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Original Article

Correlation of TNF-α, Cys C, and NLRP3 inflammasomes with venous ulcers in patients with lower extremity varicose veins

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Abstract: Objective: To explore the correlation of tumor necrosis factor α (TNF-α), cystatin C (Cys C), and NLR family pyrin domain containing 3 (NLRP3) inflammasomes with venous ulcers from lower extremity varicose veins. Methods: In this retrospective analysis, 135 patients with primary varicose veins of lower extremities were selected and divided into a varicose ulcer group (n=32) and a non-varicose ulcer group (n=103) according to clinical ulcer presence. Healthy adults with similar general information during the same period were included as a healthy controls (n=30). The levels of TNF-α, interleukin-1β (IL-1β), Cys C, and NLRP3 inflammasomes were statistically analyzed among the three groups. Logistic regression was used for analyzing the risk factors for venous ulcers in patients with varicose veins of the lower extremities. Spearman correlation was applied for correlation analysis. The area under the receiver operating characteristic (ROC) curve (AUC) was found to disclose the predictive value of TNF-α, Cys C, and NLRP3 inflammasomes for venous ulcers. Results: (1) Logistic regression analysis showed that TNF-α, IL-1β, and NLRP3 inflammasomes were risk factors for venous ulcers in patients with varicose veins of the lower extremity, and Cys C in ulcer wound tissue was a protective factor. (2) TNF-α was significantly correlated with IL-1β and Cys C in ulcer wound tissue, and NLRP3 in plasma (r=0.256, -0.290, 0.305; *P*=0.003, 0.001, <0.001). IL-1β was significantly correlated with CysC in ulcer wound tissue and plasma (r=-0.251, -0.193; *P*=0.003, 0.025). (3) The AUC, sensitivity, and specificity of TNF-α and NLRP3 inflammasomes for predicting varicose veins were high, with AUC of 0.881 and 0.712, sensitivity of 0.875% and 0.875%, and specificity of 0.893% and 0.738%, respectively. Conclusion: TNF-α in plasma, Cys C in ulcer wound tissue and plasma, and NLRP3 inflammasomes in plasma were closely related to the occurrence of venous ulcers in patients with varicose veins of the lower and may serve as new targets for treatment.

Keywords: TNF-α, Cys C, NLRP3 inflammasome, varicose veins of lower extremity, venous ulcer, correlation

Introduction

Varicose veins are a common venous disease of lower extremities, mostly caused by venous valve insufficiency, increased luminal pressure and blood regurgitation [1]. The incidence of varicose veins is higher in women than in men. The main clinical manifestations of this disease are pain and swelling of lower limbs, beaded veins, earthworm protrusions, and possible skin pigmentation. Complications may occur in severe cases [2]. Venous ulcers are one of the serious late complications of varicose veins of lower extremity. They are characterized by persistent and recurrent attacks, with an incidence of about 1.5% per year [3]. Venous hypertension can induce a chronic inflammatory response, promote the entry of white blood cells into the dermis, increase the tension of the dermis, and raise the iron ion level, which change the structure and morphology of macrophages and destroy the tissue structure, thus reducing the skin's ability to repair itself and inducing ulcers [4]. At present, the main principles of clinical treatments for varicose venous ulcers are to eliminate venous pressure and promote the healing of ulcer wounds. However, the healing time of an ulcer is relatively long, and the

continuous venous pressure and inflammatory reaction easily lead to a co-infection, which increases the difficulty of treatment and the risk of disability. Coleridge et al. [5] proposed the theory of leukocyte capture and pointed out that venous ulcers on the lower extremity were caused by a systemic inflammatory reaction, in which blood cells, blood components, and blood flow characteristics were involved. Subsequently, in accordance with many studies, the inflammatory reaction was found to be related to the occurrence of venous lesions and venous ulcers [6]. Tumor necrosis factor α (TNF-α) is a major inflammatory cytokine. Cystatin C (Cys C) and its fragments may affect the phagocytosis and chemotactic function of granulocytes, and it participate in the inflammatory process [7]. As a protein complex, the NLR family pyrin domain containing 3 (NLRP3) inflammasome regulates the synthesis of inflammatory mediators [8]. Therefore, we hypothesized that TNF-α, Cys C, and NLRP3 inflammasome may affect the occurrence of venous ulcers on the lower extremities, but there have been few relevant studies. For the above reasons, this study analyzed the clinical data of patients with primary varicose veins of the lower extremity, aiming to explore the correlation of TNF-α, Cys C and NLRP3 inflammasome with venous ulcers on the lower extremities, so as to provide a reference for further improving the prognosis of venous ulcers on the lower extremities.

Materials and methods

Patient sources

In this retrospective analysis, 135 patients with primary varicose veins of the lower extremity treated in the Cardiovascular and Cerebrovascular Disease Hospital, General Hospital of Ningxia Medical University from December 2019 to November 2021 were included. Patients were required to meet the following conditions for inclusion: (1) The diagnosis of primary varicose veins of lower extremity was confirmed by venography and ultrasound Doppler examination [9]; (2) The disease was first diagnosed as unilateral; (3) The patients were 18 years old or older; (4) No lower extremity soft tissue trauma and fracture occurred during the same period. Patients were excluded if they had (1) a definite history of limb varicose veins, (2) other lower extremity diseases such as diabetic foot or pressure ulcers, (3) incomplete medical records or laboratory examination data. The diagnostic criteria of venous ulcers were based on the *Diagnostic criteria of lower extremity venous ulcers* in *Modern Wound Repair* [10]. The patients were divided into a varicose ulcer group (n=32) and a non-varicose ulcer group (n=103) according to whether they were complicated with venous ulcers. Healthy adults with similar general information during the same period were included in a healthy group (n=30). The procedures of this research are shown in **Figure 1**. This study was approved by the Ethics Committee of the Cardiovascular and Cerebrovascular Disease Hospital, General Hospital of Ningxia Medical University.

Collecting data

By consulting the electronic medical records of patients, clinical data were collected, including: (1) general data: sex, age, course of disease, injured limb, pain of injured limb, calf swelling, wound area, hypertension, diabetes, alanine transaminase (ALT), creatinine, total cholesterol (TC), blood glucose, and albumin; (2) research indicators: TNF-α, interleukin-1β (IL-1β), Cys C, and NLRP3 inflammasome.

Determination of relevant indexes

(1) Determination of plasma TNF-α: The level of plasma TNF-α was determined by avidin biotin complex-enzyme linked immunosorbent assay (ELISA) with double antibody sandwich. First, anti-human TNF-α mAb was coated on the enzyme-labeled plate. Biotinylated anti-human TNF-α was added to form an immune complex connected to the plate. Next, horseradish peroxidase labeled Streptavidin was bound to the biotin. Then, the enzyme substrate TMB was added to terminate the reaction with sulfuric acid, and the absorbance value was measured at 450 nm. (2) Determination of Cys C: The level of Cys C in wound tissue and plasma was measured at admission. First, phosphate buffer saline (pH 7.4) was added to the venous tissue to homogenize the specimens, which were subjected to centrifugation at 3000 r/min for 20 min. Next, the supernatant was collected and reloaded to be tested. Then, the linear regression equation of the standard curve was plotted according to the mean absorbance (optical density) value following the human Cys C ELISA

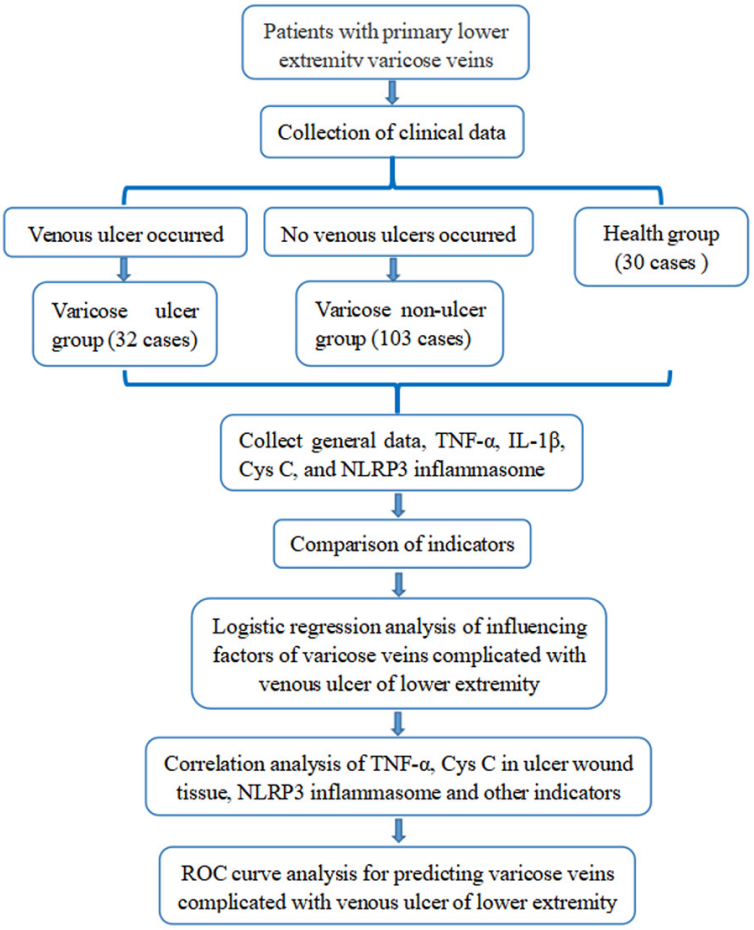

Figure 1. Procedures of this research. Note: TNF-α: tumor necrosis factor α, IL-1β: interleukin-1β, Cys C: cystatin C, NLRP3: NLR family pyrin domain containing 3.

kit instruction and the standard concentration. The concentration was calculated according to the absorbance value of the sample, and the final concentration was the actual measured concentration multiplied by the dilution ratio [11]. (3) NLRP3 inflammasome: 3 mL of fasting venous blood was collected on the day of admission and centrifuged for 10 min at 3000 r/min. The supernatant was isolated and measured for the levels of NLRP3 inflammasome (lot number ab214185, Abcam, UK) and interleukin (IL)-1β (lot number JEB-13503, Nanjing Jinyibo Biotechnology Co., LTD.) by ELISA.

Statistical approach

This study applied the SPSS 22.0 for statistical analyses. Measured data in line with a normal distribution were expressed as mean ± standard deviation and compared between two groups using t-test. Measured data did not conform to a normal distribution were expressed as median values (interquartile moments) and compared between two groups using Mann-Whitney U test. F test was used for comparisons among three or more groups. The enumerated data were expressed as percentage and compared using the Chi-square test. When the theoretical frequency was less than 1, the exact probability method was used for calculation. Spearman correlation coefficient was used for correlation analysis. Logistic regression was applied to analyze the risk factors for venous ulcers in patients with lower extremity varicose veins. The area under the receiver operating characteristic (ROC) curve (AUC) was used to analyze the predictive value of TNF-α, Cys C, and NLRP3 inflammasome for venous ulcers. Test level α=0.05.

Results

Baseline information

There was no significant difference in the general data among the varicose ulcer group, the non-varicose ulcer group and the healthy group, or between the varicose ulcer group and the non-varicose ulcer group (P>0.05). See **Table 1**.

Comparison of the research indexes

The levels of TNF-α, IL-1β, Cys C, and NLRP3 inflammasomes were compared among the varicose ulcer group, the non-varicose ulcer group and the healthy group, and between the varicose ulcer group and the non-varicose ulcer group, all P<0.05. See **Table 2** and **Figure 2**.

Logistic regression analysis of risk factors for venous ulcers in patients with lower extremity varicose veins

With venous ulcer as the dependent variable, and TNF-α, IL-1β, Cys C, and NLRP3 inflammasome as the independent variables, logistic

Table 1. Baseline information

General information	Varicose ulcer group (n=32)	Non-varicose ulcer group (n=103)	Healthy group (n=30)	χ^2/t/F	*P*
Sex (n, male/female)	13/19	33/70	11/19	0.869	0.648
Age ($\bar{x}$±s, years)	51.26±12.38	50.67±11.64	48.56±14.29	0.439	0.646
Course of disease ($\bar{x}$±s, d)	78.52±15.68	75.55±18.92	-	0.806	0.422
Injured limb (n, left side/right side)	14/18	49/54	-	0.143	0.705
Pain of injured limb (n, yes/no)	32/0	98/5	-	2.765	0.096
Calf swelling (n, yes/no)	26/6	71/32	-	1.832	0.176
Wound area ($\bar{x}$±s, cm^2)	4.45±1.01	4.32±1.06	-	0.613	0.541
Complicated hypertension (n, yes/no)	7/25	26/77	-	0.150	0.699
Complicated diabetes (n, yes/no)	10/22	21/82	-	1.628	0.020
ALT ($\bar{x}$±s, U/L)	34.52±7.58	35.12±9.28	36.03±9.02	0.225	0.798
Creatinine ($\bar{x}$±s, mmol/L)	70.08±11.56	73.12±6.62	71.03±9.25	1.983	0.141
TC ($\bar{x}$±s, mmol/L)	4.29±1.32	4.81±1.33	4.52±1.42	2.014	0.137
Blood glucose ($\bar{x}$±s, mmol/L)	7.76±2.01	7.82±1.71	8.53±1.77	2.041	0.133
Albumin ($\bar{x}$±s, g/L)	38.56±5.57	40.03±5.53	38.61±5.12	1.354	0.261

Note: ALT: alanine transaminase, TC: total cholesterol.

Table 2. The research indexes were compared among each group ($\bar{x}$±s)

Group	TNF-α (pmol/L)	IL-1β (pmol/L)	Cys C (mg/L)		NLRP3 inflammasome (ng/mL)
			in the wound tissue of the ulcer	in plasma	
Varicose ulcer group (n=32)	97.50 (80.78)*,#	107.99 (32.89)*,#	1.06±0.35*,#	0.60 (0.22)*,#	14.62 (8.79)*,#
Non-varicose ulcer group (n=103)	47.07 (9.84)*	72.87 (18.11)	1.52±0.33	0.85±0.32	12.62±3.25
Healthy group (n=30)	33.19±6.28	60.22±14.38	1.75±0.36	1.42±0.40	8.13±3.37
F	53.158	30.197	40.580	28.783	31.136
P	<0.001	<0.001	<0.001	<0.001	<0.001

Note: *denotes comparison with the healthy group, #denotes comparison with varicose the non-varicose ulcer group, *P*<0.05. NLRP3: NLR family pyrin domain containing 3.

regression analysis was performed. TNF-α, IL-1β, and NLRP3 inflammasomes were found to be risk factors for venous ulcers in patients with lower extremity varicose veins, and Cys C in ulcer wound tissue was a protective factor. See **Table 3**.

Correlation analysis among TNF-α, IL-1β, Cys C and NLRP3 inflammasome in patients with lower extremity varicose veins

TNF-α was significantly correlated with IL-1β and Cys C in ulcer wound tissue, and NLRP3 in plasma (r=0.256, -0.290, 0.305; *P*=0.003, 0.001, <0.001). IL-1β was significantly correlated with Cys C in ulcer wound tissue and in plasma (r=-0.251, -0.193; *P*=0.003, 0.025). See **Figure 3**.

ROC curve analysis for predicting venous ulcers in patients with lower extremity varicose veins

The AUC, sensitivity, and specificity of TNF-α and NLRP3 inflammasome for predicting venous ulcers in patients with lower extremity varicose veins were high, with AUC of 0.881 and 0.712, sensitivity of 0.875% and 0.875%, specificity of 0.893% and 0.738%, respectively. See **Table 4** and **Figure 4**.

Discussion

The incidence of varicose ulcers of the lower extremity is increasing year by year, showing recurrence and difficulty in treatment. At present, medical researchers have not reached a consensus on the pathologic mechanism of

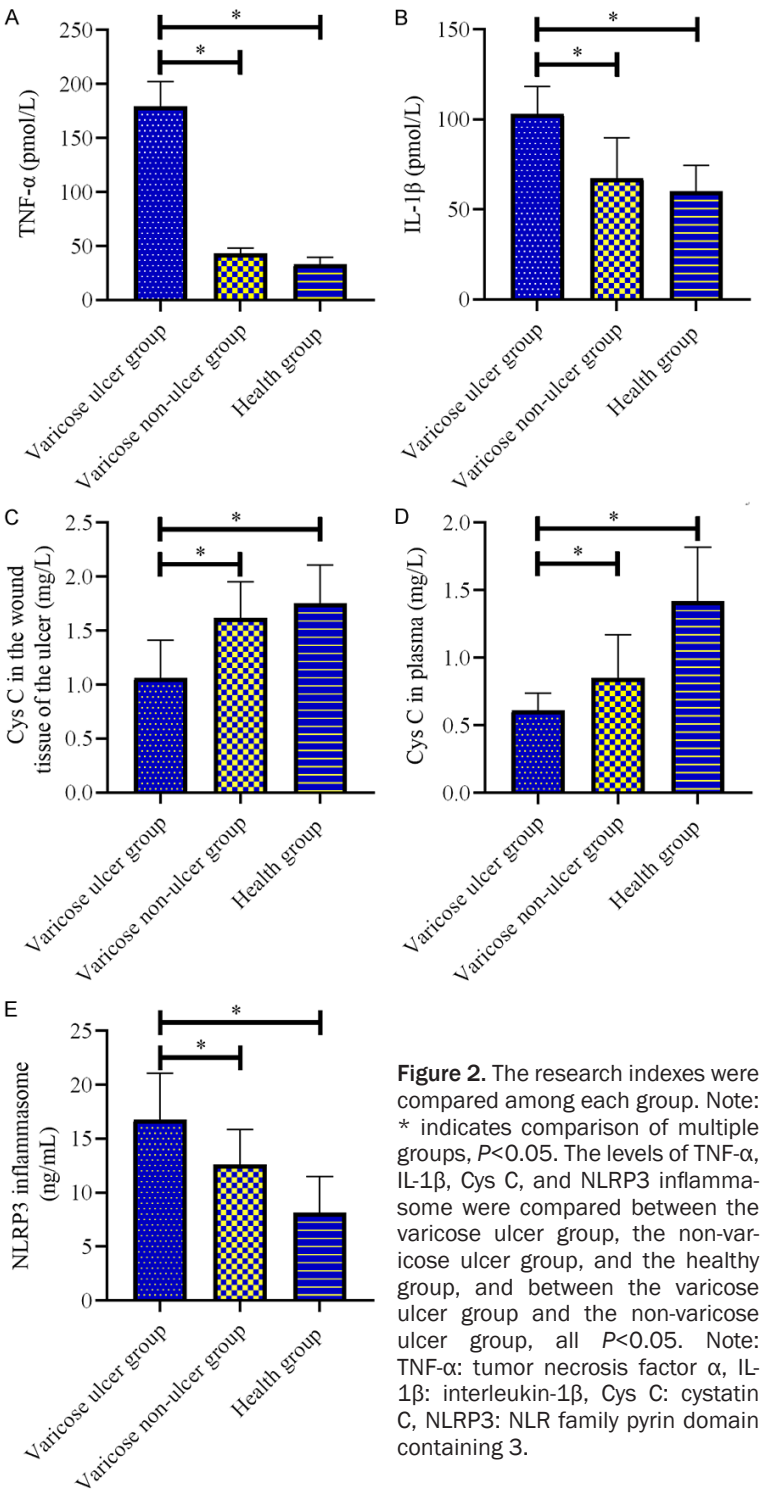

Figure 2. The research indexes were compared among each group. Note: * indicates comparison of multiple groups, $P<0.05$. The levels of TNF-α, IL-1β, Cys C, and NLRP3 inflammasome were compared between the varicose ulcer group, the non-varicose ulcer group, and the healthy group, and between the varicose ulcer group and the non-varicose ulcer group, all $P<0.05$. Note: TNF-α: tumor necrosis factor α, IL-1β: interleukin-1β, Cys C: cystatin C, NLRP3: NLR family pyrin domain containing 3.

venous ulcers. Studies have found that an inflammatory reaction is key in the occurrence and development of venous ulcers [12]. Lower extremity venous hypertension promotes the adhesion of leukocyte induced adhesion molecules to endothelial cells, thereby activating and releasing inflammatory mediators and oxygen free radicals and destroying the normal process of wound healing. Lower extremity venous ulcers are a result of a systemic inflammatory reaction [13]. In addition, venous ulcers on the lower extremity can activate mononuclear phagocytes and upregulate the level of interleukin inflammatory proteins such as IL-1β, leading to persistent wound inflammation and granulation tissue regeneration disorder, resulting in delayed or even nonhealing ulcer wounds. This suggests that excessive inflammatory reaction in the state of lower extremity venous hypertension may be one of the important mechanisms causing wound healing disorders of lower extremity venous ulcers [14]. TNF-α, Cys C, and NLRP3 inflammasomes are all key factors in an inflammatory response. Therefore, it is necessary to explore the correlation of TNF-α, Cys C, and NLRP3 inflammasomes with venous ulcers in patients with lower extremity varicose veins.

In the study we found that TNF-α, IL-1β, and NLRP3 inflammasomes were risk factors for venous ulcers in patients with lower extremity varicose veins, and Cys C in ulcer wound tissue was a protective factor ($P<0.05$). These results indicated that TNF-α, IL-1β, Cys C, and NLRP3 inflammasomes were closely related to venous ulcers on the lower extremity. Possible reasons are as follows: When the venous valves of the lower extremities are dysfunctional, venous reflux is accelerated, and a large amount of blood pools in the lower legs, resulting in blood stasis and high pressure in

Table 3. Logistic regression analysis

Variable	B	Standard error	Wals	*P*	OR	95% CI	
						lower limit	upper limit
TNF-α	0.018	0.008	4.947	0.026	1.019	1.002	1.035
IL-1β	0.047	0.013	14.167	<0.001	1.049	1.023	1.075
Cys C							
in the wound tissue of the ulcer	-4.608	1.229	14.067	<0.001	0.01	0.001	0.111
in plasma	-1.019	0.705	2.088	0.148	0.361	0.091	1.438
NLRP3	0.274	0.101	7.389	0.007	1.315	1.079	1.602

Note: TNF-α: tumor necrosis factor α, IL-1β: interleukin-1β, Cys C: cystatin C, NLRP3: NLR family pyrin domain containing 3.

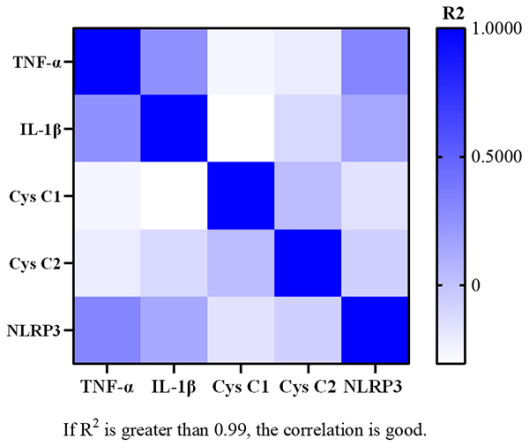

Figure 3. Spearman correlation heat map. Note: TNF-α: tumor necrosis factor α, IL-1β: interleukin-1β, Cys C1: cystatin C in the wound tissue of the ulcer, Cys C2: cystatin C in plasma, NLRP3: NLR family pyrin domain containing 3. The darker the color, the stronger the correlation between the two indicators.

the venous system of the lower extremities, which activates the secretion and release of TNF-α and IL-1β by monocytes [15]. TNF-α and IL-1β are important inflammatory cytokines for the inflammatory response [16]. With the increase of TNF-α and IL-1β levels, the inflammatory reaction aggravates varicose veins of the lower extremities, leading to an increased risk of venous ulcers. Cys C is a low molecular weight protein, and Cys C and its fragments may participate in the inflammatory process by affecting the phagocytosis and chemotactic function of granulocytes [17]. Previous studies found that compared with normal great saphenous vein, cathepsin L expression was enhanced and Cys C expression was decreased in smooth muscle cells in the media and intima of varicose great saphenous vein [18]. Therefore, it is speculated that a low level of Cys C in the ulcer wound tissue may induce a large number of leukocytes to infiltrate into local tissues and release cytokines, which may be one of the important reasons for venous ulcers. Relevant studies had found that the expression of Cys C in patients with postoperative lower extremity chronic venous insufficiency was significantly increased, and the recurrence rate of ulcers in patients with a low postoperative Cys C expression was higher than that of patients with a high postoperative Cys C expression [19]. A low level of Cys C in the plasma of patients indicated that the secretion of Cys C by endothelial cells was decreased. The results showed that leukocytes and platelets were more important in local blood stasis, venous congestion was increased, and venous hemodynamics of local tissues was impaired. The lower the level of Cys C, the more severe the venous dysfunction and the higher the incidence of venous ulcers. In addition, Cys C was negatively correlated with TNF-α and IL-1β. An increase of TNF-α and IL-1β levels increases the stimulation of an inflammatory reaction in local tissues, inhibits the synthesis and secretion of Cys C in ulcer wound tissue, and promotes the chemotaxis of leukocytes, thus creating favorable conditions for the occurrence of venous ulcers [20]. The NLRP3 inflammasome, which consists of NLRP3, caspase-1, and ASC, is one of the cores of an inflammatory response. There are a few reports on its correlation with venous ulcers. A study had shown that NLRP3 was significantly correlated with disease activity in patients with ulcerative colitis [21]. NLRP3 inflammasome activation under the induction of exogenous stimuli (such as bacteria and viruses) could induce the production of cytokines [22]. Another study found that in comparison with the inactive ulcer group and

Table 4. ROC curve analysis for predicting venous ulcers in patients with lower extremity varicose veins

Variable	AUC	Standard error	*P*	95% CI		Cutoff value	Sensitivity (%)	Specificity (%)
				lower limit	upper limit			
TNF-α	0.881	0.036	<0.001	0.810	0.953	56.00	0.875	0.893
IL1-β	0.843	0.040	<0.001	0.764	0.923	1.225	0.219	0.223
Cys C in ulcer wound tissue	0.159	0.043	<0.001	0.075	0.244	18.465	0.406	0.961
NLRP3	0.712	0.055	<0.001	0.604	0.819	83.755	0.875	0.738

Note: ROC: receiver operating characteristic, TNF-α: tumor necrosis factor α, IL-1β: interleukin-1β, Cys C: cystatin C, NLRP3: NLR family pyrin domain containing 3.

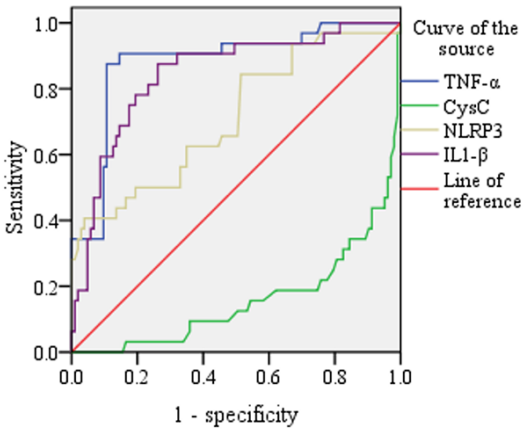

Figure 4. ROC curve analysis of TNF-α, Cys C in ulcer wound tissue, and NLRP3 inflammasome in plasma for predicting venous ulcers. Note: The AUC, sensitivity, and specificity of TNF-α and NLRP3 inflammasome for predicting venous ulcers were high. TNF-α: tumor necrosis factor α, IL-1β: interleukin-1β, Cys C: cystatin C, NLRP3: NLR family pyrin domain containing 3.

the traumatic wound group, the expression level of NLRP3 in the wound tissue of venous ulcers of the lower extremity in patients with an active ulcer was higher, and the relative mRNA expression levels of caspase-1 and ASC were also higher [23]. This suggests that the high-level TNF-α and IL-1β in the wound tissue of patients with lower extremity venous ulcers may be related to activation of the NLRP3 inflammasome. NLRP3 inflammasome is continuously activated under venous hypertension, and the expressions of proinflammatory cytokines are increased, which aggravates the inflammatory response, putting the body tissues in a chronic inflammatory state. This promotes the formation of ulcers. It was found in this study that the AUC, sensitivity, and specificity of TNF-α and NLRP3 inflammasomes for predicting venous ulcers in patients with lower extremity varicose veins were high, suggesting that they are markers for venous ulcers. Although Cys C in ulcer wound tissue was related to the occurrence of venous ulcers, the prediction efficiency was low. Therefore, it may be of more clinical significance to monitor TNF-α and NLRP3 inflammasomes in patients with lower extremity varicose veins.

Strengths and limitations

This study showed that the levels of TNF-α, Cys C, and NLRP3 inflammasome were closely related to venous ulcers in lower extremity varicose veins, which may provide new guidance for the treatment of venous ulcers. However, this study could only determine the relationship of TNF-α, Cys C, and NLRP3 inflammasomes with venous ulcers of the lower extremity, but it failed to determine a causal relationship between them. Furthermore, our study was a single-center and small-sample study, which may affect the results. Future research should involve a multi-center large-sample study to provide more clinical evidence about venous ulcers in patients with lower extremity varicose veins.

Conclusions

TNF-α in plasma, Cys C in ulcer wound tissue and plasma, and plasma NLRP3 inflammasomes are closely related to venous ulcers in patients with lower extremity varicose veins. They may serve as new targets for treatment.

Disclosure of conflict of interest

None.

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