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Amino acids and wound healing in people with limb-threatening diabetic foot ulcers

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ABSTRACT

Background: Amino acids are associated with wound healing in traumatic wounds and burns, although their effects on healing in patients with diabetic foot ulcers (DFUs) are limited. This study aimed to evaluate and identify specific amino acids associated with healing outcomes of patients with DFUs.
Methods: Sixty-two out of 85 patients who completed the in-hospital treatment for limb-threatening DFUs were enrolled. All ulcers had epithelialization without clinical evidence of infection at discharge. The patients and their families were instructed on foot-care techniques and committed to regular follow-up for 1 year. Baseline characteristics, PEDIS wound classification, laboratory data and serum amino acid levels were used to analyze their predictive power.
Results: Fifty-seven patients completed the study in which 38 had healed and 19 had unhealed ulcers. The unhealed group had higher incidence of coronary artery disease and larger wound size. Most patients received endovascular therapy (81.6% healed group; 78.9% unhealed group) before enrollment. Following adjustments for clinical factors, the serum levels of arginine (326.4 μmol/L vs. 245.0 μmol/L, $P = 0.045$), isoleucine (166.7 μmol/L vs. 130.1 μmol/L, $P = 0.019$), leucine (325.8 μmol/L vs. 248.9 μmol/L, $P = 0.039$), and threonine (186.7 μmol/L vs. 152.0 μmol/L, $P = 0.019$) were significantly higher in the healed group.
Conclusions: The amino acids associated with wound healing in DFUs differ from those reported for traditional traumatic wounds. These findings affirm the necessity for future large-scaled studies for the application of these amino acids in DFU healing, either as prognostic predictors or supplemented regimens.

1. Introduction

Diabetic foot ulcers (DFUs) are one of the most common causes of admission and may result in limb loss and premature death for patients with diabetes.[1–3] The treatment for limb-threatening DFUs involves management of infection and limb ischemia. Nevertheless, even following the efforts of multidisciplinary treatment for acute treatment, the long-term wound healing for such patients is difficult to predict[4]; accordingly, more aggressive management is required to improve DFU healing, especially for those with concomitant peripheral artery disease (PAD).[5]

Wounding increases the overall state of catabolism,[6] and protein deficiency further hampers wound healing by mechanisms including decreased wound tensile strength,[7] decreased T-cell function,[7] decreased phagocytic activity,[8] and decreased complement and antibody levels,[8] ultimately diminishing the body's ability to defend the wound against infection.[8]

Various amino acids have been reported to have specific benefits on traumatic wound healing or burns.[9] In particular, arginine improves the collagen synthesis, antimicrobial activity, and blood flow by nitric oxide (NO) synthesis.[10] Glutamine serves as an energy source and protects tissue from inflammatory injury by inducing the expression of heat shock proteins.[11] Hydroxy methylbutyrate (HMB), the leucine metabolite, decreases the proteolysis, increases the protein synthesis, decreases the apoptosis and increases the cell proliferation.[12] A recent randomized study compared specific amino acids (arginine, and glutamine, and

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HMB) with total protein supplementations in patients with chronic DFUs; this, however, reported unsatisfactory results.[13]

The differences of pathophysiology in wound healing between chronic diabetic foot ulcers and traumatic wounds or burn may result in different requirements of amino acid supplementation. Hyperglycemia impairs the orderly progression of the healing process in patients with diabetes including the complex biological and molecular events of cells migration, proliferation, remodeling, and extracellular matrix deposition.[14,15] Furthermore, DFUs usually involve complex factors such as infection, distal circulation, and microvascular complications that might further affect healing. In order to verify the role of amino acids in DFU healing, we evaluated wound healing by thoroughly evaluating levels of serum amino acids in patients with limb-threatening DFUs.

2. Subjects and methods

2.1. Patients

From January 2015 to February 2017, 85 patients with type 2 diabetes and limb-threatening foot ulcers treated in the diabetic foot care unit at Chang Gung Memorial Hospital were studied, a diabetic foot care center accredited by the International Diabetes Federation West-Pacific Region in Taiwan. These patients had no major organ disease, including end-stage renal disease, liver cirrhosis, heart failure with a New York Heart Association (NYHA) score ≥ 3, or active malignancy. When wounds showed epithelialization without clinical evidence of infection, patients were asked to enter this prospective observational study before they were discharged. Two patients who receive major lower extremity amputation (LEA) were excluded because those wounds were sutured. This study was approved by the Institutional Review Board of Chang Gung Memorial Hospital (No. 102-2544B). Sixty-two patients were enrolled and written informed consent was obtained from each patient.

2.2. Wound recording

Wound assessment was recorded using the PEDIS classification system for perfusion, extension (size), depth, infection, and sensation.[16] The PEDIS classification scores at initial assessment of limb-threatening foot ulcers and at enrollment were used for analysis. All the wounds at enrollment should demonstrate infection score 1 and tissue epithelialization, regardless of their size and depth. A healed DFU was defined as complete epithelialization without skin ulceration, as determined by a plastic surgeon during the year of follow-up.

2.3. Clinical factors of the patients at enrollment and during treatment of limb-threatening foot ulcers

Clinical demographics, associated comorbidities, factors of PEDIS wound grading, and major procedures of endovascular therapies and levels of foot amputation for limb-threatening ulcers were recorded and compared. Laboratory data of routine hematology tests and chemistry profile at enrollment were analyzed.

2.4. Metabolite analysis

Blood samples were immediately separated and frozen at −80 °C after an overnight fast (>8 h). Twenty-one amino acids (19 proteinogenic amino acids, citrulline and ornithine) were assayed and quantified using an AbsoluteIDQ™ p180 kit (BIOCRATES Life Sciences AG, Innsbruck, Austria).

This targeted metabolomic approach was based on liquid chromatography-tandem mass spectrometry (LC-MS/MS) assay. Measurements were performed using a Waters Acquity Xevo TQ-S (Waters Corp., MA, USA) instrument according to the manufacturer's instructions. The AbsoluteIDQ™ p180 kit has been proven to conform to the FDA Guidelines[17] It also provides semi-quantification of free carnitine, acylcarnitines, biogenic amines, hexose, glycerophospholipids, and sphingolipids.

2.5. Statistical analysis

Categorical variables were reported as numbers with percentages, and continuous variables were reported as medians and interquartile ranges. Comparisons between healed and unhealed groups were performed using Pearson's chi-square test or the Mann–Whitney *U* test as indicated. The levels of amino acids were analyzed using receiver operating characteristic (ROC) curves corresponding to whether the wound healed or not. All statistical tests were carried out at a two-tailed significance level of 0.05 using SPSS version 23 (IBM SPSS Inc., Chicago, IL, USA). Our manuscript was prepared using the STROBE guidelines for observational studies.[18] Receiver operating characteristic curves were calculated, and the areas under the ROC curves (AUCs) with 95% CIs were used to assess the ability of the considered variables to predict wound healing. The Youden index was used to define the optimal cutoff point. Kaplan-Meier method was used to examine the time to wound healing for target amino acid.

3. Results

3.1. In-hospital limb-preservation treatment

Among the 85 patients who received multidisciplinary managements, 52 had endovascular therapy. At the end of the acute treatment, 33 patients had LEAs (2 had major LEA). When looking for the factors that determine the LEA treatment outcomes (Fig. 2), lower serum albumin level (3.1 [2.8–3.4] vs. 3.5 [3.0–3.8] mg/dL, $P = 0.027$) as well as coronary artery disease (CAD), PAD, deeper wound, and more severe infection status were noted.

3.2. Baseline characteristics

Sixty-two patients were enrolled for the amino acid analysis and five patients were lost to follow-up in this study (Fig. 1). The median age of the 57 patients who completed the study was 73.8 (66.9–80.4) years, and 63.2% were female. The median duration of diabetes was 15.0 (10.0–20.0) years (Table 1).

Thirty-eight patients had healed wounds and the other 19 had unhealed foot ulcers after one year of follow-up. There were no

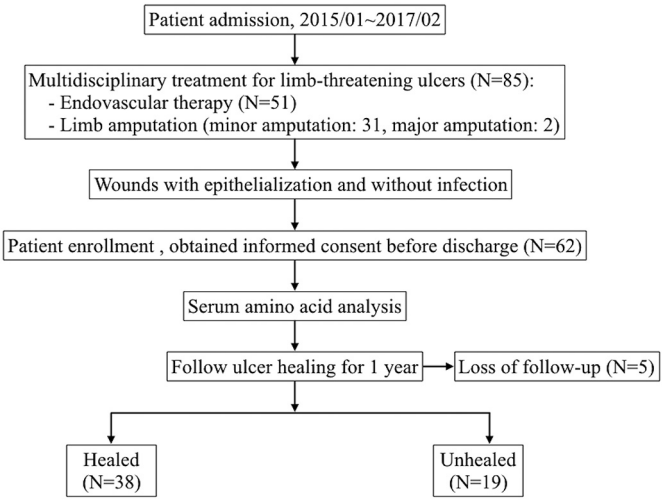

Fig. 1. Flowchart of the participant selection.

Table 1
Baseline characteristics and wound assessment at enrollment

	Healed	Unhealed	
	$N = 38$	$N = 19$	P value
Age (year)	72.9 (63.3–80.4)	76.0 (68.6–80.7)	0.229
Male gender (%)	42.1%	26.3%	0.192
BMI (kg/m^2)	25.1 (22.5–28.0)	24.7 (21.7–25.8)	0.180
DM duration (year)	14.5 (9.8–20.0)	20.0 (11.0–23.0)	0.071
Cardiovascular disease (%)	57.9%	78.9%	0.100
Coronary artery disease (%)	18.4%	52.6%	0.010
Stroke (%)	13.2%	10.5%	0.571
Previous diabetic foot ulcer (%)	36.8%	63.2%	0.055
Previous endovascular therapy (%)	19.4%	26.7%	0.411
Plantar site (%)	52.6%	68.4%	0.505
PEDIS classification			
Perfusion			0.348
Grade 1 (%)	81.6%	73.7%	
Grade 2 (%)	18.4%	21.1%	
Grade 3 (%)	–	5.3%	
Wound size (cm^2)	0.8 (0.1–6.0)	5.5 (1.2–21.5)	0.016
Depth			0.201
Grade 1 (%)	92.1%	84.2%	
Grade 2 (%)	7.9%	5.3%	
Grade 3 (%)	–	10.5%	
Infection			1.000
Grade 1 (%)	100.0%	100.0%	
Sensation			0.254
Grade 1 (%)	45.7%	31.3%	
Grade 2 (%)	54.3%	68.6%	
Minor amputation (%)	42.1%	42.1%	1.000
Endovascular therapy (%)	81.6%	78.9%	0.537

statistical differences in age, gender, BMI, and comorbidities between the two groups. However, the unhealed group had a higher incidence of coronary artery disease (18.4% vs. 52.6%, $P = 0.010$) (Table 1).

In the follow-up period, there was no significant difference in insulin usage (63.2% in healed group and 50% of patients in the unhealed group ($P = 0.256$)). Common commercial dressings were used for ulcers (such as, sulfasil, purilon gel, iodine solution, polymem Ag, or neomycin ointment). None of these patients received topical application of epidermal growth factor or hyperbaric oxygen therapy. No patient needed further surgical debridement.

3.3. PEDIS wound scoring at enrollment

More than half of the wounds were located over the plantar site (52.6% in the healed group and 68.4% in the unhealed group, $P = 0.505$). All of the participants received parenteral antibiotic treatment, and most of them (81.6% of the healed and 78.9% of the unhealed participants, $P = 0.537$) had endovascular therapy.

There was no significance difference of wound scores between the two groups at enrollment except for the larger size found in the unhealed group (5.5 [1.2–21.5] cm^2 vs. 0.8 [0.1–6.0] cm^2 in the healed and unhealed groups respectively, $P = 0.016$) (Table 1).

3.4. Comparisons of laboratory and amino acid data between the two groups

Table 2 demonstrates the laboratory data at enrollment. Both groups of participants had a normal leukocyte count and low C-reactive protein level, reconfirming that the wounds were not infected. The higher prevalence of CAD of participants in unhealed group had lower low-density lipoprotein (LDL) levels. The serum albumin level was similar in both groups (3.3 mg/dL in the healed group and 3.4 mg/dL in the unhealed group, $P = 0.699$).

The amino acid analysis is shown in Appendix S1. There were significant differences in six amino acids (isoleucine, threonine, arginine, serine, aspartate, and leucine) between the two groups (Table 2). We adjusted the clinical factors that may have impacted healing (Tables 1 and 2) in three models. Model 1 was adjusted for wound size and history of CAD, while model 2 was adjusted for wound size, CAD history, and duration of diabetes, and model 3 was adjusted for wound size, CAD history, duration of diabetes, and previous history of a DFU (Table 2). The independent amino acids identified in model 3 were arginine (300.0 μmol/L vs. 186.6 μmol/L, $P = 0.045$), isoleucine (143.3 μmol/L vs. 126.0 μmol/L, $P = 0.019$), leucine (272.7 μmol/L vs. 221.0 μmol/L, $P = 0.039$), and threonine (184.9 μmol/L vs. 139.9 μmol/L in the healed vs. unhealed groups, $P = 0.010$).

Model 3 yielded an AUC of 0.680 (95% CI 0.53–0.84) for arginine, 0.668 (95% CI 0.52–0.82) for isoleucine, 0.670 (95% CI 0.52–0.82) for leucine, and 0.688 (95% CI 0.53–0.84) for threonine (Appendix S2). The best cutoff points in the ROC curves of arginine, isoleucine, leucine, and threonine were 187.8 μmol/L, 135.5 μmol/L, 243.4 μmol/L, and176.7 μmol/L respectively. When time to wound healing was

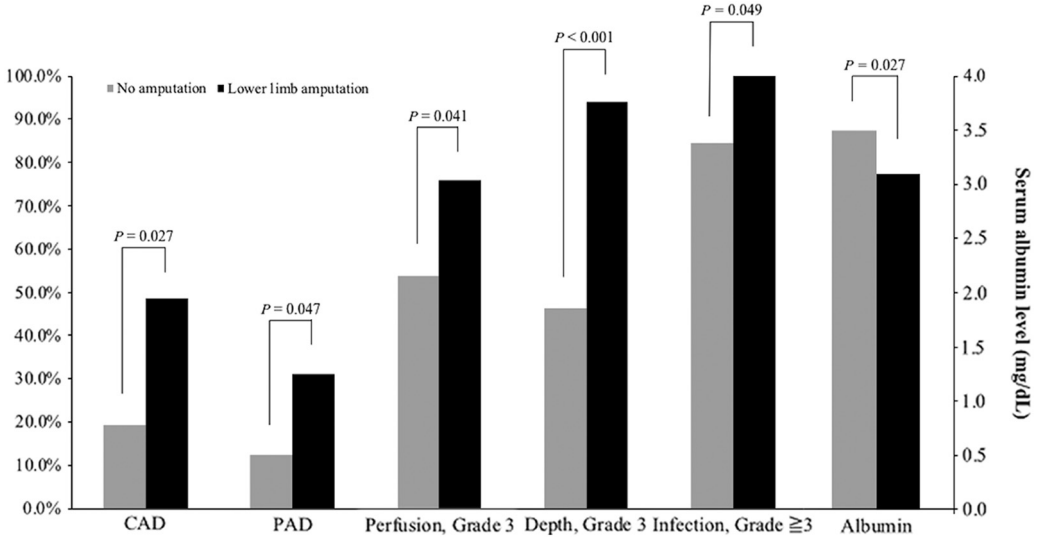

Fig. 2. Comparison of clinical features and lower limb amputation outcome. The amputation group had lower serum albumin level (3.1 [2.8–3.4] vs. 3.5 [3.0–3.8] mg/dL, $P = 0.027$), as well as higher incidence of CAD (48.5% vs. 19.2%, $P = 0.005$), PAD (31.0% vs. 12.5%, $P = 0.047$), grade 3 perfusion (75.8% vs. 53.8%, $P = 0.041$), grade 3 depth (93.9% vs. 46.2%, $P < 0.001$), and grade ≥ 3 infection (100.0% vs. 84.6%, $P = 0.049$).

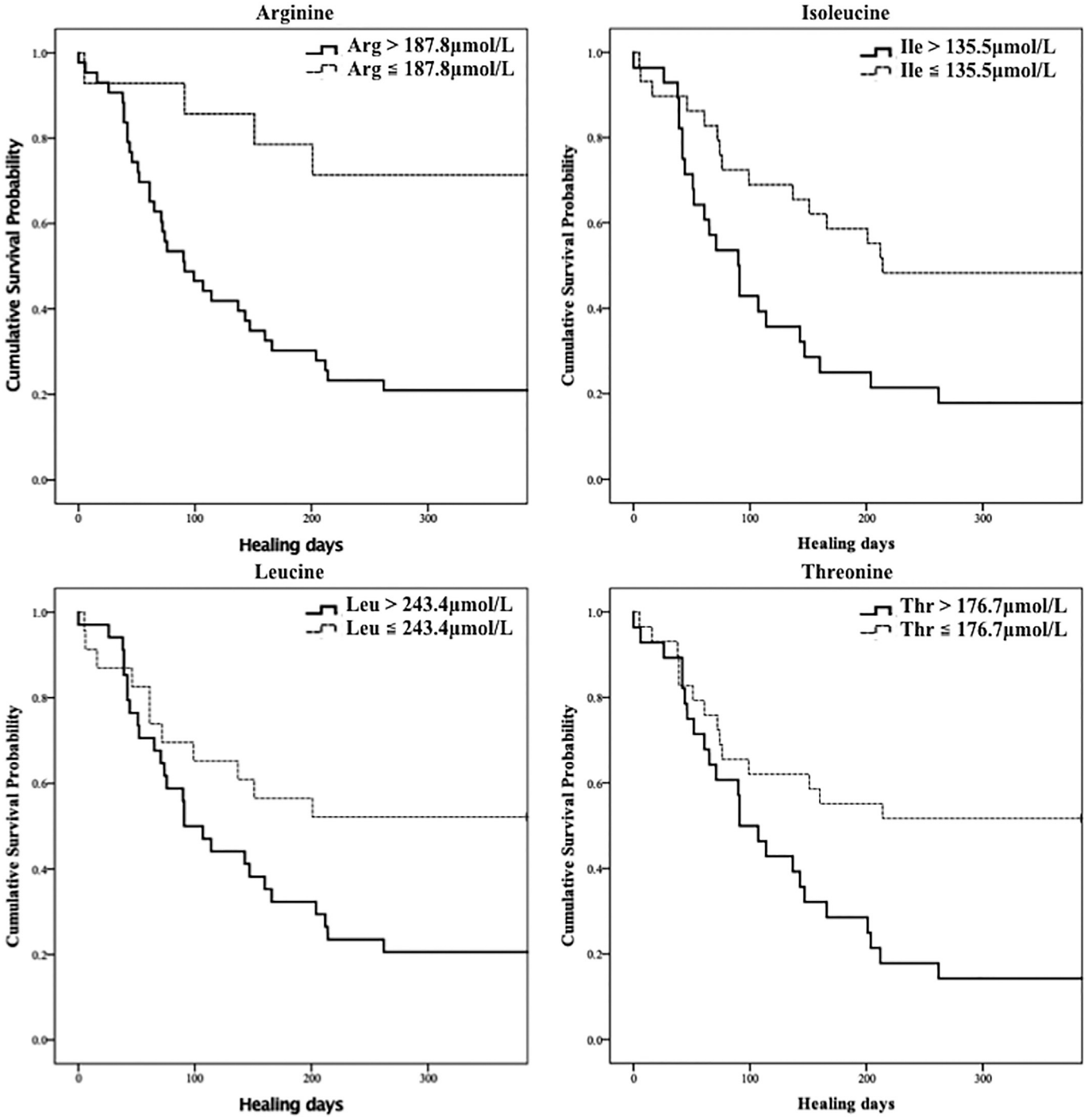

Fig. 3. Kaplan–Meier survival curves for wound healing using the cutoff points in ROC curves of arginine, isoleucine, leucine, and threonine.

examined by Kaplan-Meier method (Fig. 3), patients with higher levels of each of the four amino acids showed better wound healing outcome during the follow-up period.

4. Discussion

Our results demonstrated significant differences in several amino acids between participants with healed and unhealed DFUs, including arginine, leucine, isoleucine, and threonine. In addition, the serum albumin levels appeared to be lower in both groups, which may indicate wound catabolism[6] and the existence of inflammation.[19] Of note, there was no difference in serum albumin level between the two groups in the present study, suggesting that albumin level cannot be used to predict future wound healing. We previously reported that serum albumin level is a marker for limb preservation during acute treatment for limb-threatening foot ulcers[20,21]; however, it was not a marker for chronic wound healing in the present study.

Arginine has long been proposed to have a vascular effect because it plays the central role in NO synthesis.[22] The increase in NO and other free radicals during acute wounding can result in severe matrix destruction, which may impair wound healing.[23] On the other hand, the higher concentration of NO has been shown to increase vascular permeability,[22] bactericide,[24] and to be a mediator of angiogenesis,[22] and these effects may be beneficial for chronic wound healing. In

Table 2
Clinical laboratory data and amino acids showing significant differences between healed and unhealed groups

	Healed	Unhealed		Model 1	Model 2	Model 3
	N = 38	N = 19	*P*	*P*	*P*	*P*
HbA_{1c} (%)	8.6 (7.5–10.3)	8.5 (7.5–9.9)	0.966			
HbA_{1c} (mmol/mol)	70.0 (58.0–89.0)	69.0 (58.0–85.0)	0.966			
Hb (mg/dL)	10.6 (9.5–12.5)	11.0 (8.8–11.6)	0.582			
eGFR[a]	53.3 (27.6–65.3)	45.5 (19.2–56.8)	0.388			
ALT (U/L)	14.5 (10.0–21.8)	15.0 (12.0–26.0)	0.246			
LDL (mg/dL)	79.5 (65.3–94.8)	62.0 (50.0–80.0)	0.013			
HDL (mg/dL)	33.0 (28.0–38.8)	34.0 (27.5–39.5)	0.850			
TG (mg/dL)	113.5 (88.3–168.5)	96.0 (60.8–146.5)	0.249			
WBC (10^3/uL)	7.5 (6.0–8.7)	7.0 (5.6–7.9)	0.267			
N/L ratio	2.7 (1.7–4.5)	3.5 (2.6–4.6)	0.145			
CRP (mg/dL)	6.7 (3.4–22.1)	10.2 (3.5–19.3)	0.946			
Albumin (mg/dL)	3.3 (3.0–3.7)	3.4 (3.1–3.8)	0.699			
Threonine (μmol/L)	184.9 (138.7–220.5)	139.9 (104.5–175.6)	0.022	0.011	0.011	0.010
Isoleucine (μmol/L)	143.3 (118.0–194.1)	126.0 (88.6–139.0)	0.041	0.011	0.013	0.019
Leucine (μmol/L)	272.7 (217.6–403.3)	221.0 (190.0–269.5)	0.037	0.023	0.023	0.039
Arginine (μmol/L)	300.0 (210.8–431.9)	186.6 (146.0–352.0)	0.028	0.014	0.022	0.045
Serine (μmol/L)	293.2 (250.3–360.3)	233.0 (192.1–266.6)	0.009	0.014	0.022	–
Aspartate (μmol/L)	83.0 (68.3–96.1)	61.7 (50.9–88.4)	0.034	0.023	0.033	–

Model 1: Adjusted for wound size and CAD history.
Model 2: Adjusted for wound size, CAD history, and duration of diabetes.
Model 3: Adjusted for wound size, CAD history, duration of diabetes, and previous DFU.
Abbreviations: Hb: haemoglobin, HbA_{1c}: haemoglobin A_{1c}, eGFR: estimated glomerular filtration rate, ALT: alanine transaminase, LDL: low-density lipoprotein, HDL: high-density lipoprotein, TG: Triglyceride, WBC: Leukocyte count, N/L ratio: neutrophil-lymphocyte ratio, CRP: C-reactive protein.
[a] Calculated by the Modification of Diet in Renal Disease (MDRD) study equation.

addition, ornithine, the arginine metabolite, can subsequently be converted to proline for collagen synthesis,[25] cell growth and differentiation,[23] and help with matrix formation.[25]

Isoleucine and leucine are branched-chain amino acids (BCAAs). They account for 35% of the total content of essential amino acids in muscle proteins.[26] BCAAs, and especially leucine, has been reported to activate the mammalian target of the rapamycin signaling pathway,[27] thereby promoting muscle-protein synthesis. Elderly subjects and those with diabetes[28] or PAD[29] are more likely to have sarcopenia, and the long-term presence of a DFU can hamper limb activity and might have resulted in further loss of lower extremity muscle mass in our population. Nevertheless, there is currently no direct evidence suggesting a correlation between BCAA supplements and wound healing.

Threonine has been reported to participate in the formation of collagen and elastin.[30] It has also been shown to serve as a structural constituent of proteins and to feed into the biosynthetic pathway for isoleucine.[31] However, the mechanism by which threonine participates in the healing of DFUs is still unclear.

Delayed wound healing in DFUs involves many complex mechanisms in addition to nutritional factors. In this study, we found that the levels of several amino acids were apparently lower among participants with unhealed DFUs. Supplements of these amino acids may thus enhance wound healing in people with DFUs.

This study was designed to compare baseline clinical data and levels of amino acids for wound healing. Data on longitudinal changes in amino acids and peripheral artery circulation were not provided. Furthermore, the design was intended to study people at higher risk of non-healing wounds. The clinical applications of these findings should be limited to the subgroup of people with PAD or larger wounds.

In summary, this study found an association between arginine, isoleucine, leucine, and threonine with wound healing of DFUs. Future application of these amino acids in DFU healing, either as prognostic predictors or supplemented regimens, needs validation through further large-scaled studies.

Declaration of Competing Interest

All authors declare that there is no duality of interest associated with their contribution to this manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jdiacomp.2019.06.008.

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