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Does preoperative muscle biopsy predict the outcome of lower extremity amputation in diabetic patients? a prospective observational study

Ihsan Akan[1*], Tayfun Bacaksiz[1], Mehmet Maden[2], Arzu Avci[3], Mahmut Tuncez[2] and Cemal Kazimoglu[1]

Abstract

Background Determining the most appropriate level of amputation in patients with diabetes mellitus has not been well established. The purpose of this study is to determine whether muscle biopsy reveals predictive information about the success rate of patients undergoing diabetic major lower limb amputation.

Methods A prospective observational study was conducted among diabetic patients who underwent below-knee amputation. Skin-subcutaneous and muscle biopsy samples were obtained during the operation from 62 patients who undergo major limb amputation. Depending on the complications after surgery, patients were assigned into three groups: Group 1 consisted of patients with adequate wound healing without any complications; Group 2 included patients with prolonged wound healing requiring additional interventions like debridement; and Group 3 consisted of patients who underwent reamputation at a more proximal level. Biopsy samples of the groups were compared regarding degenerative cells, inflammatory cells, and the presence of infection.

Results There was a significant difference between Groups 1 and 3 regarding the presence of abscess formation and infection ($p < 001$). Comparison of Groups 1 and 3 revealed significant differences regarding inflammatory cell count, respectively ($p < 001$). According to the results of the ROC analysis performed for histopathologic cellular evaluation, 15% for inflammatory cell ratio in muscle samples and 25% for degenerative cell ratio both in muscle and skin samples were determined as cut-off values.

Conclusions The presence of increased degenerative cell count and infection in muscle biopsy areassociated with higher rates of reoperation. The present study revealed that preoperative muscle biopsy has predictive value in patients undergoing major limb amputation.

Level of evidence Level II, Prospective observational study.

Keywords Amputation, Lower limb, Diabetus mellitus, Muscle biopsy, Re-operation

*Correspondence:
Ihsan Akan
ihsan.akan@ikc.edu.tr
[1]Department of Orthopaedics and Traumatology, Izmir Katip Celebi University, Izmir, Turkey
[2]Department of Orthopaedics and Traumatology, Izmir Ataturk Training and Research Hospital, Izmir, Turkey
[3]Department of Pathology, Izmir Tınaztepe University, Izmir, Turkey

Introduction

The prevalence of diabetes has been increasing over the last decades [1, 2, 3, 4]. Despite advances in medical care and patient education, more than 15% of patients with type II diabetes mellitus (DM) experience diabetic lower extremity ulceration, diabetic neuropathy, arterial insufficiency, and infections, which adversely affect wound healing, eventually leading to major lower extremity amputation in more than 5–24% of these patients [2, 3, 5, 6]. According to a previous study, the incidence of lower limb amputation is 58.7 per 100,000 of the population, and this rate is significantly increased among the diabetic population [7]. The socioeconomic cost of amputation is high, and especially inappropriate-level amputations and re-operations are a burden to both patients and society [8].

Determining the most appropriate level of amputation with a reasonable chance of healing in terms of functional prognosis and postoperative quality of life, the patient's rehabilitation potential, and avoiding surgical revision or reduction of complications is challenging [9]. The decision of the level of amputation is often made by vascular and orthopaedic surgeons, depending on the level of arterial obstruction, demarcation, infection, and necrosis, without a defined strict criteria. Besides, non-invasive diagnostic methods, including transcutaneous oxygen measurements and Doppler flowmetry, are not reliable due to the lack of absolute cutoff levels. More objective methods are needed to predict the outcome of amputation in diabetic patients [10, 11, 12].

Muscle atrophy is caused by systemic inflammation and imbalance in contractile protein synthesis and degradation [13, 14, 15]. Besides, prolonged diabetic vasculopathy leads to degenerative and inflammatory changes in the muscles, which may be used as predictors of the appropriate level of amputation. In addition, histopathological changes in muscle tissue may provide insight into delayed wound healing and residual infection, which may lead to stump revision and amputation level elevation. The aim of this study was to evaluate the effectiveness of a muscle biopsy performed at the planned amputation level before surgery in determining the appropriate amputation level and preventing the need for repeat surgery.

Materials and methods

A prospective observational study was conducted among the diabetic patients who underwent below-knee amputation at a reference hospital (Izmir Katip Celebi University Ataturk Training and Research Hospital) between 2021 and 2024. The study was initiated after obtaining ethics committee approval and continued until the planned sample size was achieved.

Patients

Patients who were followed up with a diagnosis of diabetic foot had at least Wagner stage 3 or 4, and whose clinical council had decided to undergo below-knee amputation were included in the study. Surgical procedures were performed by a skilled and experienced senior orthopaedic surgeon team with at least 10 years of postgraduate specialisation in diabetic foot surgery. Written informed consent was obtained from each participant before inclusion.

Patients with other comorbidities such as chronic renal failure, rheumatological diseases, or immunosuppression that affect wound healing regardless of the level of amputation, or patients who did not comply with medical therapy for glycaemic control and died during clinical follow-up, were excluded. In addition, patients with vascular pathology that could be revascularised by percutaneous intervention were excluded from the study because their recovery process after amputation would be affected and would create intergroup bias.

Procedure

All patients underwent glycaemic control assessments by a consultant endocrinologist before and after surgery. Demographic data, presence of chronic diseases other than DM, time between surgical treatment decision and surgery, and wound care follow-up were recorded. In addition, fasting blood sugar levels, glycated haemoglobin (HbA1c), haemoglobin (Hb), and albumin values, inflammatory markers, and wound culture analyses were also documented.

During amputation surgery in all patients, skin and muscle tissue samples in the form of 1*1 cm cubes were taken from the medial part of the gastrocnemius muscle, 2 cm proximal to the planned stump level, under spinal anaesthesia. Thereafter, the standard amputation surgery protocol was carried out. The antibiotic regime was conducted by an infectious disease specialist regarding the wound cultures. All patients were followed up at regular intervals in the outpatient clinic with a standard wound care protocol until complete wound healing was achieved. No complications were observed in any patient related to the biopsy site.

The participants were divided into three groups regarding the condition of the stump wound healing. Group 1: Patients with adequate wound healing without any complications; Group 2: Patients with prolonged wound healing that require additional interventions such as debridement; Group 3: Patients who underwent reamputation at a more proximal level.

Histopathological examination

Hematoxylin Eosin (H/E) and Masson Trichrome-stained sections of the skin, subcutaneous, and muscle

tissue samples were examined under a light microscope at 10x and 40x magnifications. The examined parameters were evaluated simultaneously by 2 researchers (a clinician and a pathologist) who were unaware of the clinical patient details, and a consensus decision was reached in the presence of any disagreement. Percentage values were obtained by examinations at 40x magnification. High Power Field (HPF) was determined as the region where the most intense parameters were observed. The ratio of this area to the entire cross-sectional area provided the percentage values. The percentage evaluation was used for degenerative cells, inflammatory cells and cellular steatosis data. Cellular swelling, pale cytoplasm, ballooning degeneration, vacuolar degeneration, and hyaline droplet presence were evaluated as signs of degeneration, and the presence of mixed-type inflammatory cells, especially polymorphonuclear leukocytes (PNLs), as a sign of inflamation. The presence of vasculopathy, abscess formation, and bacterial microorganisms in any area of the examined slides was noted. An area with inflammatory infiltrate with abundant neutrophils and necrotic debris was accepted as abscess formation. Vasculopathy was accepted as the presence of vasculitis and fibrosis with disintegrity of the vascular wall. Verhoeff-Van Gieson elastic stain and Periodic acid-Schiff (PAS) stain were utilized in order to evaluate vascular structures. The presence of microorganisms was investigated with additional Giemsa staining.

Statistical analysis

The sample size of the study was calculated using the G*Power (Ver.3.9.1.6©Franz-Faul-Germany) program. Since a similar study in which microscopic examination was performed could not be found before, calculations were made according to the PCT (procalcitonin) level, which was one of the secondary endpoints [16]. The median procalcitonin value, which was routinely measured preoperatively in all patients, was predicted to be 1.72 [Interquartile range (IQR): 0.81–5.51] in reamputees and 0.105 (IQR: 0.05–0.44) in the control group, and it was aimed to reach at least 60 people for 80% power and a type 1 error level of 5%.

Statistical analysis was performed using the Statistical Package for the Social Sciences 22 (released 2013 NY: IBM Corp.) program. Normality analysis of data distribution was evaluated with the Kolmogorov-Smirnov test. If the numerical scores were normally distributed, the independent T-test (student T-test) was applied, and if they were not normally distributed, the Mann-Whitney U test was applied. The Kruskal-Wallis test was used for comparisons of more than two independent groups. The Chi-square test was used since the categorical data of the groups were compared. Receiver operating characteristic (ROC) analysis was carried out to establish the diagnostic performance of histopathological findings for reamputation risk. Several parameters, including sensitivity, specificity, and area under the curve (AUC), were employed. Typically, AUC > 0.6 was considered acceptable. For all analyses, significance was accepted as a p value < 0.05.

Results

A total of 62 patients, 12 (19%) female and 50 (81%) male, who underwent below-knee amputation due to diabetic foot between 2022 and 2024 were included in the study. The mean age was 64.47 ± 11.21 (35–91). The baseline characteristics reveal no statistically significant differences in the preoperative interval after surgical decision, fasting blood glucose (FBG), glycated hemoglobin (HbA1c), hemoglobin (Hb), white blood cell count (WBC), and inflammatory markers across the three groups, as detailed in Table 1.

The distribution of inflammatory cells, degenerative cells, and fatty degeneration rates in histopathological evaluations by groups is given in Table 2. The histopathological evaluation of the skin and subcutaneous tissue revealed no significant difference between Group 1 and

Table 1 Preoperative laboratory values and demographic data of the groups

	Total (*n* = 62)	Group 1 (*n* = 25)	Group 2 (*n* = 18)	Group 3 (*n* = 19)	*P* value
Age	64,47 ± ± 11,21 (35–91)	64,68 ± 9,73 (47–86)	68,11 ± 11,13 (50–91)	60,74 ± 12,43 (35–86)	0,152*
Gender (male/female)	50/12	21/4	15/3	14/5	0,653°
Preoperation time	3,66 ± 2,23 (1–10)	3,04 ± 1,79 (1–7)	4,44 ± 2,64 (1–10)	3,74 ± 2,20 (1–10)	0,679*
HbA1c	7,94 ± 2,40 (4,7–13,7)	8,94 ± 2,55 (4,7–13,7)	7,70 ± 2,34 (5,3–13)	7,08 ± 2,00 (5–13,1)	0,061*
ESR	79,62 ± 33,42 (13–142)	79,53 ± 35,21 (13–138)	76,25 ± 36,43 (22–142)	83,33 ± 29,42 (31–115)	0,752*
CRP	110,46 ± 72,55 (1,88–281)	119,39 ± 80,49 (1,88–281)	109,47 ± 66,08 (6,7-214)	100,60 ± 70,73 (2,40–259)	0,755*
FBG	180,30 ± 112,94 (72–617)	179,12 ± 91,09 (75–412)	165,82 ± 101,70 (72–466)	194,79 ± 147,78 (87–617)	0,742*
Hb	11,01 ± 2,16 (7,0–15,6)	11,28 ± 2,44 (7,0–15,6)	10,77 ± 2,22 (7,9–14,6)	10,87 ± 1,74 (7,0–14,2)	0,768*
Albumin	30,74 ± 7,64 (17–47)	32,32 ± 8,45 (17–47)	28,89 ± 8,67 (18–42)	30,67 ± 5,05 (22–38)	0,378*
WBC	12,75 ± 5,01 (4,8–25,2)	14,05 ± 4,91 (6,0–24,6)	12,31 ± 5,29 (4,8–25,2)	11,45 ± 4,72 (5,9–21,6)	0,141*

BMI: Body Mass Index; HbA1c: Glycated Hemoglobin; ESR: Eritrocyte Sedimantation Rate; CRP: C Reactive Protein; FBG: Fasting Blood Glycose; Hb: Hemoglobin; WBC: White Blood Cell

*Kruskal Wallis test, °Pearson chi square

Table 2 Relationship between groups in degenerative cells, inflammatory cells, and fatty degeneration in samples taken from skin and muscle

		Group 1	Group 2	Group 3	*P** value
		mean ± SD (min-max)			
Skin/Subcutaneous	Degenerative cell ratio	13,4 ± 7,32 (0–30)	17,5 ± 12,39 (0–40)	37,4 ± 18,51 (20–80)	**< 0,001**
	Inflammatory cell ratio	7,4 ± 6,14 (0–20)	10,6 ± 9,38 (0–30)	21,8 ± 25,99 (0-100)	0,061
	Fatty degeneration ratio	18,0 ± 12,67 (0–50)	18,9 ± 7,96 (5–30)	13,7 ± 12,12 (0–50)	0,172
Muscle	Degenerative cell ratio	17,0 ± 8,66 (0–40)	29,7 ± 17,19 (5–80)	33,4 ± 21,22 (5–80)	**0,002**
	Inflammatory cell ratio	8,2 ± 6,44 (0–20)	10,8 ± 13,20 (0–50)	35,5 ± 21,79 (5-100)	**< 0,001**
	Fatty degeneration ratio	16,8 ± 8,77 (5–40)	21,1 ± 13,99 (5–60)	12,9 ± 9,62 (0–30)	0,163

SD: Standard Deviation, *: Kruskal-Wallis test

Table 3 ROC analysis results regarding the risk of reamputation

	AUC (%95)	Cut-Off	*P* value	Sensitivite (%)	Spesifite (%)
Inflammatory cell ratio(muscle)	0.913 (0.834–0.992)	**15**	< 0.001	94.7	79.1
Degenerative cell ratio(muscle)	0.665 (0.512–0.817)	**25**	0,04	57.9	65.1
Degenerative cell ratio(skin)	0.896 (0.819–0.973)	**25**	< 0.001	78.9	88.4

AUC: Areas Under the Curve ROC: Receiver operating characteristic

Table 4 Relationship between the groups in the presence of abscesses, bacteria and vasculopathy in skin and muscle samples

		Group 1 (*n* = 25)	Group 2 (*n* = 18)	Group 3 (*n* = 19)	*P** value
Skin/Subcutaneous	Abscess	1	4	5	0,097
	Bacteria	-	-	3	**0,028**
	Vasculopathy	3	-	1	0,278
Muscle	Abscess	1	3	7	**0,018**
	Bacteria	-	1	3	0,106
	Vasculopathy	2	0	1	0,481

*: Pearson Chi-square test

Group 2 in terms of degenerative cell count ($p = 0.527$), while a significant relationship was observed between Group 1 and Group 3 ($p < 0.001$) and also between Group 2 and Group 3 ($p = 0.002$). In the histopathological assessment of the muscle tissue, no significant difference was observed in the degenerative cell count between Group 2 and Group 3 ($p = 0.917$). However, a significant relationship was observed between Groups 1–2 ($p < 0.025$) and Groups 1–3 ($p = 0.013$). No significant relationship was found between groups in terms of inflammatory cell rates in skin-subcutaneous samples ($p = 0.061$). In addition, a significant difference was found between Group 1 and Group 3 ($p < 0.001$) and Group 2 and Group 3 ($p = 0.001$) in terms of inflammatory cell rates, while no significant difference was observed between Group 1 and Group 2 ($p = 0.826$). No statistical difference was found between the groups in terms of fatty degeneration in both skin-subcutaneous tissue and muscle tissue ($p = 0.172$, $p = 0.163$). ROC analysis was performed for the parameters found significant in the histopathologic cellular evaluation, 15% for inflammatory cell ratio in muscle samples and 25% for degenerative cell ratio in both muscle and skin samples were determined as cut-off values regarding the risk of reamputation (Table 3).

The distribution of prevalence of abscesses, bacteria, and vasculopaty in histopathological evaluations by groups is given in Table 4. While there was no significant difference between the groups in terms of the presence of abscesses in the tissues taken from the skin-subcutaneous ($p = 0.097$), a significant difference was found between the groups in the samples taken from the muscle ($p = 0.018$). While no bacteria were found in skin-subcutaneous tissue samples of Groups 1 and 2, bacteria were seen in 3 of 19 patients in Group 3. A significant difference was found between the groups ($p = 0.028$). Bacteria were found in muscle tissue samples in one of 18 patients in Group 2 and in three of 19 patients in Group 3. There was no statistically significant difference between the groups ($p = 0.106$).

No statistically significant difference was found between the groups in terms of the presence of vasculopathy in samples taken from both skin-subcutaneous and muscle ($p = 0.278$, $p = 0.481$).

Discussion

The findings of the present study reveal that skin-subcutaneous and muscle biopsy provide significant predictors for patients undergoing lower limb amputation with DM. Histopathological changes occur before clinical markers,

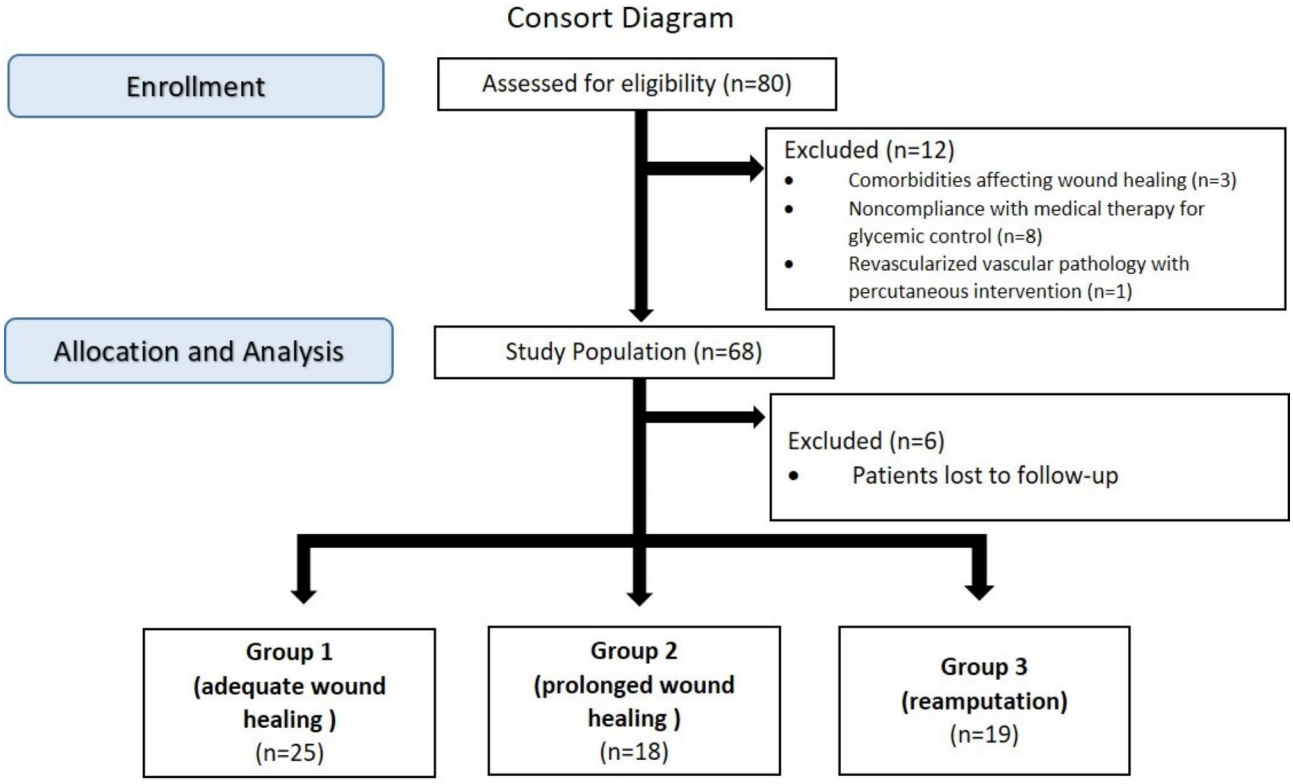

Fig. 1 Flowcharts of the study

and biopsy samples allow the evaluation of muscle morphology and early identification of inflammation and mitochondrial abnormalities. In this way, tissue healing after amputation can be predicted. This study has shown that increased inflammatory and degenerative cells in muscle tissue samples taken from the determined amputation level, substantially influence stump healing. In fact, reamputation was required in patients with an inflammatory cell rate above 15% and a degenerative cell rate above 25%. In light of our findings, a muscle biopsy before lower limb amputation may prevent unnecessary reoperations and improve patient care, which poses a major burden on both the patients and healthcare systems [2, 3, 4]. Besides, it may also be useful regarding legal issues in developing countries where most of the patients are unwilling to have lower limb amputation at the appropriate level after clinical evaluation. The procedure is easy and swift and can be carried out as an outpatient procedure in many clinics.

An increased neutrophil count in biopsy material has been found to increase the risk of reamputation in this cohort. In normal wounds, recruited neutrophils will eventually undergo apoptosis and be engulfed by macrophages, initiating a resolution program that terminates the inflammatory response [17].However, current research has indicated that neutrophils may have both positive and negative effects on the healing process of chronic wounds through multiple mechanisms [17, 18, 19, 20].The inflammatory response activated by cytokines disrupts the development mechanisms of new tissue and blood vessels in diabetic wounds and prevents healing by repairing and replacing damaged cells [10, 20]. While activated neutrophils produce reactive oxygen species, proteases, and antimicrobial peptides that help prevent infection, at the same time these protective mediators are released extracellularly, causing tissue damage. This leads to further inflammation, creating a vicious cycle that halts the wound-healing process and prevents complete wound closure [17, 18, 19].In the present study, we found that increased neutrophil count is a negative predictor of amputation success in DM patients. Recent research has shown that neutrophils may also have a detrimental effect on diabetic wound healing by producing neutrophil extracellular traps (NETs) during the healing process, which is well correlated with our results [21, 22, 23].

Diabetes also impacts the ability of injured skeletal muscle to regenerate, which may contribute to the manifestation of complications such as chronic limb-threatening ischemia. Degenerative cells manifest as abnormal mitochondrial morphology [24]. Diabetes not only leads to muscle degeneration, but also impairs the regenerative ability of the muscle. the regenerative process, which involves both muscle stem cells (MuSCs) and inflammatory cells, is hindered by excessive fibrosis and delayed

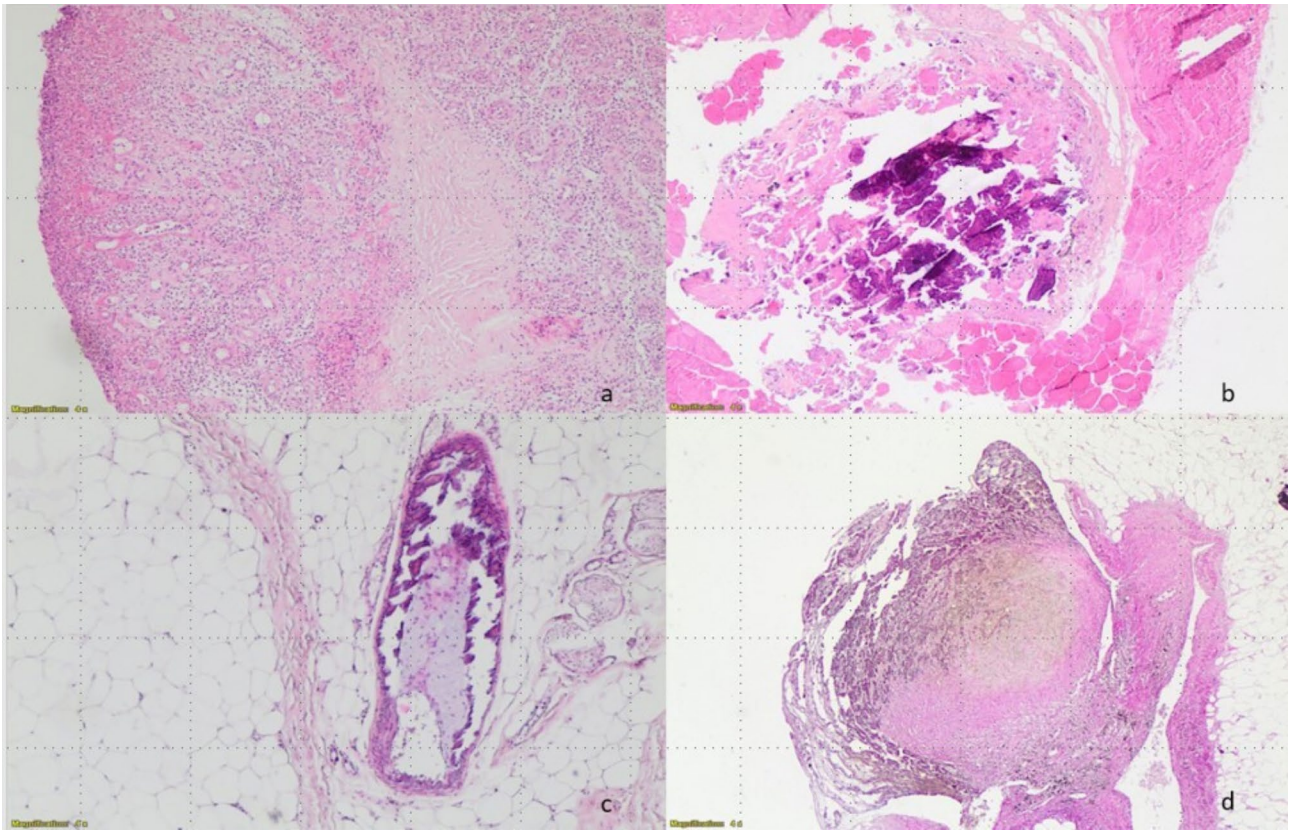

Fig. 2 Microscopic images of the biopsy material taken from the initially planned amputation level of a 52-year-old male who underwent reamputation. (**a**) Inflammatory cells, ×4, H&E (**b**) Degenerative cells, ×40, H&E (**c**) Fatty degeneration, ×10, H&E (**d**) Necrosis, ×10, PAS

myofiber formation [13]. Another study conducted in individuals undergoing below-knee amputation surgery, with and without diabetes, revealed that those with diabetes showed signs of muscle degeneration such as myofiber necrosis, myofiber splitting, and hypercontracted myofibers [25]. In this study, the high rate of degenerative cells in histopathological muscle samples taken from the amputation level was found to be associated with reamputations. This is consistent with the impairment of skeletal muscle healing ability, which is closely related to complications such as stump healing problems seen in diabetic patients as reported in the literature [26].

According to this study, the inflammatory cell rate in muscle tissue is over 15% in biopsy examinations taken from the area where amputation is planned, and the degenerative cell rate in skin and muscle samples over 25% predicts a higher risk of re-amputation. The level at which amputation will be performed should be re-evaluated in these patients.

Our results indicate that patients with abcess formation and deep infection have higher rates of reopoeration. This result is well correlated with the current related literature. Pickwell K. et al. reported that the presence of a moderate infection substantially increased the risk of failure for any amputation [27]. Ugwu E. et al. also reported that diabetic patients have a heightened risk of re-amputation due to increased infection risk [28]. Besides, diabetic patients are associated with a lower survival rate after major amputations, which leads orthopedic surgeons to search for predictive factors that may influence the level of amputation to be selected [29].

It is known that microvascular and macrovascular vasculopathy as complications of diabetes can affect the onset of tissue damage [30]. The iliac and femoral arteries are mostly affected and are usually associated with poor outcomes that may lead to amputation [31]. Diabetes mainly affects medium-large arteries but also impairs the circulation of the microvascular system [32]. This is closely related to prolonged wound healing and tissue regeneration [33]. However, in our study, no significant difference was observed between the groups in terms of vasculopathy. We believe that the main reason for this is that the homogeneous patient group in our study did not have involvement of large arteries, which are known to require revascularization. However, it was notable that endothelial dysfunction and focal necrosis, which are signs of early stage vasculopathy, were observed in most of the patients who underwent re-amputation in our study [34].

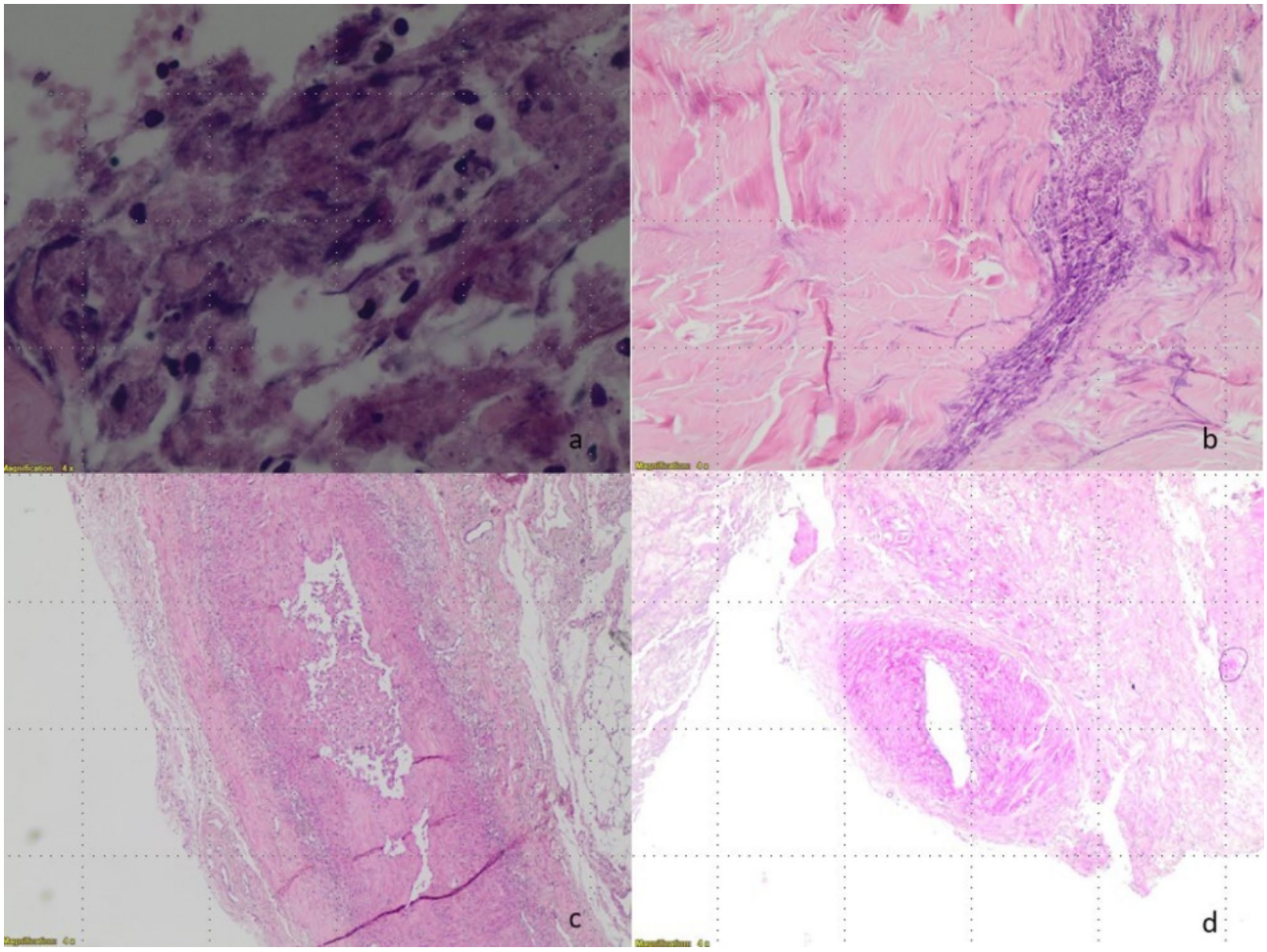

Fig. 3 Microscopic images of biopsy material from a patient in Group 3. (**a**) Microorganism, ×40, H&E (**b**) Abscess, ×10, H&E (**c**) Vasculopathy and vascular thrombosis, ×20, H&E (**d**) Vaskulopathy, ×20, PAS

This study had several limitations despite the promising results obtained. Complications related to wound healing can occur after invasive procedures such as biopsies in patients with diabetes or vascular disease. The most important of these complications are infection and delayed wound healing. Although this may seem like a limitation of our study, the data obtained will minimize the possibility of repeat surgery and therefore greater complications. Another limitation of our study is the lack of subgroups that include patients with other comorbidities that may affect the results or those with poor glycemic control. Therefore, multicenter studies with more patients are needed to confirm our findings. Besides, the pathological evaluation with an electron microscope could be more meaningful.

Conclusion

The present study revealed that a biopsy taken from the clinically planned amputation level before or during surgery may provide important clues to surgeons in determining the optimum level of lower extremity major amputation in diabetic patients. Increased inflammatory and degenerative cells and the presence of infection in muscle tissue samples have a significantly negative impact on stump healing. Re-amputation is required in patients with inflammatory cell ratios over 15% and degenerative cell ratios over 25% in biopsy sections.

Abbreviations

ROC	Receiver operating characteristic
DM	Diabetes mellitus
HbA1c	Glycated hemoglobin
Hb	Hemoglobin
WBC	White blood cell count
H/E	Hematoxylin eosin
HPF	High power field (HPF)
PNLs	Polymorphonuclear leukocytes
PAS	Periodic acid-schiff
PCT	Procalcitonin
IQR	Interquartile range
FBG	Fasting blood glucose
NETs	Neutrophil extracellular traps
BMI	Body mass index
ESR	Eritrocyte sedimantation rate
CRP	C-reactive protein
SD	Standard deviation
AUC	Areas under the curve

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Author contributions
All the authors contributed to the study's conception and design. IA and TB: Conceptualization; data curation; formal analysis; investigation; methodology; visualisation; writing-original draft; writing-review and editing. MM: Formal analysis; investigation; visualisation; writing-original draft; writing-review and editing. MT: Conceptualization; investigation; methodology. AA: Conceptualization; investigation; methodology; resources; writing-original draft; CK: Conceptualization; investigation; supervision; writing-original draft; writing-review and editing. All the authors read and approved the final manuscript.

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Data availability
The datasets generated during or analysed during the current study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate
All methods were carried out in accordance with relevant guidelines and regulations. This study was performed in accordance with the Declaration of Helsinki. Ethics approval was obtained by the Izmir Katip Celebi University Atatürk Training and Research Hospital Clinical Studies Institutional Review Board (Approval number:92). The ethical standards from the 1964 Helsinki declaration and its later amendments were upheld. Informed consent was obtained from all participants for this study.

Consent for publication
Authors obtained written consent for participation and publication, including from participants in photographs.

Competing interests
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Clinical trial number
Not applicable.

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