-related lower limb amputations originate from DFU.[4] This condition imposes substantial medical, economic, and social burdens on patients, families, and society. Reflecting this burden in China, the average total cost per patient with DFU soared from RMB

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15,535.58 in 2014 to RMB 42,040.60 in 2020.[5] Consequently, preventing amputations has become a pivotal goal in the comprehensive management of type 2 diabetes mellitus (T2DM).

Vitamin D deficiency remains a significant public health concern, particularly in China where its prevalence reaches 34.3%.[6] Vitamin D is not only essential for calcium and phosphorus metabolism, but also participates in suppressing inflammation and autoimmune responses, alleviating insulin resistance, and enhancing insulin synthesis and secretion.[7,8] Preclinical studies demonstrate that 25-(OH)D exerts multiple effects, specifically protecting β cells via attenuation of oxidative stress, inhibiting inflammation through regulation of pancreatic stellate cells, and enhancing insulin signaling by promoting receptor gene transcription.[9–11] Consequently, Vitamin D deficiency may reduce insulin sensitivity and exacerbates glucose dysregulation, increasing the risk of diabetic complications.[12] Notably, Vitamin D plays a direct role in the occurrence and development of diabetic microvascular and macrovascular complications. Observational studies reveal that decreased serum 25-(OH)D concentrations correlate with increased incidence of both microvascular and macrovascular diabetic complications.[13] In microvascular pathophysiology (retinopathy, neuropathy, nephropathy), Vitamin D deficiency potentiates endothelial dysfunction through upregulated pro-inflammatory cytokines (IL-6, TNF-α) and dysregulated angiogenesis via impaired VEGF signaling pathway.[14–17]Regarding macrovascular complications (such as myocardial infarction and stroke), insufficient 25-(OH)D levels associate with increased arterial calcification, impaired nitric oxide-mediated vasodilation, and hyperactivation of the renin-angiotensin-aldosterone system (RAAS), accelerating atherosclerosis progression.[18] Current evidence suggests that maintaining adequate vitamin D status is beneficial to prevent diabetic vascular complications.

Vitamin D deficiency may further elevate DFU risk by impairing immune function and delaying ulcer healing, potentially culminating in amputation. Serum 25-(OH)D levels negatively correlate with novel inflammatory markers,[19] such as the platelet-to-lymphocyte ratio (PLR) and neutrophil-to-lymphocyte ratio (NLR), which are reliable predictors of post-amputation mortality.[20] 25-(OH)D enhances wound healing through stimulating cell differentiation, suppressing hyperproliferation, and modulating critical repair mechanisms. Adequate 25-(OH)D concentrations not only curb pro-inflammatory cytokines and boost anti-inflammatory responses to fortify infection defense, but also improve endothelial function to enhance foot perfusion and accelerate tissue repair.[21] For example, vitamin D can induce antimicrobial peptides (AMPs) in DFU cells, strengthening skin barrier function and facilitating ulcer healing.

Despite possible associations between 25-(OH)D and DFU, data on 25-(OH)D deficiency and DFU minor amputation in Chinese diabetic patients remain scarce. Therefore, this study aimed to compare 25-(OH)D levels in Chinese patients with DFU minor amputation and controls and to explore the role of 25-(OH)D deficiency in DFU and its association with minor amputation.

Materials and Methods

Patients and Samples

This study retrospectively analyzed the clinical characteristics of 185 patients with T2DM hospitalized in the Second Affiliated Hospital of Soochow University from January 2023 to June 2024. All patients in the control group met the following inclusion criteria: (1) Diagnosis of type 2 diabetes mellitus; (2) Age above 18 years; (3) Complete and reliable clinical history and clinical data; (4) Serum 25-(OH)D level was measured during hospitalization; (5) Follow-up data. All patients in the surgical group met the following inclusion criteria: (1) Diagnosis of DFU as defined by the International Working Group on the Diabetic Foot (IWGDF); (2) Diagnosis of type 2 diabetes mellitus; (3) Age above 18 years; (4) Complete and reliable clinical history and clinical data; (5) Serum 25-(OH)D level was measured during hospitalization; (6) Follow-up data. While the exclusion criteria included: (1) Diagnosis of type 1 diabetes; (2) Patients with severe systemic immunodeficiency; (3) Patients with malignant tumors; (4) Patients who died during hospitalization. Ninety-four patients without DFU in the control group received general treatment for diabetes in the endocrinology department and the other ninety-one patients with DFU in the surgical group received minor lower limb amputation in the orthopedics department. Besides, 40 healthy controls were included. This study was approved by the Second Affiliated Hospital of Soochow University. Written informed consent was obtained from all patients in accordance with the Declaration of Helsinki.

Data Collection

General clinical characteristics including sex, age, height, weight, medical history and compliance were directly extracted from electronic medical record system. Laboratory data were obtained within 24 hours of hospitalization, included white blood cell (WBC), neutrophil count, lymphocyte count, monocyte count, platelet (PLT) count, C-reactive protein (CRP), D-dimer, serum albumin (ALB), serum creatine (Scr), serum calcium, serum phosphorus, glycosylated hemoglobin (HbA1c), and 25-(OH)D. In addition, neutrophil to lymphocyte ratio (NLR), platelet to lymphocyte ratio (PLR), systemic inflammatory index (SII), and systemic inflammatory response index (SIRI) was calculated, respectively. Estimated glomerular filtration rate (eGFR) was calculated using the abbreviated Modification of Diet in Renal Disease (MDRD) equation: $186\times(\text{Scr})^{-1.154}\times(\text{age})^{-0.203}\times(0.742 \text{ if female})$.

25-(OH)D Measurements

After hospitalization, fasting blood samples from patients were collected after hospitalization and centrifuged at 4000 r/min for 10 min to separate the serum, which was used to measure 25-(OH)D2 and 25-(OH)D3 levels. Stable isotope dilution-high performance liquid chromatography-tandem mass spectrometry was used to detect the levels of 25-(OH)D2 and 25-(OH)D3, and the sum of the two was taken as the total serum 25-(OH)D level. Meanwhile, we supplemented the matching samples of serum calcium, phosphorus and renal function. The normal range of 25-(OH)D is 20.0–100.0 ng/mL, and 25-(OH)D deficiency is defined as serum 25-(OH)D levels below the lower limit of normal range.

Pathogen Detection

The lesion site was cleaned and rinsed with sterile saline. Secretions, lesion tissue or bone fragments were scraped from the wound base and deep sinus tract with cotton swabs during the surgery. The specimens were immediately sent to clinical laboratory center for bacterial identification through routine culture methods and drug sensitivity analysis.

Statistical Analysis

Statistical analysis was performed in SPSS 27.0 (Armonk, NY, USA) software and GraphPad software 9.0 (La Jolla, CA, USA). The Kolmogorov–Smirnov test was used to determine the normality of continuous variables. Normally distributed variables are reported as mean ± standard deviation (SD), while non-normally distributed variables are expressed as median (interquartile range, IQR). Categorical variables were presented as absolute frequencies with relative proportions N (%). Homogeneity of variance was determined using the Levene test. Student's *t* test was applied to analyze normally distributed variables with homogeneity of variance, while Mann–Whitney *U*-test was used to analyze variables with non-normal distribution or heterogeneity of variance. Categorical variables were analyzed by the chi-square test. Receiver operating characteristic (ROC) curve analysis with area under the curve (AUC) quantification was performed for diagnostic accuracy evaluation. Logistic regression analysis was used to identify the interrelationships between the variables. A two-tailed $P<0.05$ defined statistical significance for all analyses.

Results

Clinical Characteristics of Patients with T2DM Enrolled in This Study

In total, 185 patients with T2DM and 40 healthy donors were retrospectively enrolled in this study. As is shown in Figure 1A, 25-(OH)D levels in patients with diabetes decreased compared with normal controls ($P<0.001$). The median age of the study was 64 years (range: 22–89 years), of which 58.4% were elderly people over 60 years old. The entire T2DM cohort was divided into two groups. The control group consisted of 94 patients diagnosed T2DM without DFU, while the surgical group consisted of patients with DFU accompanied by minor amputation. Male patients predominated, with a male to female ratio of 2.96:1 in the surgical group and 1.14:1 in the control group ($P=0.002$). The median age was 60 years (range: 28–79 years) in the surgical group and 66 years (range: 22–89 years) in the control group. The comparison of the baseline clinical characteristics of the two groups is shown in Table 1. Compared with the control group, the patients in the surgical group showed significantly lower 25-(OH)D levels ($P<0.001$), serum calcium ($P<0.001$), and albumin levels (all $P<0.001$). Meanwhile, the white blood cell count, platelet count, and inflammatory

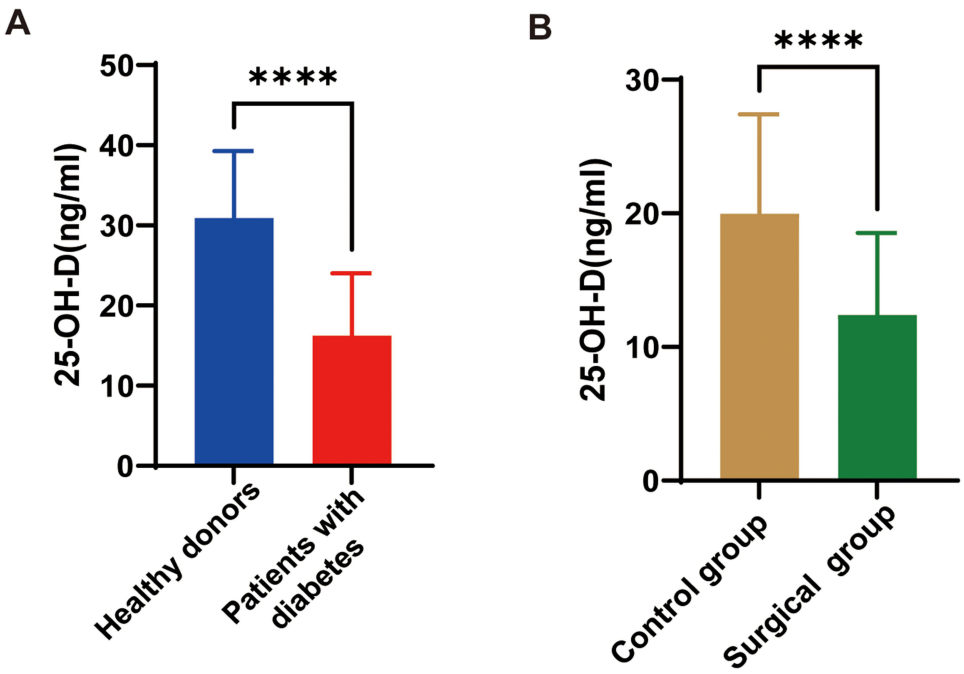

Figure 1 (**A**) 25-(OH)D levels of type 2 diabetes mellitus (T2DM) group and in healthy donor group. (**B**). 25-(OH)D levels of patients in the control group and surgical group. **** means that *P* value <0.001.

markers (including CRP, NLR, PLR, SII, and SIRI) in the surgical group were significantly higher than those in the control group (all $P<0.001$). Glycosylated hemoglobin is an important indicator reflecting short-term blood sugar control. The proportion of patients with HbA1c>7% in the two groups was similar (79.8% vs 73.6%), reflecting the poor blood

Table 1 Clinical Characteristics of 185 Patients with Diabetes in the Study

Characteristics	Control Group (N=94)	Surgical Group (N=91)	*P*-Value
Gender			**0.002****
Male	50 (53.2%)	68 (74.7%)	
Female	44 (46.8%)	23 (25.3%)	
Age (year)	64(55,71)	60(53,58)	**0.011***
WBC (× 10^9)	5.9(4.8,7.0)	7.6(5.3,10.8)	**<0.001*****
PLT (× 10^9)	199.5(172,238)	266(199,340)	**<0.001*****
CRP (mg/L)	5.0(1.9,5.3)	18.2(5.0,83.9)	**<0.001*****
LDH (U/L)	166(145,189)	164(145,187)	0.826
25-(OH)VD (ng/mL)	18.8 (15.0, 23.9)	10.71 (8.2,16.7)	**<0.001*****
BMI (kg/m^2)			0.078
High/Very high	44 (46.8%)	31 (34.1%)	
Low/Intermediate	50 (53.2%)	60(65.9%)	
ALB (g/L)	42.1±3.9	35.1±5.7	**<0.001*****
Serum calcium (mmol/L)	2.2±0.1	2.1±0.1	**<0.001*****
Serum phosphorus (mmol/L)	1.2±0.2	2.2±0.2	0.646
eGFR (mL/min/1.73 m^2)	87.9±28.1	81.8±37.9	0.176
NLR	2.1(1.6,2.6)	3.6(2.3,6.6)	**<0.001*****
PLR	124.1(94.8,155.8)	187.5(143.3,258.8)	**<0.001*****
SII	440.3(278.1,616.1)	944.0(513.2,2168.1)	**<0.001*****

(*Continued*)

Table 1 (Continued).

Characteristics	Control Group (N=94)	Surgical Group (N=91)	*P*-Value
SIRI	0.84(0.6,1.3)	1.5(0.8,4.9)	**<0.001*****
HbA1c (%)			0.321
≥7%	75 (79.8%)	67 (73.6%)	
<7%	19 (20.2%)	24 (26.4%)	

Notes: *P* values in bold represent variables with statistically differences. *, **, *** means that *p* value <0.5,0.01 and 0.001, respectively.

Abbreviations: WBC, white blood cell count; PLT, platelet; CRP, C-reactive protein; LDH, lactate dehydrogenase; BMI, body mass index; ALB, albumin; eGFR, estimated glomerular filtration rate; NLR, neutrophil to lymphocyte ratio; PLR, platelet to lymphocyte ratio; SII, systemic inflammatory index; SIRI, systemic inflammatory response index; HbA1c, glycosylated hemoglobinA1c.

sugar control, but no statistical difference was observed between the two groups (P=0.321). In addition, we found no significant differences in LDH, serum phosphorus, eGFR, and BMI.

The overall incidence of 25-(OH)D deficiency (defined as the value <20 ng/mL) was 73.0%. Amputation group exhibited significantly higher prevalence versus control group (85.7% vs 60.6%, P<0.001), indicating that 25-(OH)D deficiency was more common and severe in the surgical group. As is shown in Figure 1B, the median 25-(OH)D level in the surgical group (10.71 ng/mL; range: 2.47–29.78 ng/mL) was significantly lower than that in the control group (18.98 ng/mL; range: 10.30–51.24 ng/mL) (P<0.001). This further confirms that the significant decrease in Vitamin D levels is associated with the risk of DFU and subsequent amputation in diabetic patients.

Comparison of the Correlation Between 25-(OH)D Deficiency, Serum 25-(OH)D Levels and Clinical Characteristics in the DFU Surgical Group

We systematically analyzed the effects of 25-(OH)D deficiency and 25-(OH)D levels on patients with DFU in the surgical group. Based on the standard vitamin D deficiency threshold (serum 25(OH)D <20 ng/mL), surgical patients were stratified into 25-(OH)D normal group (N=13) and 25-(OH)D deficiency group (N=78). The analysis revealed that 25-(OH)D deficiency was not significantly associated with gender, age, CRP, LDH, BMI, albumin levels, eGFR, or other inflammatory markers (all P >0.05), except for SII. However, significant differences were found with elevated WBC count (P =0.002), platelet count (P=0.011), D-dimer (P=0.041), HbA1c (P=0.033), and SII (P=0.023) (Table 2), indicating a potential link between vitamin D deficiency and abnormalities in infection control or glucose metabolism in DFU. To further investigate the clinical relevance, we utilized the median of 25-(OH)D levels (10.71ng/mL) as the optimal cutoff value to stratify patients in the surgical group. Subgroup analysis of minor amputation cases demonstrated that the low 25-(OH)D group exhibited significantly higher WBC (P=0.016), D-dimer (P=0.004), and SIRI (P=0.042), along with lower albumin levels (P=0.009) compared to the high-expression group (Table 3). This also reinforced the

Table 2 Relationship Between Clinical Characteristics and 25-(OH)D Deficiency in 91 DFU Patients with Minor Amputation

Characteristics	25-(OH)D Deficiency Group (N=78)	25-(OH)D Normal Group (N=13)	*P*-Value
Gender (Male)	58 (85.3%)	10 (14.7%)	0.844
Age (year)	60.5(53,68)	60(57,68)	0.678
WBC ($\times 10^9$)	7.8(5.7,11.5)	4.7(3.8,7.4)	**0.002****
PLT ($\times 10^9$)	266.5(212,352)	175(148,212)	**0.011***

(*Continued*)

Table 2 (Continued).

Characteristics	25-(OH)D Deficiency Group (N=78)	25-(OH)D Normal Group (N=13)	*P*-Value
CRP>3.5 mg/L	68 (87.2%)	10 (12.8%)	0.328
LDH (U/L)	266.5(213,352)	168(137,186)	0.892
BMI≥24	26 (83.9%)	5 (16.1%)	0.718
D-dimer>0.5μg/mL	53 (91.4%)	5 (8.6%)	**0.041***
ALB (g/L)	34.7±5.8	37.5±4.7	0.098
eGFR (mL/min/1.73 m^2)	93.5(65.8,105.3)	87(26.5,102.0)	0.496
NLR	3.6(2.2,7.3)	3.4(2.6,4.1)	0.379
PLR	199.8(143.4,262.2)	175.0(145.0,212,5)	0.315
SII	978.0(570.8,2268.1)	600.0(389.9,1050.0)	**0.023***
SIRI	1.6(0.9,5.3)	0.9(0.6,2.4)	0.111
HbA1c (%)	8.3(7.1,10.1)	6.2(5.4,9.2)	**0.033***

Notes: *P* values in bold represent variables with statistically differences. *, ** means that *p* value <0.5 and 0.01, respectively.
Abbreviations: WBC, white blood cell count; PLT, platelet; CRP, C-reactive protein; LDH, lactate dehydrogenase; BMI, body mass index; ALB, albumin; eGFR, estimated glomerular filtration rate; NLR, neutrophil to lymphocyte ratio; PLR, platelet to lymphocyte ratio; SII, systemic inflammatory index; SIRI, systemic inflammatory response index; HbA1c, glycosylated hemoglobinA1c.

Table 3 Clinical Characteristics of 91 Patients with Minor Amputation in Relation to the Level of 25-(OH)D

Clinical Characteristics	Patients (%)	High Level Group (%) N=26	Low Level Group (%) N=65	*P*-Value
Gender (male)	68 (74.7)	23 (33.8)	45(66.2)	0.057
Age>60 years	45 (49.5)	15 (33.3)	30 (66.7)	0.320
WBC>9.5*10^9	29 (31.9)	2 (6.9)	27 (93.1)	**0.002**
NLR>4	36 (39.6)	2 (5.6)	34 (94.4)	**0.003**
CRP>3.5 mg/L	78 (85.7)	20 (25.6)	58 (74.4)	0.130
D-dimer>0.5 μg/mL	58 (63.7)	53 (91.4)	5 (8.6)	**0.007**
HbA1c≥7%	69 (75.8)	16 (23.2)	53 (76.8)	**0.044**
BMI index≥24	31 (34.1)	10 (32.3)	21 (67.7)	0.576
25-(OH)D deficiency	78(85.7)	-	-	-

Note: P values in bold represent variables with statistically differences.
Abbreviations: WBC, white cell count; NLR, neutrophil to lymphocyte ratio; BMI, body mass index.

association between hypovitaminosis D and systemic inflammatory. Overall, the results revealed consistent trend with 25-(OH)D deficiency.

ROC curve analysis demonstrated superior diagnostic accuracy of serum 25-(OH)D for predicting minor amputation risk in DFU patients compared to inflammatory markers. 25-(OH)D achieved the highest discrimination (AUC=0.798; 95% CI 0.734–0.861) with optimal cutoff=11.12 ng/mL ($P< 0.05$). By comparison, the predictive performance of the inflammatory markers NLR, PLR, SII, and SIRI was as follows: PLR: AUC=0.782 (95% CI: 0.715–0.849), SII: AUC=0.779 (95% CI: 0.711–0.846), NLR: AUC=0.748 (95% CI: 0.677–0.820), SIRI: AUC=0.695 (95% CI: 0.621–0.774) (Figure 2). These results establish Serum 25-(OH)D as potential biomarker for minor amputation risk stratification in DFU.

Through univariate and multivariate regression analysis, potential influencing factors affecting the amputation group were screened out. In the univariate analysis, age>60 years, male, elevated WBC, NLR, CRP, elevated D-dimer, HbA1c>7, and 25-(OH)D deficiency were statistically significantly associated with surgery. Then these factors were included in the multivariate analysis, the six variables of age, gender, CRP, HbA1c, D-dimer and 25-(OH)D deficiency

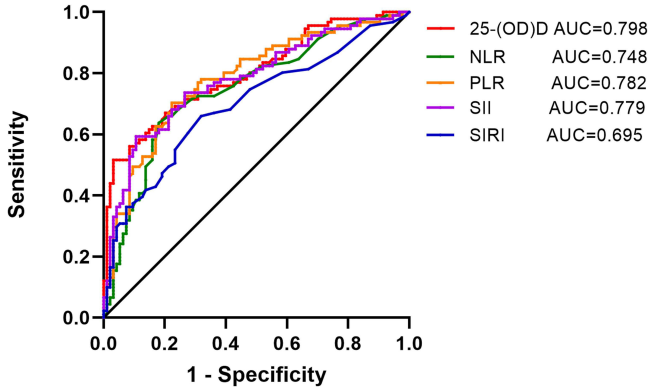

Figure 2 The ROC curve of 25-(OH)D, NLR, PLR, SII, and SIRI to indicate minor amputation in DFU patients.

were significantly associated with amputation (Table 4). In summary, 25-(OH)D deficiency is a risk factor for minor amputation in DFU.

Pathogen Distribution and Association Between 25-(OH)D Levels and DFU in the Surgical Group

All patients' specimens were collected during the operation for culture and pathogen detection. In order to clarify the etiology of tissue infection, we further compared the pathogen spectrum. As detailed in Table 5, bacterial or fungal pathogens were identified in 93.4% of surgical specimens, with sterile cultures observed in 6.6% of cases. Bacterial

Table 4 Univariate and Multivariate Logistic Regression Analysis of Patients

Clinical Characteristics	Univariate Analysis			Multivariate Analysis		
	OR	95% CI	*P* Value	OR	95% CI	*P* Value
Gender (male)	2.602	1.396–4.849	**0.003**	2.709	1.199–6.121	**0.017***
Age>60 years	0.481	0.266–0.873	**0.016**	0.443	0.205–0.955	**0.038***
WBC>Normal	6.860	2.687–17.513	**<0.001**	5.252	1.359–20.290	**0.016***
CRP>3.5 mg/L	2.812	1.355–5.836	**0.005**	2.308	0.968–5.503	0.059
D-dimer>Normal	6.503	3.384–12.496	**<0.001**	6.439	2.895–14.325	**<0.001****
HbA1c≥7%	4.394	1.739–11.099	**0.002**	0.223	0.089–0.560	**0.001****
BMI index≥24	0.587	0.324–1.063	0.079	-	-	-
25- (OH)D	3.895	1.899–7.988	**<0.001**	3.166	1.294–7.744	**0.012***

Notes: *P* values in bold represent variables with statistically differences. *, ** means that *p* value <0.5 and 0.01, respectively.

Abbreviations: WBC, white blood cell count; CRP, C-reactive protein; BMI, body mass index; HbA1c, glycosylated hemoglobinA1c; OR, Odds Ratio; CI, Confidence Interval.

Table 5 Distribution of Characteristics of Pathogen Spectrum Features in the Minor Amputation Group

Pathogen Spectrum	Total (N)	Percentage (%)
Negative	6	6.6%
G+	39	42.9%
Staphylococcus aureus	13	14.3%
Streptococcus	11	12.1%
Enterococcus	11	12.1%
Others	4	4.4%

(*Continued*)

Table 5 (Continued).

Pathogen Spectrum	Total (N)	Percentage (%)
G-	43	47.2%
Pseudomonas aeruginosa	10	11.0%
Escherichia coli	14	15.4%
Proteus	6	6.6%
Klebsiella	2	2.2%
Acinetobacter baumannii	1	1.1%
Others	9	9.9%
Fungi	3	3.3%
Candida parapsilosis	2	2.2%
Candida albicans	1	1.1%

species predominated (96.5%) while fungal species accounted for 3.5% among pathogen-positive cases. Among bacterial infections, Gram-negative bacilli accounted for 52.4%, primarily represented by *Pseudomonas aeruginos*a, while Gram-positive cocci accounted for 47.6%, with *Staphylococcus aureus* as the predominant isolate. Moreover, 24 patients (26.4%) had complicated infection (two or more pathogens). The specific pathogens are shown in Figure 3A.

We further compared the 25-(OH)D levels in the complicated infection group with those in the single pathogen or no infection group and found that the level of 25-(OH)D was significantly lower in the complicated infection group ($P<0.001$) in Figure 3B as expected. As for treatment, patients in the surgical received systemic antibiotic therapy based on the antimicrobial susceptibility profiles. Moreover, Vancomycin-impregnated bone cement implantation and Negative pressure wound therapy (NPWT) were implemented beyond minor amputations for patients with osteomyelitis. The preoperative and postoperative conditions of minor lower limb amputation group were presented in Figure 4.

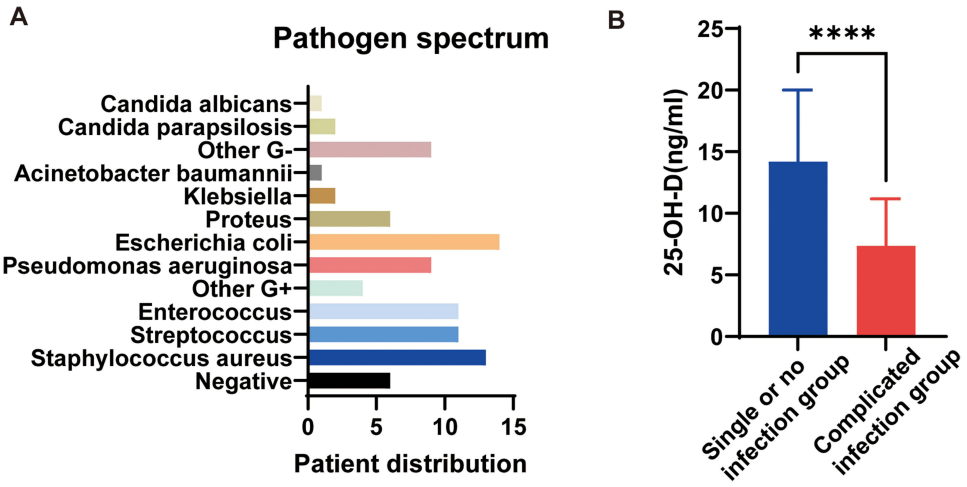

Figure 3 (**A**) Distribution of pathogens in DFU patients in the surgical group. (**B**) Differences in 25-(OH)D levels between patients with single or no infection and patients with complicated infection in the surgical group. ****means that *P* value <0.001.

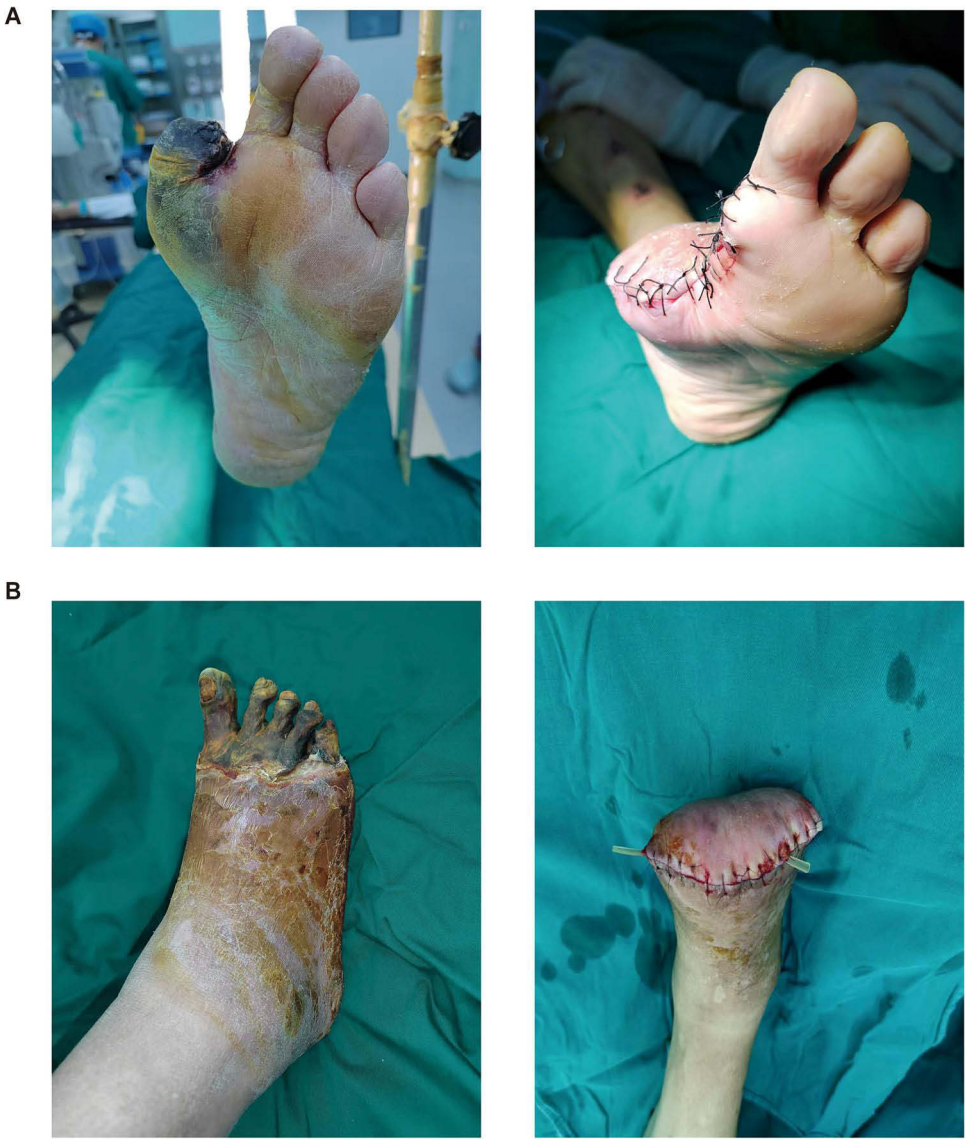

Figure 4 Preoperative and postoperative conditions of two DFU patients with minor lower limb amputation in the surgical group (**A** and **B**).

Discussion

25-(OH)D deficiency adversely affects the progression of diabetes and its complications, especially DFU. Because of amputation represents one of the most severe outcomes of DFU, understanding the role of Vitamin D in this pathologic cascade is critical. In this study, we analyzed the clinical characteristics of 185 diabetic patients stratified by 25-(OH)D levels and explored the relationship between 25-(OH)D deficiency and amputation risk of DFU.

Our data reveal markedly depleted serum 25-(OH)D levels in T2DM versus healthy controls, with further reduction in DFU patients relative to non-DFU diabetics (median: 10.71 vs 18.98 ng/mL, $P<0.001$). This deficiency of 25-(OH)D correlates with diabetes progression and DFU complications, confirming prior epidemiological patterns.[22–24]Vitamin D deficiency correlated with elevated amputation risk in the surgical group, consistent with other cohort studies showing higher all-cause mortality in DFU patients with hypovitaminosis D and increased amputation rates in peripheral arterial disease cohorts.[25,26] Several studies have shown that the incidence of Vitamin D deficiency in patients with DFU is significantly higher than that in ordinary diabetic patients or diabetic patients without foot ulcers.[23,27] This study revealed Vitamin D deficiency in 85.7% of amputated DFU patients, aligning with published deficiency rates (55.7–86.8%),[28–30] confirming its strong association with DFU severity. Retrospective analyses indicate that 25-(OH)D insufficiency

elevates osteomyelitis risk.[31] Consistently, our minor amputation cases progressed to Wagner grade of 3–4 with concurrent osteomyelitis. This condition may be exacerbated by 25-(OH)D deficiency through disruption of calcium homeostasis and impaired bone remodeling. Mechanistically, related studies have shown that Vitamin D levels are negatively correlated with ulcer severity and healing time, which may be related to the fact that Vitamin D deficiency weakens the function of immune cells or affects vascular endothelial repair, aggravates inflammatory response, enhance microcirculation disorders, and delays ulcer healing. Overall, these findings underscore that measures should be taken to prevent vitamin D deficiency in T2DM patients to reduce the risk of potential complications, especially the risk of amputation in DFU.

25(OH)D deficiency amplifies infection-driven inflammation through dysregulated myeloid activation, correlating with elevated markers across multiple hematologic axes. In this study, patients in the surgical group were diagnosed with osteomyelitis by imaging and eventually performed minor amputation of lower limb. We included several easily accessible inflammatory biomarkers to assess infection of DFU and found that patients undergoing minor amputation demonstrated significantly elevated systemic inflammatory markers than those in the control group, including WBC, CRP, NLR, PLR, SII, and SIRI. In the surgical DFU cohort, 25-(OH)D deficiency was associated with increased WBC and SII and the low 25-(OH)D expression group was significantly related to elevated WBC and SIRI. These findings indicate that composite inflammatory indices may offer superior amputation risk stratification over isolated WBC evaluation. Su et al proposed that NLR can objectively reflect the inflammatory indicators of patients with DFU and the higher the preoperative NLR value may indicate worse disease outcomes in patients with diabetic foot, including amputation and death.[32] Combined with the data in our amputation group, we tend to believe that different inflammatory markers may be more valuable for the prognosis of patients with DFU. In subsequent studies, we can also include novel indicators such as PHR and NHR to further explore the in-depth relationship between infection and 25-(OH)D deficiency in the diabetic foot amputation group, which were also explored in other studies.[19]

Given that the microbiota of DFU wounds presents dynamic changes, timely and accurate identification of pathogenic bacteria in clinical practice is a favorable factor for implementing precise and individualized treatment. Pathogen analysis revealed polymicrobial infections in 26.4% of DFU cases, predominantly *Staphylococcus* aureus and *Escherichia coli*.[33,34] Patients with polymicrobial infections exhibited lower 25-(OH)D levels compared to those with single-pathogen or no infections. Severe infections in DFU patients with 25-(OH)D deficiency may be attributable to arise from interdependent immunological and metabolic disruptions. For example, the lower level of 25(OH)D is associated with impaired innate immune defense, macrophage dysfunction, cytokine dysregulation, enhanced pathogen virulence, and increased risk of refractory osteomyelitis, thereby increasing susceptibility to systemic infection.[31,35,36] While conventional culture methods often miss pathogens in severe cases of DFU, metagenomic next-generation sequencing (mNGS) significantly enhances detection sensitivity, underlining its value for managing recalcitrant infections.[37] Despite its efficacy, the high cost of mNGS limits widespread clinical adoption, reserving its use primarily for suspected false-negative scenarios as a supplementary tool. Vancomycin proves particularly effective against common DFU pathogens, such as *Staphylococcus* and *Enterobacter* species, which remain largely show no drug resistance as reported.[38,39] To enhance local infection control and efficacy, surgical interventions included Vancomycin-impregnated bone cement implantation and Negative pressure wound therapy. Comprehensive approach addresses both systemic and localized aspects of DFU management.

Considering that Hb1Ac is closely related to short-term average serum glucose levels, despite over 50% of patients with HbA1c>7%, patients in the amputation group did not show higher HbA1c than controls, which seems to contradict existing studies.[40,41] The difference may stem from health care disparities. For example, rural patients often present with advanced disease due to delayed diagnosis and irregular monitoring, masking true glycemic trajectories. Notably, when analyzing amputated subgroups exclusively, lower level of 25-(OH)D strongly correlated with elevated HbA1c (P=0.033), supporting the Vitamin D's role in glucose metabolism.[42,43]

Circulating serum 25-(OH)D levels exhibit variability due to age, skin pigmentation, adiposity, and latitude-dependent sun exposure. Crucially, no universal consensus exists on optimal thresholds of 25-(OH)D for clinical outcomes. Divergent recommendations impede clinical practice, for example in patients with diabetes comorbidities such as DFU. To resolve this ambiguity, our study implemented a dual-threshold strategy: Initial stratification using

conventional cutoffs (20 ng/mL [IOM standard] and 10.17 ng/mL [population median]), ROC-derived optimization establishing 11.12 ng/mL as the evidence-based diagnostic threshold. This methodology transcends prespecified deficiency definitions by integrating population characteristics and clinical outcomes.

Inevitably, our research has some limitations. Firstly, due to the less abundant data content of samples in the cohort, there was some potential selection bias. Secondly, patients with confirmed 25-(OH)D deficiency have received standard supplementation to increase serum 25(OH)D concentration. However, therapeutic efficacy was not evaluated. Meanwhile, we did not further compare the relationship between the 25-(OH)D levels of the patients and the depth, grade and healing time of the ulcer according to the Wagner grade due to sample data limitations. In the future, prospective randomized trials are needed to confirm our findings and further analyze the role of Vitamin D in wound healing in DFU patients. Moreover, we also need to conduct more mechanistic investigation to delineate the pathobiological impact of Vitamin D deficiency on DFU progression.

In conclusion, this study found that 25-(OH)D deficiency occurs in 85.7% of patients with minor amputation in DFU, and the decreased levels of 25(OH) D was significantly associated with several clinical characteristics. 25-(OH)D deficiency is a vital risk factor for minor amputation and measures should be taken to prevent 25-(OH)D deficiency in T2DM patients with DFU.

Data Sharing Statement

The authors declare that all data and materials are available on reasonable request.

Ethics Approval and Consent to Participate

This study was approved by the Second Affiliated Hospital of Soochow University. And written informed consent was obtained from all patients according to the Declaration of Helsinki.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare no conflicts of interest in this work.

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