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## ORIGINAL PAPER

## Haemoglobinopathies

# Impaired microvascular function in patients with sickle cell anemia and leg ulcers improved with healing

Judith Catella<sup>1,2,3</sup>   | Etienne Turpin<sup>2,3</sup> | Philippe Connes<sup>2,3</sup>  | Elie Nader<sup>2,3</sup> | Romain Carin<sup>2,3</sup> | Marie Martin<sup>2,3</sup> | Hamdi Rezigue<sup>4,5</sup>  | Christophe Nougier<sup>4,5</sup> | Yesim Dargaud<sup>5,6</sup> | Audrey Josset-Lamaugarny<sup>7</sup> | Justine Dugrain<sup>7</sup> | Muriel Marano<sup>5</sup> | Alexandre Leuci<sup>5</sup> | Camille Boisson<sup>3,8</sup> | Celine Renoux<sup>2,3,8</sup>  | Philippe Joly<sup>2,3,8</sup>  | Solène Poutrel<sup>1,2,3</sup> | Arnaud Hot<sup>1,2,3</sup> | Nicolas Guillot<sup>2,3</sup> | Berengère Fromy<sup>7</sup>

<sup>1</sup>Service de Médecine Interne et Vasculaire, Hôpital Edouard Herriot, Hospices Civils de Lyon, Lyon, France

<sup>2</sup>Laboratoire d'Excellence du Globule Rouge (Labex GR-Ex), Sorbonne, Paris, France

<sup>3</sup>Laboratoire Interuniversitaire de Biologie de la Motricité (LIBM) EA7424, Equipe «Biologie Vasculaire et du Globule Rouge», Université Claude Bernard Lyon 1, Université de Lyon, Lyon, France

<sup>4</sup>Service d'hématologie-hémostase, Hospices civils de Lyon, Bron, France

<sup>5</sup>EA 4609-Hémostase et cancer, UFR Laennec, Université Claude Bernard, Lyon 1, France

<sup>6</sup>Unité d'hémostase Clinique, Hôpital Cardiologique Louis Pradel, Lyon, France

<sup>7</sup>Laboratoire de Biologie Tissulaire et Ingénierie Thérapeutique (LBTI UMR 5305), CNRS/Université Claude Bernard Lyon 1, Lyon, France

<sup>8</sup>Service de Biochimie et Biologie Moléculaire, Laboratoire de Biologie Médicale Multi-site, Hospices Civils de Lyon, Lyon, France

**Correspondence**

Judith Catella, Service de Médecine Interne et Médecine Vasculaire, Hôpital Edouard Herriot, Hospices Civils de Lyon, Lyon 69003, France.  
 Email: [judith.catella@chu-lyon.fr](mailto:judith.catella@chu-lyon.fr)

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**Summary**

Leg Ulcer (LU) pathophysiology is still not well understood in sickle cell anaemia (SCA). We hypothesised that SCA patients with LU would be characterised by lower microvascular reactivity. The aim of the present study was to compare the microcirculatory function (transcutaneous oxygen pressure (TcPO<sub>2</sub>) on the foot and laser Doppler flowmetry on the arm) and several blood biological parameters between nine SCA patients with active LU (LU+) and 56 SCA patients with no positive history of LU (LU-). We also tested the effects of plasma from LU+ and LU- patients on endothelial cell activation. We observed a reduction of the TcPO<sub>2</sub> in LU+ compared to LU- patients. In addition, LU+ patients exhibited lower cutaneous microvascular vasodilatory capacity in response to acetylcholine, current and local heating compared to LU- patients. Inflammation and endothelial cell activation in response to plasma did not differ between the two groups. Among the nine patients from the LU+ group, eight were followed and six achieved healing in 4.4 ± 2.5 months. Among thus achieving healing, microvascular vasodilatory capacity in response to acetylcholine, current and local heating and TcPO<sub>2</sub> improved after healing. In conclusion, microcirculatory function is impaired in patients with LU, and improves with healing.

**KEY WORDS**

endothelial cell, leg ulcers, microcirculation, sickle cell disease, transcutaneous oxygen pressure

Nicolas Guillot and Berengère Fromy contributed equally and co-last authors.

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## INTRODUCTION

Sickle cell disease (SCD) is an autosomal recessive genetic disorder secondary to a single mutation in the  $\beta$ -globin gene. Sickle cell anaemia (SCA) is the most common and the most severe form of SCD, and is characterised by the presence of two  $\beta^S$ -globin alleles resulting in the production of an abnormal haemoglobin (Hb) called HbS.<sup>1</sup> HbS may polymerise under deoxygenated conditions,<sup>2</sup> leading to sickling and chronic haemolytic anaemia.<sup>3</sup> The clinical expression of the disease is highly variable from one patient to another.<sup>4</sup>

Leg ulcer (LU) corresponds to spontaneous or traumatic wound, which failed to heal within 2 weeks. The prevalence of LU varied from 1.4% in Brazil to 29.5% in Jamaica.<sup>5–9</sup> Usually located towards the ankle and very painful, sickle LU pathophysiology is still not well defined. It has been proposed that LU would be secondary to a local vaso-occlusion<sup>10</sup> through several factors such as increased blood viscosity,<sup>11</sup> chronic haemolysis,<sup>4</sup> increased oxidative stress,<sup>12</sup> increased inflammation,<sup>13</sup> increased coagulation<sup>14</sup> and decreased nitric oxide (NO) bioavailability<sup>15</sup> promoting microvascular dysfunction. While compromised microvascular reactivity has been reported in SCD population,<sup>16,17</sup> no study has compared patients with and without LU.

We hypothesised that SCA patients with LU would be characterised by lower microvascular reactivity than patients without LU. The first aim of the present study was to compare the cutaneous microvascular vasodilatory function assessed by laser Doppler flowmetry and transcutaneous oxygen pressure (TcPO<sub>2</sub>) between SCA patients with active LU and those without LU (who never experienced LU). Using iontophoresis or heating, laser Doppler flowmetry assesses the ability of vessels to dilate in response to external stimuli and helps to characterise the signalling pathways and cell types involved in vasodilatation, such as NO, cyclooxygenase (COX) or neural mechanisms. In addition, TcPO<sub>2</sub> measures the amount of oxygen carried out by the cutaneous microcirculation and the oxygen consumed locally. The second aim of this study was to follow patients with active LU and compare their microcirculatory function before and after healing. Since it has been proposed that inflammation and haemolysis would be involved in LU occurrence in SCA,<sup>11,18</sup> we suspected that the plasma from patients with LU would have more deleterious effects on endothelium than the plasma from patients without LU.

## METHODS

The DrepanO<sub>2</sub> study was a single-centre, prospective and controlled study conducted in Lyon University Hospital between February 2022 and September 2023, and included 65 adults with SCA (HbSS or HbS $\beta$ 0 genotypes) regularly followed by the Sickle Cell Centre at the Hospital Edouard

Herriot (Lyon, France). Patients belonged to the LU+ group if they had currently an active ulcer on the legs. Those who never experienced LU were included in the LU– group. Those without active LU but with a positive history of leg ulcers were not included.

Patients with acute episodes (infection, vaso-occlusive crisis (VOC), acute chest syndrome (ACS), stroke and priapism) in the past 4 weeks were excluded. At the time of the study, patients were undergoing systemic treatments (see Table 2): patients were included regardless of their disease-modifying treatment. A transfusion was not an exclusion criterion, but inclusion was carried out as far as possible from the previous transfusion.

Based on the microvascular response values obtained by our team in sickle cell patients with or without vaso-occlusive complications,<sup>17,19,20</sup> and the prevalence of leg ulcers in sickle cell patients, a minimum of six subjects per group (i.e. with and without LU) would be necessary (with an alpha risk of 0.05 and a power of 90%, two-sided test). We observed that approximately 10% of the sickle cell population in Lyon is affected by leg ulcers. As the number of patients included should reflect this proportion, we decided to include a total of 70 patients (with a minimum of seven in the group of sickle cell patients with ulcers).

All patients were informed and gave their written informed consent. The study was conducted in accordance with the guidelines set by the Declaration of Helsinki and was approved by the Regional Ethics Committee (CPP Sud-Est IV, Lyon, France, registration number: 2021-A01605-36).

## Follow-up

Patients with active LU+ were scheduled for a second visit at 6 months, or earlier if the ulcer healed before that time. According to the healing status at the second visit, patients were divided into 'LU+ healed' or 'LU+ still active'.

## General parameters

Data concerning medical history were collected: sickle cell genotype, age at first LU,  $\alpha$ -thalassaemia status, clinical phenotype of SCA, cardiovascular risk factors (tobacco status, diabetes, treated hypertension and diabetes mellitus) and previous history of venous thrombo-embolism. Body mass index was calculated ( $BMI = \text{weight}/\text{height}^2$  (Kg/m<sup>2</sup>)) for all patients. Charts were retrospectively reviewed by one physician to record all ACS and VOC episodes, within the 2 previous years. Treatments were classified as specific for hydroxyurea, pyruvate kinase activator and Voxelator and non-specific for the other treatments. Analgesics were classified as level 1 (paracetamol), level 2 (tramadol, codeine, nefopam chlorhydrate) and level 3 for opioids.

LU area was measured (based on greatest width and length) and presence of leg oedema was assessed. Need of

technical assistance for mobilisation was reported as it can influence venous return.

Venous blood was taken from the antecubital vein and collected into citrate, EDTA, heparin and dry tubes for whole blood and plasma analysis.

## Routine biology, biochemistry and coagulation

Several routine biological and biochemical parameters were determined.

Citrate blood was used for D-Dimers, prothrombin time, activated partial thromboplastin time (APTT) ratio, fibrinogen, chromogenic protein C activity (%) and protein S free antigen level.

## Pro-inflammatory cytokines and matrix metalloproteinases

Concentration of Interleukin-1 alpha (IL-1 $\alpha$ ), IL-1 $\beta$ , IL-6, IL-8, IL-10, IL-15, tumour necrosis factor-alpha (TNF- $\alpha$ ), vascular endothelial growth factor-A (VEGF-A), matrix metalloproteinase-1 (MMP-1), MMP-3, MMP-8, MMP-9 and tissue inhibitor of metalloproteinase-1 (TIMP-1) were assessed in plasma from LU- and LU+ SCA patients. Quantification was performed based on the Luminex xMAP technology, using the Human ProcartaPlex Mix and Match Panel (Thermo Fisher Scientific), according to the manufacturer's instructions. Plates were analysed on a Bio Plex 200 analyser (AniRA, Lyon, France).

## Assessment of skin sensory function

Since neurovascular interactions are impaired by neuropathic disorders, all patients and controls underwent a sensory assessment based on the examination of cutaneous modalities (thermal sensitivity, tactile and vibration) on upper and lower limbs.<sup>21</sup> Thermal sensitivity was analysed in response to hot (around 45°C) and cold (around 4°C) stimulation applied to the skin. A set of 20 Von Frey monofilaments was used, decreasing force from 300 g to 0.008 g, to assess cutaneous tactile threshold.<sup>22</sup> Vibration sensitivity was assessed on trapeze and distal extremity of radius for upper limbs and on distal extremity of tibia and on the base of the first metatarsus for lower limbs. Irrespective of the leg ulcer status, all other tests were performed on the internal face of forearms for upper limbs and around the ankle and on the feet for lower limbs. For each individual, three determinations were carried out.

## Vascular evaluation

Systolic and diastolic blood pressures (BP) were measured as recommended<sup>23</sup> before and after microvascular evaluation.

BP values of 120/70 mmHg were considered as normal. Patients with BP values >120/70 mmHg at the two measures were considered as having hypertension.<sup>24,25</sup>

Ankle pressure and/or toe systolic pressure were measured according to standard operating protocols, on the leg with the ulcer when applicable. Patients with Ankle brachial index <0.90 or Toe brachial index <0.70 were considered to have peripheral artery disease.<sup>26</sup> TTcpO<sub>2</sub> measurements were performed using TCM 400 Monitor (Radiometer, Neuilly-Plaisance, France) set to default temperature at 43°C.<sup>27</sup> The measurements were taken while the patient was lying down, after removing compression. Probes were placed at the first intermetatarsal space. TcpO<sub>2</sub> values were collected after 20 min of recording and after stabilisation.

Cutaneous microvascular function was analysed by laser Doppler flowmetry (Laser Doppler PF5010, Perimed) on the volar aspect of forearms for all patients and controls in supine position.<sup>28</sup> To assess endothelium-mediated vasodilatation, cutaneous blood flow was measured in response to anodal iontophoretic delivery (100  $\mu$ A, 20 s) of acetylcholine (Ach, 2%) and to cathodal current application (100  $\mu$ A, 60 s) of deionised water. Local heating to 42°C was also performed with the laser Doppler probe at a third site of the forearm. A stable baseline blood flow was first recorded for a few minutes before the iontophoretic current, or heat was applied. Modifications of cutaneous blood flow were continuously recorded for 30 min by the laser Doppler on each of the three sites, measuring the effect of the stimuli on skin microcirculation in real time.

## Cell culture

Human Dermal Microvascular Endothelial Cell (HDMEC) were purchased from PromoCell (#C-12262). HDMEC were cultured in Endothelial Cell Growth Medium MV2 (PromoCell, #C-39221) at 37°C and 5% CO<sub>2</sub>. The culture cells were maintained until passage 5 according to the manufacturer's protocol. For experiments, HDMEC were treated with culture medium containing 5% plasma from LU- or LU+ patients.

## Total RNA/DNA isolation and qPCR

RNA was analysed on HDMEC incubated with plasma of LU- and LU+ patients. Total RNA from HDMEC was isolated using a chloroform/phenol procedure (TRIzol Reagent, Thermo Fisher Scientific, #15596018). mRNAs were reverse-transcribed into cDNA using PrimeScript RT Reagent kit (Takara Bio Europe, #RR037A) and primers specific to genes of interest (Data S1), and analysed in real-time qPCR using TB Green Premix ExTaq (Takara Bio Europe, #RR42WR). Results were normalised to TATA-box binding protein (TBP) housekeeping gene as previously published.<sup>29</sup>

## Western blotting

Western blot was performed on HDMEC incubated with the plasma of LU<sup>-</sup> and LU<sup>+</sup> patients. Total proteins from HDMEC were extracted using RIPA buffer (Cell Signaling #9806S) containing protease and phosphatase inhibitors cocktail (Thermo Fisher Scientific, #A32955). After quantification with the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, #23227), 20 µg of proteins was loaded on a 4–20% Precast Protein Gel (Bio Rad, #4561095) and transferred onto a nitrocellulose membrane (Bio Rad, #1704272) by a semi-dry transfer. The membrane was incubated for 1 h at room temperature (RT) in blocking buffer (TBS-Tween-20 0.1%, 5% BSA) and immunoblotted overnight at 4°C with primary antibodies targeting eNOS, phospho-eNOS (Ser1177), ERK and phospho-ERK1/2 (Thr202/Tyr204) (Data S2). After washing, HRP-linked secondary antibodies (Data S2) were applied on the membrane for 1 h at RT. Membrane was revealed using Chemiluminescent Substrate (Bio Rad, #1705060) and the signal was detected by the ChemiDoc (LYOS, Lyon, France).

## Statistical analysis

Data are expressed as mean ± standard deviation or *n* (%). Comparisons between LU<sup>-</sup> and LU<sup>+</sup> SCA patients were achieved by using independent Student's *t*-test and Mann-Whitney *U*-test, when appropriate, using Prism software (version 10.1.1, GraphPad Software, San Diego, CA, USA). Paired *t*-tests were used to compare microcirculatory function before and after healing in the LU<sup>+</sup> healed patients. A *p* value <0.05 was considered to be statistically significant.

## RESULTS

### Characterisation of LU and characteristics of patients with and without LU

Sixty-five patients were enrolled at the time of the analysis. Five patients had HbSβ0 genotype in the LU<sup>-</sup> group, while all other patients had HbSS genotype (Table 1). Nine patients composed the LU<sup>+</sup> group (Figure 1), of whom seven patients had one active LU, one patient had two active LUs and one patient had three active LUs. Mean age at first ulcer was 35.5 ± 18.8 years old. The mean area of LU was 10.3 ± 10.1 cm<sup>2</sup>. There were no significant predominant side (4LU on the right side and 8LU on the left side) nor predominant malleoli (eight on the external malleoli and four on the internal malleoli). The mean LU duration was 8.3 ± 7.5 months. The LU<sup>+</sup> group was older than the LU<sup>-</sup> group (*p* < 0.0001; Table 1). There was no difference in sex ratio between the two groups. LU<sup>-</sup> group had more frequent VOCs than LU<sup>+</sup> group during the last 2 years (*p* 0.002; Table 1). There was no other significant association between the occurrence of sickle LU and other complications of SCA (i.e. acute chest syndrome, pulmonary hypertension, ischaemic stroke,

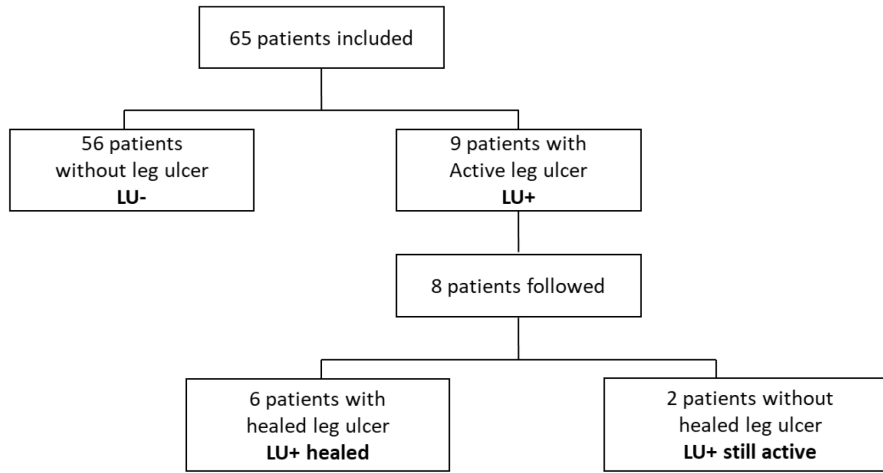
**TABLE 1** Comparison of clinical phenotypes between SCA patients with (LU<sup>+</sup>) and without (LU<sup>-</sup>) leg ulcer.

	LU <sup>+</sup> <i>n</i> = 9	LU <sup>-</sup> <i>n</i> = 56
HbSS genotype	9 (100)	51 (91.1)
Age (years)	48.5 ± 9.2	31.8 ± 10.7***
α-thalassaemia	4 (50.0)/8	23 (41.1)/50
Male	4 (44.0)	22 (39.3)
Body mass index (kg/m <sup>2</sup> )	23.6 ± 3.4	21.8 ± 3.6
SCD complications		
VOCs last 2 years	0.6 ± 0.7	1.9 ± 2.3**
ACSs last 2 years	0.3 ± 0.5	0.4 ± 0.7
History of ACS	6 (66.7)	33 (58.9)
Pulmonary hypertension	0	0
Ischaemic stroke	1 (11.1)	1 (1.8)
Priapism	1 (25.0)	4 (18.2)
Osteonecrosis	4 (44.0)	15 (26.8)
Retinopathy	0	5 (8.9)
Respiratory insufficiency		
Medical history		
Current smoker	1 (11.1)	10 (17.9)
Dyslipidaemia	1 (11.1)	1 (1.8)
Treated hypertension	0	4 (7.1)
Mellitus diabetes	0	0
Venous thromboembolism	6 (66.7)	7 (12.5)**
Measured hypertension	3 (33.3)	7 (12.7)
Peripheral arterial disease	1 (11.1)	0
Technical assistance for mobilisation	2 (22.2)	1 (1.8)*
Leg oedema	8 (88.9)	4 (7.3)***

Note: Values represent mean ± standard deviation or *n* (%). Significant difference: \**p* < 0.05; \*\**p* < 0.01; \*\*\**p* < 0.001.

Abbreviations: ACS, acute chest syndrome; LU, leg ulcers; VOC, vaso occlusive crisis.

priapism, osteonecrosis and retinopathy). Venous thromboembolisms were more frequent in the LU<sup>+</sup> group compared to the LU<sup>-</sup> group (*p* 0.001; Table 1). Leg oedema and need of technical assistance for mobilisation were more frequent in the LU<sup>+</sup> group than in the LU<sup>-</sup> group (*p* < 0.0001 and *p* 0.048 respectively) (Table 1). Recent RBC transfusion was more frequent in the LU<sup>+</sup> group compared to the LU<sup>-</sup> group (*p* 0.005; Table 2). The mean time from study day to last transfusion was 82 ± 25 days for the 3LU<sup>-</sup> transfused patients in the past 4 months and 48 ± 22 days for the 4LU<sup>+</sup> transfused patients in the last 4 months. All patients were treated with hydroxyurea except two patients with Voxelator in the LU<sup>+</sup> group and one patient without any treatment in the LU<sup>-</sup> group. The mean dose of hydroxyurea per week was significantly higher in the LU<sup>-</sup> group. There was no significant difference in the non-specific treatment except more analgesic level 3 (opioids) and compression therapy



**FIGURE 1** Flow chart.

**TABLE 2** Comparison of treatment between SCA patients with (LU+) and without (LU-) leg ulcer.

	LU+ n=9	LU- n=56
No transfusion < 4 months	5 (55.6)	53 (94.6)**
Disease modifying treatment		
Hydroxyurea	6 (66.7)	53 (94.6)*
Hydroxyurea + pyruvate kinase activator	0	1 (1.8)
Hydroxyurea + voxelotor	1 (11.1)	2 (3.6)
Voxelotor	2 (22.2)	0*
None	0	1 (1.8)
Mean hydroxyurea dose per week ± SD (mg)	6028 ± 2917	8508 ± 2696*
Other treatments		
Iron chelator	2 (22.2)	2 (3.6)
Analgesic		
None	3 (33.3)	35 (63.6)
Level 1	2 (22.2)	12 (21.8)
Level 2	2 (22.2)	8 (14.5)
Level 3	2 (22.2)	0*
Antiplatelet therapy	1 (11.1)	2 (3.6)
Anticoagulant treatment	3 (33.3)	7 (12.5)
Statin	1 (11.1)	1 (1.8)
RAAS blocker	2 (22.2)	5 (8.9)
Oxygen therapy	2 (22.2)	2 (3.6)
Leg compression therapy	8 (88.9)	4 (7.3)***

Note: Values represent n (%). Analgesics were classified in level 1 (paracetamol), level 2 (tramadol, codeine, nefopam chlorhydrate) and level 3 for opioids. Significant difference: \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001. Abbreviations: LU, leg ulcer; RAAS, renin angiotensin aldosterone system.

in the LU+ group compared to the LU- group (*p* 0.017 and *p* < 0.0001 respectively). No difference in anticoagulant therapy was observed between the two groups.

**TABLE 3** Comparison of peripheral sensory parameters between SCA patients with (LU+) and without (LU-) leg ulcer.

	LU+ n=9	LU- n=55
Pain analogue visual scale (except ulcer)	5.1 ± 4.1	1.3 ± 2.3*
Intermetatarsal TcPO <sub>2</sub> (mmHg)	53.0 ± 10.0	67.5 ± 13.5***
Abnormal warm sensitivity		
Upper limb	0	0
Lower limb	2 (22.2)	1 (1.8)*
Abnormal cold sensitivity		
Upper limb	0	0
Lower limb	3 (33.3)	1 (1.8)**
Abnormal pinprick test		
Upper limb	0	1 (1.8)
Lower limb	1 (11.1)	1 (1.8)
Abnormal vibration test		
Upper limb	0	0
Lower limb	1 (11.1)	2 (3.6)
Von Frey		
Upper limb	0.02 ± 0.02	0.008 ± 0.002
Lower limb	1.0 ± 2.8	0.11 ± 0.54

Note: Values represent mean ± standard deviation or n (%). One LU- patient did not perform a physical examination due to lack of time by the patient. Significant difference: \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001. Abbreviations: LU, leg ulcer.

### Patients from the LU+ group had lower oxygen pressure and impaired sensitivity

The TcPO<sub>2</sub> in the LU+ group was lower than in the LU- group (*p* < 0.001, Table 3). There was no difference regarding pinprick, vibration and Von Frey tests between the two groups at lower and upper limbs. Sensitivity response to warm and cold on the lower limb was more impaired in LU+ compared

to LU- patients ( $p=0.0048$  and  $0.007$ , respectively, [Table 3](#)) but no difference was observed on upper limb between the two groups.

### Patients from the LU+ group had decreased albumin concentration, and inflammation level was not different between the two groups

LU+ group had higher haemoglobin, neutrophils and fibrinogen levels than LU- group ( $p$  0.035, 0.041 and 0.042 respectively) ([Table 4](#)). Albumin was lower in the LU+ group ( $p<0.001$ ; [Table 4](#)). Exclusion of patients who have received RBC transfusion in the preceding 4 months (four in LU+ group and three in LU- group) did not modify the biological results, except for neutrophils (see [Table S1](#)).

Cytokines (TNF- $\alpha$ , IL-1a, IL-1b, IL-6, IL-8, IL-10, IL-15), VEGF-A, metalloproteinases (MMP-1, MMP-3 and MMP-9) and TIMP-1 levels did not differ between the two groups ([Figure S1](#)). MMP-8 values were under the threshold of detection.

### Patients from the LU+ group had lower microvascular reactivity in response to various stimuli

Using Laser Doppler flowmetry, microvascular experiments showed no difference regarding basal skin blood flow between LU+ and LU- groups ([Figure 2A](#)). In contrast, the cutaneous vasodilatation in response to acetylcholine, current and local heating was reduced in the LU+ group compared to LU- group ( $p<0.001$  for all) ([Figure 2A](#)). We did not find any significant negative correlation between age and the different markers of microcirculatory function in SCD patients (data not shown).

### Effect of plasma from patients on endothelial cells cultures

Endothelial cells incubated with the plasma of patients from the LU+ group show no significant difference regarding mRNA expression of eNOS, COX-1, COX-2, VCAM-1 and E-selectin compared to endothelial cells incubated with the plasma of the LU- group ([Figure 3](#)). Using western blot, we found no significant difference regarding P-eNOS/eNOS and P-ERK/ERK ratios between the two groups ([Figure 3](#)).

### Follow-up

Among the nine patients with active LU, eight patients were followed ([Figure 1](#)). The mean time until second visit was  $8.6\pm 4.7$  months. Some visits had to be delayed due to intercurrent vaso-occlusive crises, to achieve a steady state.

**TABLE 4** Comparison of biological parameters between SCA patients with (LU+) and without (LU-) leg ulcer.

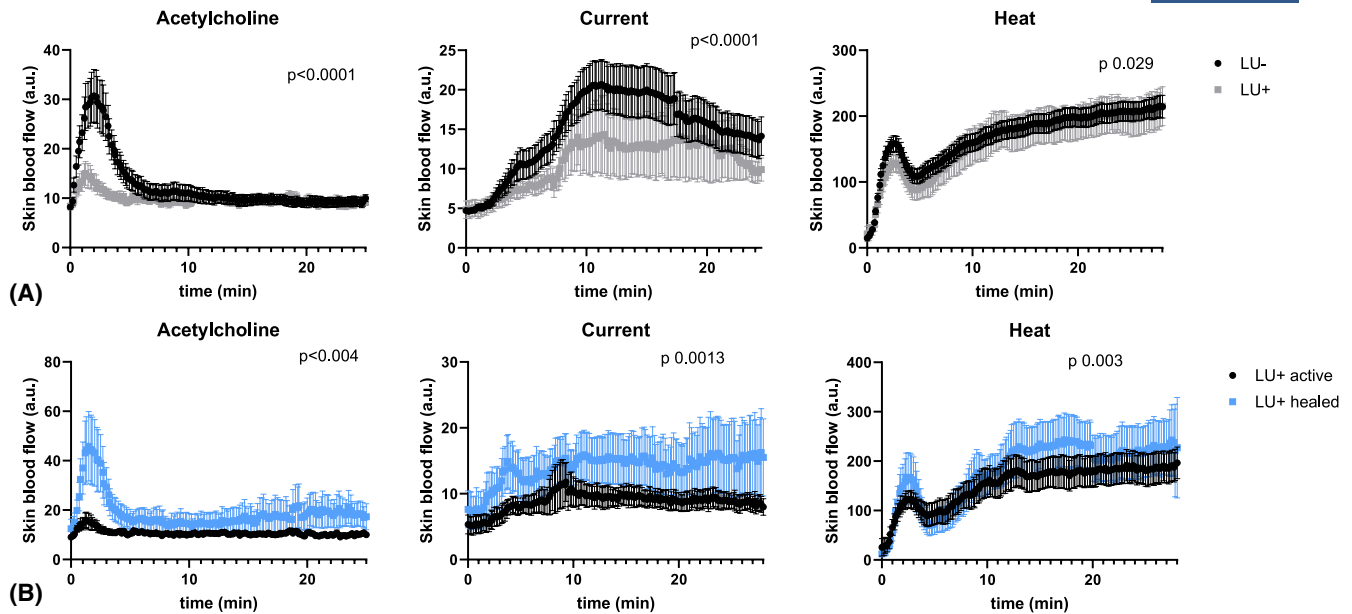
	LU+	LU-
	n=9	n=56
<b>Haematology</b>		
Haemoglobin (g/dL)	10.4 $\pm$ 1.0	9.3 $\pm$ 1.5*
MCV (fl)	96.7 $\pm$ 17.7	91.4 $\pm$ 16.2
MCHC (g/100mL)	340 $\pm$ 3.5	347 $\pm$ 13
White blood cells (G/L)	9.0 $\pm$ 2.6	7.1 $\pm$ 2.8
Neutrophils (G/L)	5.8 $\pm$ 2.4	4.1 $\pm$ 2.3*
Eosinophils (G/L)	0.15 $\pm$ 0.12	0.16 $\pm$ 0.16
Basophils (G/L)	0.04 $\pm$ 0.02	0.08 $\pm$ 0.12
Monocytes (G/L)	0.99 $\pm$ 0.39	0.70 $\pm$ 0.59
Lymphocytes (G/L)	2.0 $\pm$ 0.82	2.3 $\pm$ 0.86
Platelets (G/L)	388 $\pm$ 164	381 $\pm$ 245
Reticulocytes (G/L)	165 $\pm$ 39	163 $\pm$ 47
Reticulocytes (%)	7.6 $\pm$ 4.2	8.0 $\pm$ 4.9
<b>Coagulation</b>		
D Dimers (ng/L)	1437 $\pm$ 740	2152 $\pm$ 1406
Prothrombin time (sec)	14.4 $\pm$ 5.0	15.6 $\pm$ 15.0
APTT ratio	1.06 $\pm$ 0.17	0.97 $\pm$ 0.13
Fibrinogen (g/L)	3.8 $\pm$ 1.2	3.1 $\pm$ 0.97*
Protein C activity (%)	90.4 $\pm$ 13.9	84.5 $\pm$ 14.7
Protein S free antigen (%)	67.2 $\pm$ 29.5	71.6 $\pm$ 19.6
<b>Biochemistry</b>		
Creatinine ( $\mu$ mol/L)	54.8 $\pm$ 26.8	54.9 $\pm$ 12.2
Albumin (g/L)	38.7 $\pm$ 4.0	44.0 $\pm$ 4.0***
Prealbumin (g/L)	0.19 $\pm$ 0.04	0.22 $\pm$ 0.05
LDH (UI/L)	442 $\pm$ 94	451 $\pm$ 134
ASAT (UI/L)	68.3 $\pm$ 40.3	47.7 $\pm$ 21.9
Total bilirubin ( $\mu$ mol/L)	47.9 $\pm$ 32.4	36.7 $\pm$ 18.8
Haptoglobin (g/L)	0.09 $\pm$ 0.14	0.08 $\pm$ 0.23
Ferritin (ng/mL)	354 $\pm$ 420	291 $\pm$ 300
C reactive protein (mg/L)	12.1 $\pm$ 14.5	10.1 $\pm$ 26.2

Note: Values represent mean  $\pm$  standard deviation. Significant difference: \* $p<0.05$ , \*\*\* $p<0.001$ .

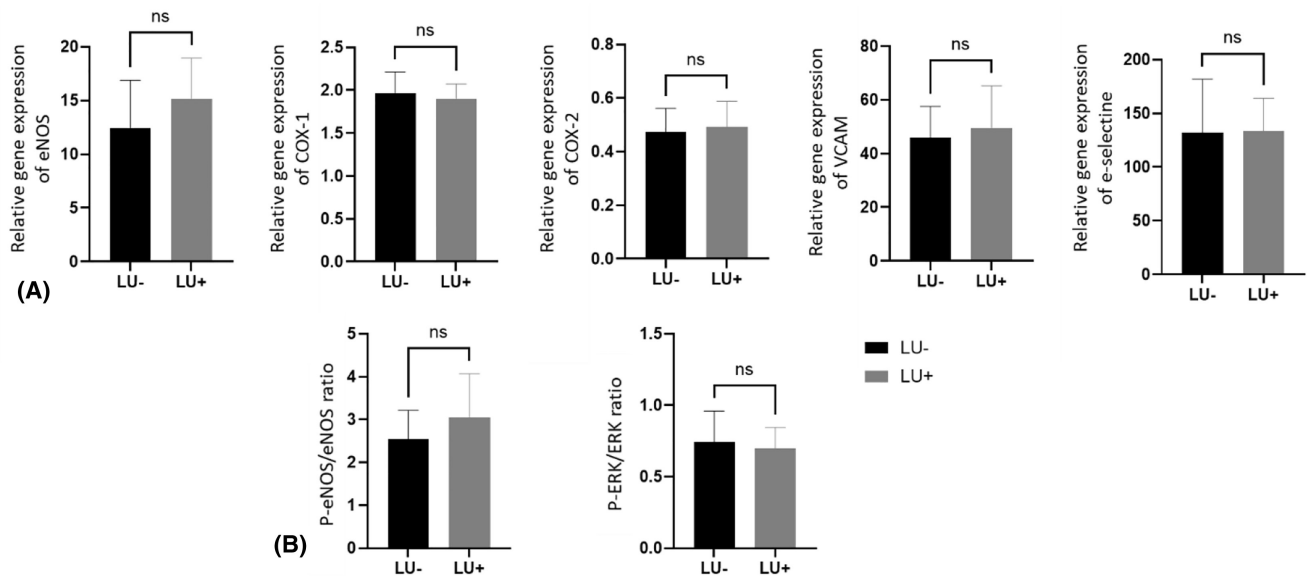
Abbreviations: aPTT, activated partial thromboplastin time; ASAT, aspartate aminotransferase; LDH, lactate dehydrogenase; LU, leg ulcer; MCHC, mean corpuscular haemoglobin concentration; MCV, mean corpuscular volume.

Six patients were healed at second visit (LU+ healed) with a mean delay of healing of  $4.4\pm 2.5$  months. The remaining two patients (LU+ still active) experienced a reduction in the size of their ulcers: one patient exhibited a decrease from  $6.3\text{ cm}^2$  to  $3.1\text{ cm}^2$ , while the other patient had three ulcers, with their sizes decreasing from  $21.2\text{ cm}^2$  to  $7.9\text{ cm}^2$  for ulcer 1,  $27.5\text{ cm}^2$  to  $16.5\text{ cm}^2$  for ulcer 2, and  $25.5\text{ cm}^2$  to  $5.5\text{ cm}^2$  for ulcer 3.

The mean ulcer size at the initial visit was  $5.7\pm 4.8\text{ cm}^2$  for the patients who achieved healing and  $20.7\pm 9.6\text{ cm}^2$  for those who did not heal. The mean duration since ulcer occurrence at the initial visit was  $4.6\pm 4.8$  months for patients who achieved healing, and  $15.0\pm 6.0$  months for the other two



**FIGURE 2** Skin blood flow in SCA patients in responses to various stimuli. (A) Patients with leg ulcer (LU+, grey) or without leg ulcer (LU-, black) in response to acetylcholine ( $n=9$ LU+ and 56LU-), current ( $n=9$ LU+ and 56LU-) and local heating ( $42^{\circ}\text{C}$ ) ( $n=9$ LU+ and 53LU-). All responses were significantly different between LU+ and LU- (Mann-Whitney  $U$ -test). (B) Six patients with active LU at initial visit (black,  $n=6$ ) and after healing (blue,  $n=6$ ) in response to acetylcholine, current and local heating. All responses were significantly different after healing (Wilcoxon matched pairs test).



**FIGURE 3** (A) mRNA expression on endothelial cells (HDMEC) incubated with plasma of 10 patients without leg ulcer (LU-) and eight patients with leg ulcer (LU+) determined by quantitative PCR. Expression is reported to TATA-box binding protein. Significant difference:  $*p < 0.05$ ; NS: No significant difference. (B) Phosphorylated NOS/eNOS ratio and phosphorylated ERK/ERK ratio determined on endothelial cells (HDMEC) incubated with the plasma of 11 patients without leg ulcer (LU-) and eight patients with leg ulcer (LU+) by western blot. Significant difference:  $*p < 0.05$ ; NS: No significant difference.

patients. Among the two patients who did not reach healing, only Voxelotor was administered. The remaining patients were treated solely with Hydroxyurea except one patient receiving a combination of Hydroxyurea and Voxelotor. The mean TcPO<sub>2</sub> at first intermetatarsal space was  $57 \pm 7$  mmHg for the six patients who achieved healing and  $42 \pm 9$  mmHg for the two other patients.

For the six patients who achieved healing, we compared microcirculatory function between the initial visit and the second visit (after ulcer healing). Mean TcPO<sub>2</sub> at first intermetatarsal spaces was significantly higher after healing ( $64 \pm 7$  mmHg and  $74 \pm 4$  mmHg,  $p = 0.037$ ). There was no difference regarding routine biology and biochemistry between the two times of measurement (see Table S2). The cutaneous

vasodilatation in response to acetylcholine, current and local heating was higher after healing than before ( $p$  0.004,  $p$  0.0013 and  $p$  0.003 respectively) (Figure 2B).

## DISCUSSION

The present study showed that (1) SCA patients with active LU had a reduction of their cutaneous microvascular vasodilatory capacity compared to patients who never experienced LU; (2) leg ulcer healing was accompanied by an improvement of the cutaneous microvascular vasodilatory capacity.

The patients with LU included in this study are similar to those typically described in the literature, in that they frequently had oedema,<sup>30</sup> ulcer-related ankylosis that required more often technical assistance for mobility, and less frequent VOCs.<sup>31</sup> Surprisingly, patients with LU had a higher haemoglobin level (even after excluding transfused patients) compared to those without, which could be explained by the fact that all patients (except one) were treated with disease-modifying therapy (mostly hydroxyurea), which reduces haemolysis.<sup>32</sup> Previous studies demonstrated a connection between LU and reduced albumin levels in SCA,<sup>33</sup> like in ulcers in non-SCA population.<sup>34</sup> Nevertheless, in our study, the albumin levels in the LU+ group remained within the normal range,<sup>35</sup> and it is unclear whether the lower albumin levels preceded the occurrence of LU or if they emerged during the attempt at wound healing. It could be interesting to propose nutritional supplementations for SCA patients with LU and low albumin levels to test the impact on LU healing. Besides, we found no difference regarding body weight and body mass index between the two groups, which confirms previous works.<sup>7,33</sup>

Some of the differences in baseline laboratory parameters (haemoglobin, neutrophil count and albumin) could be the result of chronic inflammation associated with leg ulcers. Despite higher levels of neutrophils and fibrinogen in the LU+ group, we found no difference in inflammatory cytokines between the LU+ and LU- groups. The majority of the patients of the LU+ group underwent compression therapy, which is considered a cornerstone in sickle LU treatment.<sup>30</sup> However, as compression therapy improves venous shear stress, it has been shown to decrease plasma inflammatory cytokines,<sup>36</sup> which might explain the lack of major difference in inflammation between the two groups. Incubation of endothelial cells with the plasma of LU+ and LU- patients did not show any difference in endothelial activation.

Interestingly, the inter metatarsal TcPO<sub>2</sub> was lower in the LU+ group compared to LU- patients, in the absence of peripheral arterial disease except in one patient of the LU+ group. The mean TcPO<sub>2</sub> reported in the LU- group was close to the known normal value while LU+ group had values 20% lower.<sup>37</sup> Leg oedema would not be responsible for this TcPO<sub>2</sub> defect, since a previous study showed that removal of oedema did not modify TcPO<sub>2</sub> values.<sup>38</sup> We suggest that TcPO<sub>2</sub> defect in LU+ patients could be due to

a microvascular insufficiency, which could contribute to peripheral neuropathy. The sensory defect observed with cold and heat stress on the lower limbs and with the Von Frey test on the four limbs in the LU+ patients compared to LU- patients strengthens this hypothesis. We also observed a statistically significant impairment of the microvascular capacity of vasodilatation in response to stimuli. It is largely admitted from human studies that local delivery of acetylcholine in the skin induces endothelium-dependent vasodilatation mediated by cyclooxygenase (COX) and NO pathways.<sup>39</sup> Current-induced vasodilatation primarily results from a stimulation of the COX-1 pathway.<sup>40</sup> Local heating is known to cause a two-phase vasodilatory response: an initial peak that mostly depends on the stimulation of afferent sensory nerve fibres and a second plateau response, which mainly depend on the production of NO by endothelial cells.<sup>28,41,42</sup> In our study, the functional exploration of the skin microcirculation in response to acetylcholine, current and heat clearly showed a decreased microvascular reactivity in the LU+ group compared to the LU- group. The lower peak response to acetylcholine and current suggests an impairment in the COX pathway,<sup>39</sup> while the lower plateau response to heat suggests an impairment of the NO pathway.<sup>28</sup> We were unable to demonstrate a difference between the two groups in the endothelial mRNA expression of eNOS and COX, or in the endothelial P-eNOS/eNOS ratio, after incubation of endothelial cells with plasma. Further studies are needed to understand why microvasodilatory capacity was lower in SCA patients with LU compared to those without.

Although the number of patients with active LU was small in the present study, we were able to follow them for several months and to perform new measurements of their microcirculatory function. We observed healing in six of the eight patients followed. Healing was well predictable by the criteria of healing prognosis of sickle LU observed in the French cohort reported by Senet et al.<sup>43</sup>; patients achieving healing had a smaller total LU area (i.e. < 8 cm<sup>2</sup>) and a shorter LU duration (i.e. < 9 weeks) compared to those who did not heal.<sup>43</sup> We observed that healing was associated with improved TcPO<sub>2</sub> value on first intermetatarsal spaces and improved microvascular vasodilatation. This result is consistent with the old hypothesis that TcPO<sub>2</sub> reflects the oxygen delivered by vessels minus the oxygen locally consumed by wound.<sup>44</sup> However, the results reported in this study raise the question of an improvement in TcPO<sub>2</sub> that could be due to the increased microcirculatory vasodilatory capacity after healing. We ignore if healing was facilitated by the microcirculatory improvement or if the microcirculatory improvement was secondary to healing. The number of patients who did not achieve healing was too low ( $N=2$ ) to compare to those who did achieve healing. To our knowledge, there is no previous study comparing the microcirculatory vasodilatory capacity by iontophoresis before and after healing, both in SCA and non-SCA population.

Our study has some limitations. The main limitation is the small sample size of LU+ group, due to a low incidence of leg ulcers in SCD patients in our centre. Moreover,

differences in baseline characteristics between the LU+ and LU- groups introduced confounding factors into the interpretation of the results. These differences included a higher age in the LU+ group and a higher proportion of osteonecrosis in the LU- group. Older sickle cell patients without ulcers were less likely to participate due to several reasons such as lack of time, lack of interest in the financial compensation and fatigue from frequent blood draws. We were therefore unable to obtain age-matched groups.

Ageing impacts on the vascular system in the general population<sup>21,45,46</sup> and one could suspect an effect of age in the loss of vasodilatory capacity of the microcirculation in the LU+ group. Nevertheless, we did not find any correlation between age and the different markers of the microcirculatory function in the present study. The loss of the vasodilatory capacity of the microcirculation in SCD is already present at early age<sup>47</sup> and although age surely contributes to the progression of vasculopathy, it is probably difficult to isolate the influence of age in the context of SCD, as chronic haemolysis, inflammation and oxidative stress play a significant role in the deterioration of the vascular function.

We also observed a higher rate of VTE in patients with ulcers. We are unsure if VTEs affect microcirculatory reactivity, but all episodes occurred more than 3 months prior to inclusion. Conversely, the higher proportion of leg oedema in LU+ patients could have contributed to impaired microvascular reactivity by increasing shear stress locally, leading to endothelial cell activation.<sup>48</sup> There was also an imbalance in the use of hydroxyurea, which was higher in the LU- patients and might affect endothelial activation. It has been shown that cutaneous microvascular dilation is greater in SCD patients treated with hydroxyurea than in those who do not receive this treatment.<sup>49</sup> It could therefore be possible that hydroxyurea minimised the difference in microvascular reactivity between the two groups.

Our study does not allow us to determine whether microcirculatory defects are a cause or a consequence of leg ulcers. It would be necessary to study patients just before the onset of leg ulcers and after ulcers appear. The timing of leg ulcer development is unpredictable and it is not possible to identify in advance which patients will develop ulcers, it is therefore not possible to conduct such a study at the present time.

In conclusion, our study demonstrated that patients with active LU had poor microcirculatory function and that those who healed improved vasodilatory capacity of the microcirculation. The impaired microcirculation could limit oxygen delivery to cutaneous tissues, increasing the risk for LU to occur or limiting LU healing process. Although laser Doppler flowmetry experiments suggest an involvement of the COX and NO pathways in the alterations of the microcirculatory capacity of the patients with LU, further studies are needed to address the exact mechanisms of LU which could improve clinical management of this complication.

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## CONFLICT OF INTEREST STATEMENT

All authors have no conflict of interest to disclose.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author, JC, upon reasonable request.

## INFORMED CONSENT

Complete written informed consent was obtained from the patient.

## CLINICAL TRIAL REGISTRATION

ClinicalTrials.gov ID NCT04983654.

## ORCID

Judith Catella  <https://orcid.org/0000-0001-8028-6827>

Hamdi Rezigue  <https://orcid.org/0009-0003-3765-3603>

Celine Renoux  <https://orcid.org/0000-0002-7263-805X>

Philippe Joly  <https://orcid.org/0000-0002-2351-9441>

## TWITTER

Judith Catella  JudithCatella

Philippe Connes  connesphilippe

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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