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Evaluation of sirtuin 1 as a predictor of cardiovascular outcomes in diabetic patients with limb-threatening ischemia

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Chronic limb-threatening ischemia (CLTI) significantly increases the risk of major adverse limb events (MALE) and major adverse cardiac events (MACE) after lower extremity revascularization (LER). This study aims to identify novel biomarkers that help to further reduce the risk of postoperative cardiovascular complications. In this prospective, nonrandomized, observational study, baseline serum levels of sirtuin 1 (SIRT1) were assessed in 147 diabetic patients scheduled for LER due to CLTI, and participants were followed for the occurrence of MALE and MACE over 12 months. Fifty-three patients experienced MALE, and 33 experienced MACE within the follow-up period. Lower baseline SIRT1 levels were significantly associated with an increased risk of MALE and MACE, independent of other risk factors. The ROC curve analysis identified a SIRT1 cutoff of 3.79 ng/mL for predicting the risk of MALE. Moreover, incorporating SIRT1 into predictive models significantly enhanced the accuracy of predicting adverse outcomes. Results suggest serum SIRT1 is a potential independent marker for predicting MALE and MACE in diabetic patients with CLTI undergoing LER. Further research is needed to clarify the mechanistic pathways in which SIRT1 may influence cardiovascular outcomes, and the role of this novel biomarker in the management of PAD and CLTI among patients with diabetes.

Keywords Diabetes mellitus, Peripheral artery disease (PAD), Sirtuin 1

Peripheral arterial disease (PAD) is a manifestation of atherosclerosis[1]. As with the other locations affected by atherosclerotic disease, including the coronary and cerebrovascular vasculature, PAD has significant patient variability. While some patients may remain symptom-free for years or even for their entire lifespan, others may develop chronic limb-threatening ischemia (CLTI), a severe form of PAD that can lead to major adverse limb events (MALE), such as acute ischemia, gangrene and amputation[2]. Type 2 diabetes mellitus (T2DM) is one of the most relevant risk factors for the development and progression of PAD, together with hypercholesterolemia and smoking, and patients with diabetes and CLTI very often experience MALE[3]. Moreover, diabetes represents the most critical cause of nontraumatic lower limb amputation, and when PAD is added to diabetes, patients have an even greater risk of MALE[4]. In addition to diabetes, multiple biomarkers for amputation risk have been named in patients with PAD, including the platelet-hemoglobin ratio, a readily obtainable parameter in all patients[5]. In the case of CLTI, lower extremity revascularization (LER) intervention is necessary, which can be performed via open surgery or the endovascular route[6]. In the period following LER, patients with PAD have an increased risk of major cardiovascular events (MACE) and MALE[7]. Current guidelines advocate for comprehensive risk-modifying therapy, including dual antiplatelet therapy (DAPT), cholesterol-lowering treatments, glucose control, and GLP-1 agonists[8,9]. Despite adhering to these advanced guided care interventions, many patients still

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experienced MACE and MALE after LER[10]. This underscores the need to identify reliable novel biomarkers that reflect alternative pathophysiological pathways and function as re-stratification tools and perhaps therapeutic targets. These biomarkers would facilitate more precise monitoring and management, aiming to enhance outcomes and reduce the incidence of severe complications[11].

Sirtuin 1 (SIRT1) has drawn increasing interest as a potential biomarker for atherosclerosis due to its crucial role in various biological functions[12]. As class III histone deacetylases, sirtuins (SIRTs) are integral to many cellular processes[12]. SIRT1 plays key roles in modulating inflammation. It inhibits the inflammatory effects of TNF-α by inactivating and decreasing its release from macrophages[13]; reduces IL-1-induced inflammation via Toll-like receptor 2[14], and mitigates IL-6-driven inflammation by lowering its levels and expression[15]. In addition to its anti-inflammatory effects, SIRT1 is crucial for glucose metabolism, enhancing glycemic control and insulin sensitivity[16]. It also participates in lipid metabolism, potentially offering lipid-lowering benefits, as shown in in vivo studies[17]. Due to its extensive influence on the fundamental aspects of atherosclerosis, SIRT1 is considered protective against the disease, notably by improving endothelial dysfunction through eNOS activation and reducing oxidative stress[18]. Moreover, SIRT1 plays a crucial role in a complex network of interactions in the metabolism of nitric oxide (NO)[19], employing both direct and indirect mechanisms to regulate the balance between oxidant and antioxidant systems[20]and angiogenesis[21]. Ultimately, SIRT1 helps to maintain endothelial function and integrity[18]. Furthermore, SIRT1 promotes the FOXO3 and p53 deacetylation, reducing cellular senescence and apoptosis and promoting cell survival[22]. Interestingly, SIRT1 is targeted by molecules such as miR-217, which are beneficial for atherosclerosis treatment, thus showing SIRT1's therapeutic potential[23].

The observed association between decreased SIRT1 levels and an increased risk of cardiovascular disease (CVD) highlights SIRT1's potential role as a risk predictor or tool for therapeutic monitoring, offering hope for more precise monitoring and management.

To evaluate SIRT1 as a potential biomarker, this study investigated the relationship between plasma SIRT1 levels in patients with diabetes and CLTI undergoing LER and their later risk of MALE and MACE.

Results

Characteristics of the study population

In this study, we followed a cohort of 147 patients until the study's conclusion. The majority were male, representing over two-thirds of the included participants. About one quarter of the patients were active smokers, and over half were former smokers. Most of the individuals had hypertension and hypercholesterolemia. About half of the participants had a history of coronary heart disease (CAD). Regarding PAD, the average ABI value was significantly reduced, and most patients fell into category 5, according to the Rutherford classification. The detailed characteristics of the study population are presented in Supplemental Table 1.

The demographic and clinical characteristics of our study population intrinsically reflect a substantial risk for cardiovascular complications, with a predominance of male patients and a significant prevalence of conventional cardiovascular risk factors such as arterial hypertension and hypercholesterolemia.

Serum levels of SIRT1 and incidence of MALE at 12 months

Follow-up 12 months after LER intervention showed 53 patients with MALE. The detailed characteristics of the patients with and without MALE are presented in Table 1. Notably, most patients who experienced MALE were younger and exhibited higher levels of HbA1c. However, no further differences were noted in other conventional cardiovascular risk factors. In evaluating the characteristics of PAD patients, no significant differences were seen in the ABI value. Nevertheless, notable differences appeared in the Rutherford classification. Specifically, most patients with MALE were classified as having Rutherford stage 5 disease, whereas stage 4 disease was less prevalent among patients who experienced MALE. Interestingly, the baseline levels of SIRT1 were lower in patients with MALE.

Logistic regression analysis revealed that baseline SIRT1 levels were an independent determinant of MALE in diabetic patients with CLTI 12 months after LER (Table 2). To identify factors significantly contributing to the risk of developing MALE, the multivariable logistic regression considered various variables, including patient demographics, clinical history, laboratory results, and disease characteristics. Age and the ABI value were identified as significant independent predictors of MALE (Table 2). SIRT1 levels and ABI values correlated across the study population (Supplemental Fig. 1). Levels of SIRT1 remained an independent predictor of MALE even after adjusting for multiple potential confounding factors throughout the follow-up period, as detailed in Table 2. This trend persisted after running a multivariate model using only the variables identified in the univariate analysis (age, Rutherford II-4, Rutherford III-5, HbA1c); SIRT1 was still a predictor of MALE (Supplemental Table 2).

ROC analysis established a specific cutoff value for SIRT1 to optimize the MALE. The identified cutoff value of 3.79 ng/mL exhibited an acceptable balance between sensitivity (49%) and specificity (95%). Utilizing this cutoff, a two Kaplan–Meier survival curve was plotted to compare the incidence and timing of MALE between groups.

Survival analysis revealed that patients with baseline SIRT1 levels below this threshold experienced a significantly greater incidence of MALE, and these adverse events occurred earlier in these patients than in their counterparts with higher SIRT1 levels (Fig. 1).

Improvement in the prediction of MALE after the addition of SIRT1 values to established clinical and laboratory risk factors

The baseline ROC model comprised age, sex, BMI, high blood pressure, diabetes duration, smoking status, Rutherford classification, history of cardiovascular and cerebrovascular events, total cholesterol, LDL cholesterol,

	MALE			MACE		
	NO ($n=94$)	YES ($n=53$)	p value	NO ($n=114$)	YES ($n=33$)	p value
Men/female, n	61:33	38:15	0.40	79:35	20:13	0.35
Age, years ± SD	76.7 ± 8.6	72.6 ± 9.2	< 0.01	75.1 ± 8.7	75.7 ± 10.2	0.75
Diabetes duration, years ± SD	15.7 ± 13.8	19.1 ± 13.3	0.16	15.2 ± 13.6	23.3 ± 12.3	< 0.01
BMI, kg/m^2 (IQR)	25.6 (23.4–28.7)	24.9 (23.2–28.4)	0.77	25.4 (22.7–28.5)	26.4 (24.0-31.1)	0.08
Smoking (current), n (%)	21 (22.3)	13 (24.5)	0.76	27 (23.7)	7 (21.2)	0.77
Smoking (former), n (%)	47 (50.0)	30 (56.6)	0.44	59 (51.7)	18 (54.5)	0.78
Never smoked, n (%)	26 (27.7)	10 (18.9)	0.23	28 (24.6)	8 (24.2)	0.97
Hypertension, n (%)	78 (83.0)	40 (75.5)	0.27	91 (78.8)	27 (81.8)	0.80
Hypercholesterolemia, n (%)	84 (89.3)	51 (96.2)	0.14	105 (92.1)	30 (90.9)	0.82
CAD, n (%)	43 (45.7)	27 (50.9)	0.54	49 (43.0)	21 (63.6)	0.04
CVD, n (%)	15 (16.1)	14 (26.9)	0.12	20 (17.5)	9 (29.0)	0.16
Metformin, n (%)	26 (28)	17 (32)	0.57	37 (32.5)	6 (18.2)	0.11
GLP-1 receptor agonist, n (%)	7 (7)	4 (8)	0.98	10 (8.8)	1 (3.0)	0.27
SGLT2 inhibitor, n (%)	8 (9)	7 (13)	0.37	13 (11.4)	2 (6.1)	0.37
DPP4 inhibitor, n (%)	14 (15)	5 (9)	0.34	17 (14.9)	2 (6.1)	0.18
Other antidiabetics, n (%)	10 (11)	4 (8)	0.54	12 (10.5)	2 (6.1)	0.44
Insulin, n (%)	38 (40)	26 (49)	0.31	44 (38.6)	20 (60.6)	0.03
Statin, n (%)	65 (69)	40 (75)	0.42	81 (71.1)	24 (72.7)	0.85
Ezetimibe, n (%)	27 (29)	21 (40)	0.18	38 (33.3)	10 (30.3)	0.74
ACE inhibitors/ARB, n (%)	54 (57)	32 (60)	0.73	70 (61.4)	16 (48.5)	0.18
Other antihypertensives, n (%)	42 (45)	26 (49)	0.61	54 (47.4)	14 (42.4)	0.62
Aspirin, n (%)	51 (54)	34 (64)	0.24	64 (56.1)	21 (63.6)	0.44
Clopidogrel, n (%)	25 (27)	19 (36)	0.24	28 (24.6)	16 (48.5)	< 0.01
Other antiplatelets, n (%)	4 (4)	3 (6)	0.70	6 (5.3)	1 (3.0)	0.60
ABI, (IQR)	0.4 (0.3–0.4)	0.4 (0.3–0.4)	0.34	0.4 (0.3–0.4)	0.4 (0.3–0.4)	0.38
Rutherford II-4, n (%)	48 (51.6)	14 (26.4)	< 0.01	52 (45.6)	10 (30.3)	0.12
Rutherford III-5, n (%)	46 (48.9)	39 (73.6)	< 0.01	62 (54.4)	23 (69.7)	0.12
HbA1c, % (mmol/mol) ± SD	6.9 ± 1.1	7.6 ± 1.4	< 0.01	7.0 ± 1.2	7.4 ± 1.3	0.12
FBG, mg/dL (IQR)	120.0 (95.0-146.0)	123.0 (101.0-159.0)	0.18	119.0 (95.0-146.0)	124.0 (113.0-166.0)	0.09
Total cholesterol, mg/dL ± SD	135.0 ± 35.6	132.3 ± 38.3	0.67	137.0 ± 36.8	123.5 ± 33.8	0.07
LDL cholesterol, mg/dL (IQR)	70.0 (51.0–88.0)	60.0 (46.0–78.0)	0.23	70.0 (52.0–87.0)	55.0 (41.0–81.0)	0.05
Triglycerides, mg/dL ± SD	118.3 ± 65.2	114.4 ± 38.6	0.69	117.9 ± 59.5	113.3 ± 46.7	0.69
Creatinine, mg/dL ± SD	1.4 ± 1.6	1.6 ± 1.7	0.39	1.3 ± 1.3	2.1 ± 2.4	< 0.01
eGFR, mL/min/1.73m^2 ± SD	83.1 ± 25.7	77.4 ± 22.5	0.18	83.1 ± 20.6	73.9 ± 34.7	0.60
Sirtuin 1, ng/mL (IQR)	5.1 (0.8–8.4)	1.6 (0.4–1.3)	< 0.01	4.1 (0.6–6.9)	2.6 (0.9–2.4)	0.04

Table 1. Demographic and clinical data of the study participants without or with MALE and MACE. The data are reported as the means ± standard deviations or median (interquartile range 25–75) for continuous variables and as numbers (percentages) for categorical variables. Statistical tests were performed with Student's t test, chi-square test or Wilcoxon Rank-Sum (Mann-Whitney) test, when appropriate. BMI, body mass index; CAD, coronary artery disease; CBVD, cerebrovascular disease; ABI, ankle brachial index; FBG, fasting blood glucose; eGFR, estimated glomerular filtration rate.

triglycerides, fasting blood glucose, and HbA1c. When comparing ROC curves, we evaluated the initial model—relying solely on clinical and laboratory factors—against a refined model that incorporated SIRT1 levels, with the findings presented in Fig. 2 for MALE. The integration of SIRT1 levels markedly enhanced the ability of the model to predict MALE. Specifically, the area under the ROC curve (AUC) increased from 0.79 in the baseline Model 1 to 0.90 in the Model 2, including SIRT1, demonstrating a significant improvement.

Serum levels of SIRT1 and incidence of MACE at 12 months

Over the 12-month follow-up period, 33 patients experienced a MACE following their revascularization procedure. Comparative analysis between patients with and without MACE revealed no significant differences in sex, age, smoking habits, history of hypertension or hypercholesterolemia, previous cerebrovascular disease (CBVD), ABI, Rutherford category, total cholesterol, LDL levels, HbA1c levels, or eGFR. However, patients with MACE had a significantly greater BMI, longer diabetes duration, greater likelihood of having a history of CAD, and greater creatinine levels than those without MACE. Conversely, individuals without MACE had higher serum levels of SIRT1. The detailed characteristics of both groups are outlined in Table 2.

	Coef.	St.Err.	t value	*p* value	[95% Conf	Interval]	Sig
Age	−0.01	0.01	-2.30	0.02	−0.02	0	**
Male sex	−0.02	0.1	-0.23	0.82	−0.23	0.18	
BMI	−0.01	0.01	-0.85	0.4	−0.02	0.01	
Diabetes duration	0	0	0.64	0.52	−0.01	0.01	
Hypertension	−0.19	0.12	-1.63	0.11	−0.42	0.04	
Hypercholesterolemia	0.12	0.17	0.70	0.49	−0.23	0.47	
CAD	−0.05	0.1	-0.46	0.65	−0.24	0.15	
CVD	0.15	0.11	1.29	0.2	−0.08	0.38	
Smoking (current)	0	.	.	.	.	.	
Smoking (former)	0.02	0.11	0.19	0.85	−0.2	0.24	
Never smoked	−0.06	0.14	-0.42	0.67	−0.33	0.21	
ABI	3.03	0.91	3.33	0	1.22	4.84	**
Rutherford II-4	−0.15	0.1	-1.54	0.13	−0.34	0.04	
Rutherford III-5	0	.	.	.	.	.	
LDL cholesterol	0	0	-1.81	0.07	−0.01	0	
FPG	0	0	-0.21	0.83	0	0	
HbA1c	0.04	0.05	0.85	0.4	−0.06	0.14	
Creatinine	−0.01	0.04	-0.15	0.88	−0.08	0.07	
eGFR	0	0	-0.73	0.47	−0.01	0	
Sirtuin 1	−0.06	0.01	-4.61	0	−0.09	−0.04	**
Constant	0.75	0.82	0.92	0.36	−0.87	2.38	
Mean dependent var		0.370		SD dependent var		0.485	
R-squared		0.428		Number of obs		100	
F test		3.605		Prob > F		0.000	
Akaike crit. (AIC)		118.348		Bayesian crit. (BIC)		165.241	

Table 2. Multivariate logistic regression for MALE. ** $p < 0.01$.

Multivariate analysis incorporating all variables revealed that the ABI value was a statistically significant independent predictor of MACE. Importantly, even after adjusting for all conventional risk factors, the serum level of SIRT1 remained an independent predictor of MACE, as detailed in Table 3.

ROC curve analysis for MACE revealed an AUC of 0.4811 (Supplemental Fig. 2). As this value is close to 0.5, it suggests that serum SIRT1 levels do not have significant discriminatory power for predicting MACE. We also generated Kaplan-Meier curves for MACE incidence, stratified by serum SIRT1 levels using the 3.79 ng/mL cutoff (Supplemental Fig. 3). However, the log-rank test yielded a chi-square value of 0.16 and a p-value of 0.6864, indicating no statistically significant difference in MACE-free survival between the two groups.

Discussion

Alongside traditional biomarkers such as low-density lipoprotein (LDL) cholesterol and glycosylated hemoglobin, recent efforts have focused on identifying new factors that could help define the risk profile for patients with diabetes and PAD[24]. This investigation examined diverse biological pathways and a wide array of molecules. Given the pivotal role of inflammation in the development of atherosclerosis, researchers have extensively studied markers such as C-reactive protein (CRP) and interleukins (IL-1 and IL-6), using various experimental scenarios[7]. Studies have confirmed that elevated levels of these inflammatory markers correlate with a greater risk of MACE and MALE following LER[11]. Additionally, tumor necrosis factor (TNF)-α has been recognized for its contribution to cardiovascular risk in patients with diabetes and PAD[25]. Although LDL cholesterol is a critical marker and target for managing risk, investigations have sought to identify other subtle risk determinants by examining additional factors involved in LDL cholesterol metabolism, such as Sortilin[26], and processes such as LDL oxidation and the development of foam cells in atherosclerotic plaques, including studies on Omentin 1[27]. Nonetheless, these cytokines alone have not fully explained the variability in vascular outcomes following LER observed among patients with diabetes and PAD.

This study sheds light on the complex relationship between serum SIRT1 levels and the occurrence of MALE and MACE in a cohort of patients with diabetes and CLTI following LER. These findings align with the recognized natural history of PAD, in which more advanced clinical stages correlate with a heightened risk of MALE[28]. Interestingly, we observed no significant differences in other cardiovascular risk factors between groups except for a variation in the levels of SIRT1, suggesting that this molecule plays a vital role in the pathophysiology and phenotypical variability of MACE and MALE in our patients[29].

The demographic and clinical characteristics of our study population intrinsically reflect a considerable risk for cardiovascular complications, with a predominance of male patients and a significant prevalence of conventional cardiovascular risk factors such as arterial hypertension and hypercholesterolemia. Interestingly, despite widespread dyslipidemia, average LDL cholesterol levels were notably low, suggesting effective

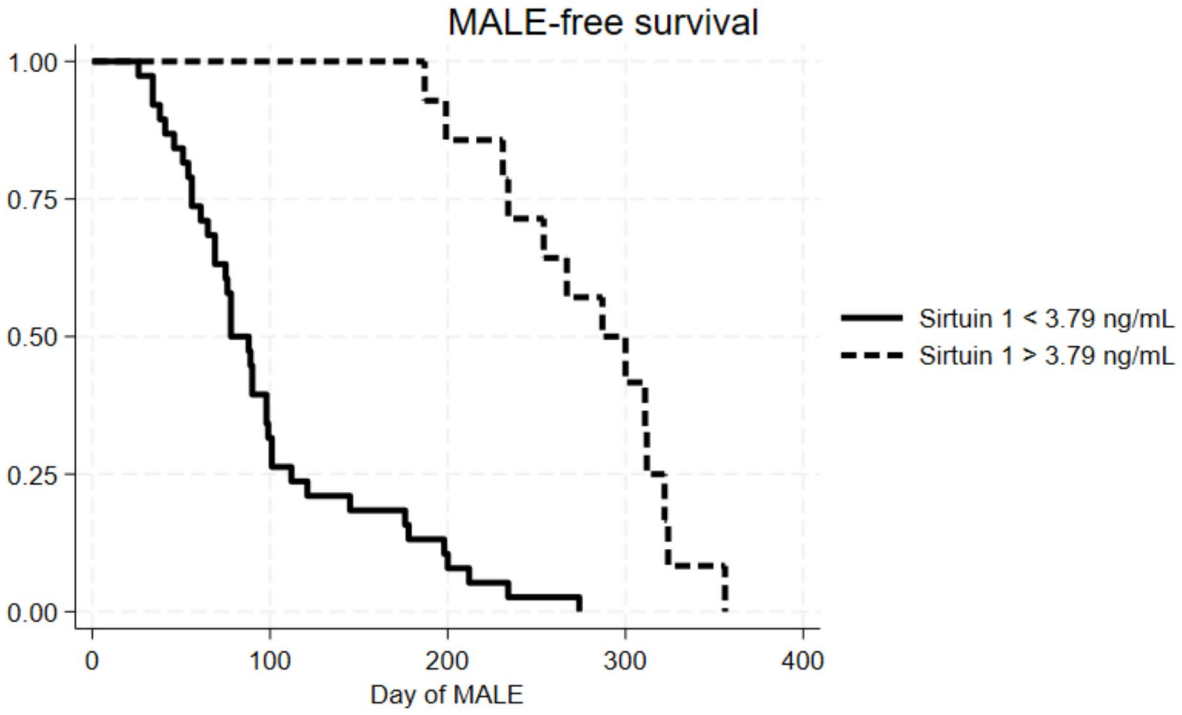

Fig. 1. The freedom from MALE according to the cutoff of Sirtuin 1 was estimated using the Kaplan–Meier method and compared using the log-rank test ($P < 0.01$). The two groups of Sirtuin 1 are listed in the pattern code. The dashed line represents a Sirtuin 1 concentration > 3.79 ng/mL, and the solid line represents a Sirtuin 1 concentration < 3.79 ng/mL.

management in line with the latest European guidelines[30]. Our findings that a quarter of the study participants never smoked and the rest were former or active smokers are consistent with published data from other similar longitudinal and observational PAD cohorts[31].

Baseline levels of SIRT1 notably influenced the incidence of MALE within 12 months post-LER. Patients who developed MALE were, on average, younger and had higher HbA1c levels than those who did not, suggesting a possible interplay between SIRT1 levels and poor glycemic control, which is a well-established risk factor for adverse limb outcomes in diabetic patients. This finding aligns with the literature, emphasizing the importance of tight glucose control in patients with diabetes to mitigate vascular complications[32]. By significantly influencing glucose metabolism, SIRT1 mediates diet and energy regulation[33]. This protein carries this out by deacetylating various transcription factors and enzymes critical to glucose production, insulin secretion, and insulin response[34]. By increasing the insulin signaling pathway, SIRT1 enhances insulin responsiveness in the liver and muscles, promoting glucose absorption and lowering blood glucose concentrations[29]. Additionally, it regulates gluconeogenesis in the liver, facilitating steady glucose levels during fasting[35]. Given the strong association between SIRT1 and glycemic regulation, it is plausible that the observed differences in patients experiencing MALE, particularly regarding HbA1c and SIRT1 levels, are interconnected in our population[28]. Our logistic regression analysis further confirmed SIRT1 as an independent determinant of MALE. The correlation between reduced SIRT1 levels and an elevated risk of MALEs contributes to the expanding body of evidence supporting the protective roles of SIRT1 in CVD. The identified relationship between SIRT1 levels and ABI values reinforces the idea that SIRT1 could play a significant role in the progression of PAD and potentially influence the prognosis of limb health. This correlation shows that the influence of SIRT1 extends beyond metabolic regulation to affect vascular health and the severity of PAD. This could, in turn, affect outcomes for patients with compromised limb vascularization. These findings suggest broader implications for SIRT1 in vascular pathology and support its potential as a biomarker for assessing disease severity and predicting limb outcomes in PAD patients. The conclusions of the survival analysis are particularly telling, revealing that individuals with SIRT1 concentrations falling below the threshold face a significantly heightened risk of encountering MALE. Furthermore, these events tend to manifest earlier than in patients with higher levels of SIRT1. This pattern highlights the predictive value of SIRT1 as a time-sensitive biomarker predictor of MALE. This finding underscores the potential of incorporating SIRT1-level measurement into clinical practice for more informed monitoring and intervention strategies to mitigate the risk of limb-threatening events in vulnerable patient populations.

Interestingly, our study also explored the predictive value of SIRT1 for MACE. In this context, in line with previous reports[27,36], both the duration of diabetes and a prior history of CAD constituted significant factors among patients who experienced MACE, although these findings were not corroborated in the multivariable

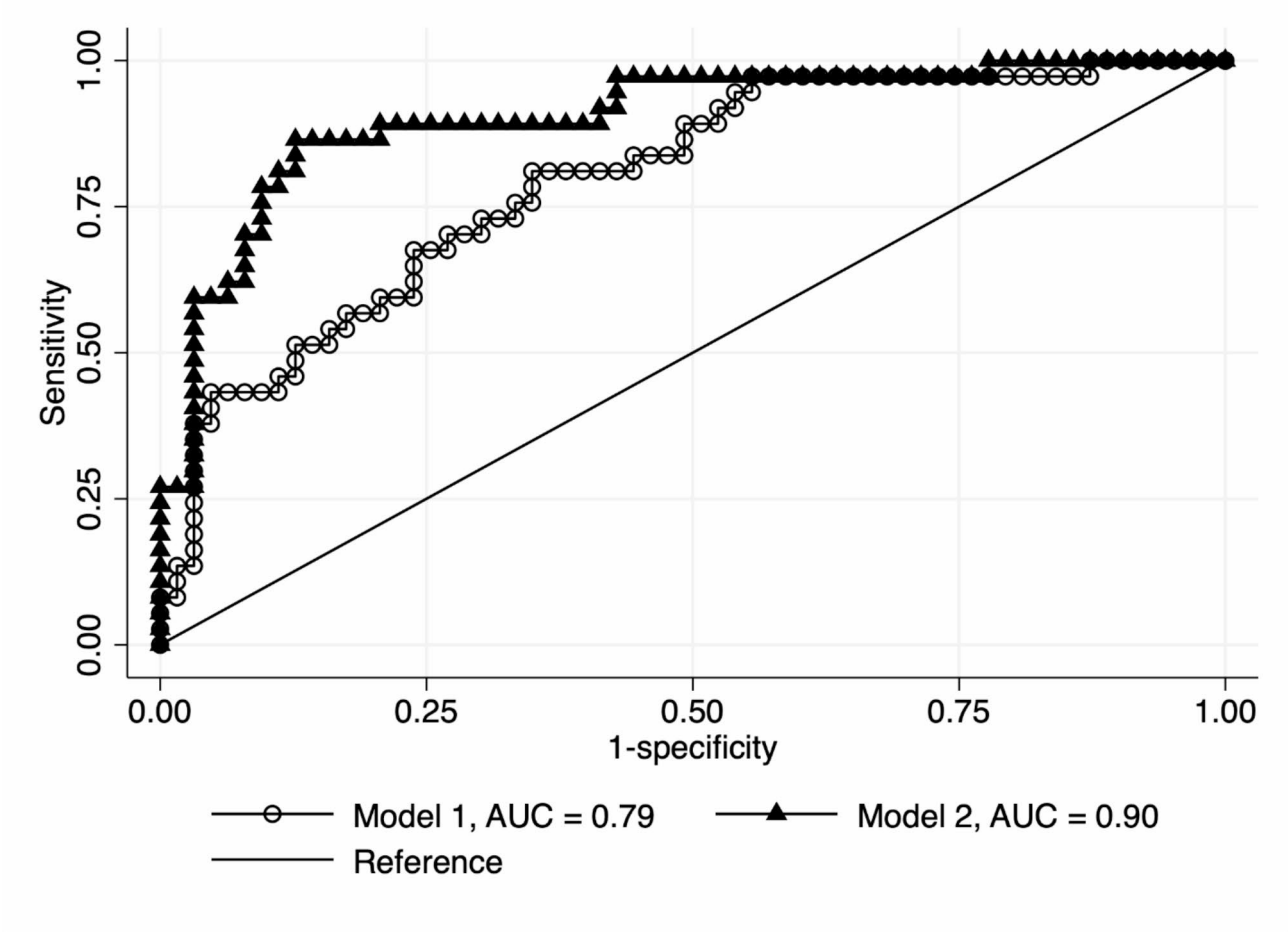

Fig. 2. Receiver operating characteristic (ROC) curves comparing the performance of a model without (Model 1) and with (Model 2) Sirtuin 1 levels in predicting major adverse limb events (MALE). The true-positive rate (sensitivity) is plotted as a function of the false-positive rate (1 - specificity). $p < 0.01$.

analysis. Similar to their predictive value observed in MALE, SIRT1 levels appeared as an independent predictor of MACE, even after adjustment for traditional risk factors. This finding suggests that the role of SIRT1 extends beyond limb health, potentially offering insights into broader cardiovascular risk stratification in this high-risk population.

Incorporating SIRT1 into a model including established clinical and laboratory risk factors significantly enhanced the prediction of MALE, as shown by an increased area under the ROC curve. This improvement shows that SIRT1 could be an additive marker, improving the predictive accuracy for adverse outcomes post-LER beyond what is possible with traditional risk factors alone.

Our study's findings underscore the role of SIRT1 measurement in the risk assessment of patients undergoing LER for CLTI. The results support its use as a novel biomarker that could refine predictive models for both limb and cardiac outcomes. The significant association between low SIRT1 levels and a greater incidence of MALE and MACE underlines the potential for targeted interventions[37]. It aims to modulate SIRT1 activity as a therapeutic strategy to reduce the burden of cardiovascular complications in this susceptible population.

This study, while showing significant promising insights, has limitations that are important to consider. The prospective study design is essential for highlighting the observed associations. Still, generalizability to a wider population may require reproducibility in a larger cohort with a broader scope of age, gender, racial-ethnic diversity, and geographical location. Furthermore, our study population was highly selective due to stringent inclusion and exclusion criteria. However, this selectivity, combined with the limited number of patients available, has resulted in a smaller sample size than in studies with broader inclusion criteria. This limitation stems from the study's execution within a single center and the distinct demographic attributes of the participant cohort. Additionally, the potential for unaccounted confounding factors exists, which may influence the observed outcome. Variables such as post-LER lifestyle modifications, adherence to prescribed medications, and the severity of coexisting health conditions—all of which could affect the risk of MALE and MACE—were not comprehensively examined. Another notable limitation is the reliance on a singular measurement of SIRT1 levels at the time of LER, which might not accurately reflect fluctuations in SIRT1 expression over time or in response to the procedure. Future longitudinal studies that monitor SIRT1 levels at various points following LER could shed light on the temporal dynamics of SIRT1 and their correlation with adverse outcomes. Moreover, the

	Coef.	St.Err.	t value	*p* value	[95% Conf	Interval]	Sig
Age	0.01	0.01	1.01	0.31	−0.01	0.02	
Male sex	−0.07	0.11	-0.63	0.53	−0.28	0.14	
BMI	0.01	0.01	1.20	0.23	−0.01	0.03	
Diabetes duration	0	0	0.30	0.77	−0.01	0.01	
Hypertension	0.09	0.12	0.76	0.45	−0.14	0.32	
Hypercholesterolemia	−0.08	0.18	-0.42	0.67	−0.43	0.28	
CAD	0.16	0.1	1.57	0.12	−0.04	0.36	
CVD	0.07	0.12	0.64	0.52	−0.16	0.31	
Smoking (current)	0	.	.	.	.	.	
Smoking (former)	−0.1	0.11	-0.92	0.36	−0.33	0.12	
Never smoked	−0.19	0.14	-1.37	0.17	−0.46	0.08	
ABI	1.88	0.93	2.02	0.05	0.03	3.72	*
Rutherford II-4	−0.03	0.1	-0.32	0.75	−0.23	0.16	
Rutherford III-5	0	.	.	.	.	.	
LDL cholesterol	0	0	0.37	0.71	0	0	
FPG	0	0	0.24	0.81	0	0	
HbA1c	0.05	0.05	0.99	0.33	−0.05	0.15	
eGFR	0.07	0.04	1.79	0.08	−0.01	0.14	
Creatinine	0	0	0.09	0.93	−0.01	0.01	
Sirtuin 1	−0.03	0.01	-2.06	0.04	−0.06	0	*
Constant	-1.61	0.83	-1.94	0.06	-3.26	0.04	
Mean dependent var		0.260		SD dependent var		0.441	
R-squared		0.284		Number of obs		100	
F test		1.917		Prob > F		0.028	
Akaike crit. (AIC)		121.506		Bayesian crit. (BIC)		168.399	

Table 3. Multivariate logistic regression for MACE. * $p < 0.05$.

relationship between ABI values and SIRT1 levels suggests that the impact of ABI values might differ depending on SIRT1 values. However, exploring this hypothesis further was impossible given the limited number of events and patients. Furthermore, the relatively short duration of the follow-up is also an important limitation. In fact, MACE typically occur over a long period, and the follow-up in this study may not have captured a sufficient number of events to yield meaningful data. Finally, the absence of external validation for the predictive value of SIRT1 limits its reliability as an effective biomarker and underscores the need for further research to confirm its utility.

The main strength of the study relies on the demonstration of SIRT1 as a novel biomarker that can be used to predict patients at risk for adverse outcomes post-LER. SIRT1 has an established role regulating inflammation, glucose metabolism, and lipid metabolism. The correlation we observed with MALE and MACE highlights the complex nature of atherosclerosis in individuals with diabetes. This study has other intrinsic design characteristics that enhance the study's relevance and applicability, including a prospective cohort design, detailed data collection methods, exhaustive clinical, laboratory, and demographic information of a cohort of patients with diabetes.

Further research is called for to elucidate the mechanisms by which SIRT1 influences cardiovascular outcomes in CLTI patients and to explore the therapeutic potential of SIRT1 modulation. Our study provides a foundation for such inquiries, highlighting the importance of integrating novel biomarkers into clinical practice to enhance the precision of risk stratification and to pave the way for personalized therapeutic approaches.

In conclusion, this study underscores the significant role of serum SIRT1 levels as a novel and independent predictor of MALE and MACE in patients with diabetes and CLTI undergoing LER. Against the backdrop of PAD and its complexities, our findings highlight the importance of incorporating novel biomarkers such as SIRT1 into clinical evaluations, especially in populations with diabetes. The study's evidence suggests that SIRT1 not only serves as a biomarker for risk stratification but also opens avenues for therapeutic interventions aimed at modulating its activity to mitigate cardiovascular complications. The potential of SIRT1 to improve prediction models for MALE, even beyond traditional risk factors, signifies a step forward in personalizing treatment strategies and enhancing patient outcomes. While our study lays the groundwork for the integration of SIRT1 into clinical practice, further research is needed to explore the underlying mechanisms and therapeutic potential of SIRT1 modulation in patients with PAD and CLTI. Through such endeavors, we can hope to refine our approach to managing this high-risk patient population, ultimately reducing the incidence of life-threatening complications post-LER.

Methods

Aim and study design

This study aimed to investigate the association between serum levels of SIRT1 at the time of LER and the occurrence of MALE and MACE in a group of patients with T2DM, PAD, and CLTI. This prospective, nonrandomized study received approval from the Ethics Committee of the Fondazione Policlinico Universitario A. Gemelli (Istituto di Ricovero e Cura a Carattere Scientifico - IRCCS), the "Comitato Etico Territoriale Lazio Area 3." Participation was voluntary, with all patients providing informed consent by the ethical standards of the Declaration of Helsinki, and all research was performed according to relevant guidelines/regulations.

Study population and clinical assessment

Participants included 147 patients with T2DM, PAD and CLT who needed revascularization procedures. They were recruited from the Fondazione Policlinico Universitario A. Gemelli IRCCS in Rome, Italy, and were enrolled sequentially from October 23, 2019, to October 27, 2022.

The inclusion criteria were as follows: 18 years or older, a diagnosis of T2DM for a minimum of 1 year; an ankle/brachial index (ABI) of less than 0.9; at least one lower extremity artery stenosis of more than 50% as identified by color Doppler ultrasound; a diagnosis of stage 4 or 5 PAD according to the Rutherford classification; and CLTI with a clear indication for LER due to target artery stenosis, as previously described[38]. The exclusion criteria included recent LER (within the past 3 months); active infection or osteomyelitis in diabetic foot ulcers; diabetic peripheral neuropathy; homozygous familial hypercholesterolemia; any contraindication to antiplatelet therapy; thrombophilia; severe anemia requiring transfusion; active cancer; ongoing autoimmune disease; liver disease classified as Child–Pugh stage B or C; a life expectancy of less than 12 months; or pregnancy.

Assessment of diabetic foot ulcers was performed with the Wound, Ischemia, and Foot Infection (WIfI) classification system. Radiographic studies were conducted as necessary to exclude osteomyelitis, and diabetic peripheral neuropathy was assessed according to established methods[7]. PAD was defined following the guidelines of the Society for Vascular Surgery and the International Society for Cardiovascular Surgery[6]. Ultrasound was used to examine all patients' lower extremities, aiding in the identification of significant arterial stenosis, especially in cases of arterial calcification where the ABI exceeded 1.40.

The study collected comprehensive clinical and laboratory data on each participant: a detailed medical history with emphasis on CAD, CBVD, hypertension, hypercholesterolemia, smoking status, body mass index (BMI), and specific blood tests.

All the patients were on lipid-lowering medication aimed at achieving an LDL cholesterol level below 55 mg/dL. At the time of revascularization, every patient was administered single antiplatelet therapy, transitioning to DAPT for 1 month post-procedure.

Endovascular revascularization procedure and follow-up

LER procedures were carried out following the methods outlined in previous reports[7,26,27,38]. The procedures, including angioplasty and arterial stenting when needed, were considered successful if postoperative arterial stenosis was reduced to less than 30% of the arterial lumen. From the initial cohort of 155 patients, 8 (5.2%) were excluded from subsequent follow-up due to failure of the primary treatment to achieve this success criterion immediately after revascularization. Notably, there were no significant perioperative complications according to criteria established by the Society of Interventional Radiology[39].

Throughout the 12-month follow-up period, patient outcomes were monitored at intervals of 1-, 3-, 6-, and 12-months post-LER to determine the incidence of MALE and MACE. The median follow-up of the patients was 13.1 months. The primary endpoint of the study was the incidence of MALE, defined as major limb-related complications, including limb amputation (above the ankle), major revascularization failure (requiring surgical or interventional intervention for graft occlusion, stenosis, or significant limb ischemia), and recurrent critical limb ischemia requiring further treatment or hospitalization. The secondary endpoints were the occurrence of MACE, defined as major cardiovascular events, including cardiovascular death (due to myocardial infarction, stroke, or other cardiovascular causes); non-fatal myocardial infarction; non-fatal stroke; and hospitalization for heart failure or urgent revascularization for unstable angina or symptomatic CAD.

Blood tests and biochemical analysis

On the LER day, blood samples were taken from all patients following an overnight fast to assess glucose, creatinine, total cholesterol, LDL cholesterol, triglycerides, and glycated hemoglobin (HbA1c) levels. Renal function was determined using the Modification of Diet in Renal Disease (MDRD) formula, which estimates the glomerular filtration rate (eGFR)[40]. After collection, the blood samples were centrifuged to separate the serum, which was then stored at -80 °C until analysis. SIRT1 concentrations were measured using an ELISA kit (EH3785 from Fine Test, Wuhan, China) following the manufacturer's instructions. The precision of these measurements was reflected in the intra-assay and inter-assay coefficients of variation, which were 3.5% and 10.5%, respectively. The assay's sensitivity was determined to be 0.188 ng/mL, based on the mean $\pm$ 3 standard deviations of the 0 standard. Serum levels were quantified twice for each patient, and the measurements were averaged for accuracy.

Statistical analysis

For the power analysis and sample size calculation, we considered an effect size (Cohen's d) of 0.4, an alpha level of 0.05, and a desired power of 0.8. Using these parameters, we calculated the required sample size to be 150. The calculation was performed using Stata's "power" command. The demographic and clinical characteristics are presented as the means with standard deviations or median (interquartile range 25–75) for continuous data and as counts with percentages for categorical data. To compare groups, chi-square tests and t tests were used

where suitable. For variables not following a normal distribution, a logarithmic transformation was applied prior to further analysis. The baseline levels of SIRT1 were compared using Mann–Whitney tests, Kruskal–Wallis tests, and Dunn's multiple comparison tests when appropriate. Multivariate stepwise logistic regression was conducted, adjusting for proven atherosclerotic risk factors as well as baseline levels of SIRT1. Additionally, two receiver operating characteristic (ROC) curves were developed to test the predictive ability of MALE: Model 1 included only traditional risk factors (age, sex, BMI, high blood pressure, duration of diabetes, smoking status, Rutherford staging, previous cardiovascular and cerebrovascular events, total cholesterol, LDL cholesterol, triglycerides, fasting blood glucose, and HbA1c); and Model 2 incorporated these risk factors, along with SIRT1, as continuous variables. The areas under the ROC curves were compared to determine the accuracy of biomarker thresholds related to MALE and MACE outcomes. To find the best threshold for SIRT1 that effectively differentiates outcomes, the cutpt function in STATA was used for ROC analysis. Following the identification of the cutoff level for SIRT1, the probability of remaining free from MALE based on the SIRT1 threshold was evaluated using the Kaplan–Meier method and compared using the log-rank test. All the statistical analyses were performed using STATA version 17.0 for MacOS and GraphPad Prism version 10.2.1 for MacOS. A p value less than 0.05 was considered as showing statistical significance.

Data availability

The datasets generated during the current study are available from the corresponding author on reasonable request.

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Author contributions

FB and MMR conceived the study, participated in the design of the study, performed data analysis and reviewed the manuscript. MMR, ER and DP participated in the study enrollment and performed statistical analyses. FA, MS and MAN carried out the immunoassays. RI performed the endovascular procedures. LE and PD reviewed the manuscript. MM, AG and AF conceived the study, participated in its design and coordination and helped draft the manuscript. All authors read and approved the final manuscript.

Declarations

Competing interests

The authors declare no competing interests.

Guarantor's statement

Dr. Andrea Flex is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Additional information

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