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# Prediction of healing in Category I pressure ulcers by skin blotting with plasminogen activator inhibitor 1, interleukin-1α, vascular endothelial growth factor C, and heat shock protein 90α: A pilot study

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ABSTRACT

The prevention of progression of Category I pressure ulcers (PUs) to Category II or higher is important, as Category II or higher PUs are open wounds and have a higher infection risk. Prognosis prediction of Category I PUs is necessary to provide successful intensive care for PUs with impaired healing. We focused on skin blotting using plasminogen activator inhibitor 1 (PAI1), interleukin-1α (IL-1α), vascular endothelial growth factor C (VEGF-C), and heat shock protein 90α (HSP90α). This pilot study was conducted at long-term-care and general hospitals to examine the applicability of DESIGN-R and thermography; the feasibility of skin blotting technique; the biomarker candidates, PAI1, IL-1α, VEGF-C, and HSP90α; and sample size for prognosis prediction for Category I PUs. Patients aged > 65 years underwent skin blotting, scoring for DESIGN-R, and took thermography images of their Category I PU site. Albumin signals were not detected in one out of three participants. PAI1, IL-1α, VEGF-C, and HSP90α were detected in 19 participants, among whom 11 participants could be followed up after one week. There was no difference in DESIGN-R score and skin surface temperature between normal and impaired healing groups, and the sample size was calculated as 16. In conclusion, the feasibility of skin blotting was confirmed. PAI1, IL-1α, VEGF-C, and HSP90α could be biomarker candidates for prognosis prediction for Category I PU and the combination of VEGF-C and HSP90α could be associated with the prognosis of Category I PU. We need to investigate 842 patients in a future study.

## 1. Introduction

A pressure ulcer (PU) is a localized injury to the skin and/or underlying tissue, usually over a bony prominence, as a result of pressure, or pressure in combination with shear [1]. The prevalence of PUs is reported to be 2.0–32.2% in nursing homes in the United States and Canada, and 9.6% in long-term-care hospitals in Japan [2–4]. PUs cause pain, discomfort, distress, deteriorating quality of life, and increasing mortality rates among patients [5–7]. PUs also increase hospitalization duration and health-care system costs in accordance with PU severity [8–10]. Therefore, patients, their families, medical staff, and the society face serious physical, psychological, and social problems.

PUs are classified by category, ranging from I to IV, according to wound depth [1]. Category II or higher PUs are open wounds with skin defects and have higher risks of infection than Category I PUs, which are still intact with non-blanchable redness [1]. Therefore, prevention of worsening wound severity to Category II or higher and promoting healing of Category I PUs are important. Category I PUs account for more than 30% of all PUs [11]. A previous study reported that 35.5% of Category I PUs worsened, and blisters and erosions occurred in patients in long-term-care hospitals in Japan [12]. Furthermore, Category I PUs are painful skin lesions. Briggs et al. reported that the prevalence of pain in Category I PU patients (72.7%) was remarkably high compared with other PUs (20.4% in Category II, 3.7% in Category III, 2.0% in Category IV, and 1.2% in unstageable PUs) [13]. Therefore, delayed healing of Category I PUs reduces the quality of life of patients. If

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delayed healing or worsening of Category I PUs could be predicted, intensive care can be provided for such PUs, including the use of high-performance air mattresses, frequent repositioning and vibration therapy [14,15].

To date, only few studies have reported the prediction of prognosis in Category I PUs. Sato et al. continuously investigated the morphological features of Category I PUs until those PUs were either healed or deteriorated, and showed that the double erythema and erythema away from the tip of the bony prominence predicted the worsening effects [12]. However, the sensitivity and specificity of these features were 36.4% and 95.0%, respectively, indicating that the worsening of the PUs was overlooked. Sanada et al. followed up 3,196 patients with PUs from two multicenter cohort studies and revealed that DESIGN-R, which is a visual inspection-based paper-and-pencil wound assessment tool, predicted the healing of PUs. The cutoff point was 9 for healing within 30 days, and the sensitivity and specificity were 82.1% and 69.8%, respectively [16]. However, the cutoff score for predicting the prognosis was decided from the analysis of all categories of PUs. It is unclear whether this cutoff score is useful in distinguishing the healing and non-healing Category I PUs, or whether we can statistically decide the specific cutoff score for Category I PUs. Cox et al. investigated the skin temperature of discolored intact skin by infrared thermography in skilled nursing facilities and revealed that the discolored intact skin with cooler temperatures as compared with the adjacent skin, was necrotic after a week [17]. However, we clinically need to predict not only the necrosis but also inflammation and pain. It is unclear whether thermography can predict such non-healing Category I PUs. Wound healing is a complicated process designed to maintain skin integrity, in which several events such as inflammation, fibrosis, vascular repair, and nerve regeneration simultaneously and/or sequentially occur [18]. Therefore, understanding the pathophysiology may contribute to the higher predictive power for the prognosis of Category I PUs.

Pathophysiologies of the skin and wound tissue are conventionally identified using histological examination of biopsy samples. However, biopsy sampling is not suitable for the repeating assessment of Category I PUs because of its invasiveness. The skin blotting method developed in this laboratory is recognized as the alternative method to skin biopsy [19,20]. In skin blotting, the hydrated nitrocellulose membrane on the skin surface attracts and captures the soluble protein from the inside of the skin tissue. Its immunostaining reflects the pathophysiological condition of the skin. In previous studies, skin blotting has already been applied for the fragile skin of elderly or obese people [19–21]. However, the feasibility of skin blotting should be proven on the morbid bony prominence, because the close attachment of inflexible nitrocellulose membrane is speculated to be harder on the morbid bony prominence than that on the relatively flat skin, such as the forearm.

To date, several biomarkers for non-healing wounds have previously been reported, such as matrix metalloproteinases, interleukins, β-catenin, c-myc, procalcitonin, and so on [22]. These studies were conducted only on open wounds, which were accompanied with a loss of skin tissue and healed through hemostasis, inflammation, granulation, epithelialization, and maturation of scar tissue. The biomarkers previously reported can also be applicable for Category II or severer PUs. However, Category I PUs are erythematous or purpuric without loss of tissue, and the healing process is different from that in open wounds. There is no report showing any biomarkers for prognosis of Category I PUs as far as we know, at least. Category I PU is caused by external force through a complex mechanism including ischemic injury, ischemia-reperfusion injury, lymphatic injury, and deformation. Activation of the fibrinolytic system, lymphangiogenesis, and inhibition of response to inflammation and cell death occur in the healing process of Category I PUs. Therefore, we focused on the secretory proteins reflecting these tissue responses as candidate biomarkers. A previous study reported that expression/secretion of PAI1 was increased under the regulation of hypoxia-inducible factor 1 by an external force [23]. IL-1α is a cytokine involved in inflammasome formation by ischemia-reperfusion injury [24]. VEGF-C secreted from macrophages is a specific growth factor that promotes lymphangiogenesis [25]. Expression and secretion of HSP90α reflect the mechanical stress of fibroblasts [26]. Kimura et al. demonstrated the skin blotting detection of the candidate biomarkers, PAI1, IL-1α, VEGF-C, and HSP90α, from the compressed dorsal skin of mice [27]. However, it is unknown whether these biomarker candidates are detectable from the human skin, which is structurally different from that of animal skin.

We conducted this pilot study to examine the applicability of DESIGN-R and thermography; the feasibility of skin blotting technique; and the biomarker candidates, PAI1, IL-1α, VEGF-C, and HSP90α, for the prognosis prediction of Category I PUs in elderly patients. Furthermore, we calculated the sample size based on these results to plan the subsequent prospective cohort study.

## 2. Materials and methods

### 2.1. Study design and settings

This pilot study was conducted cross-sectional in long-term-care and general hospitals in Ishikawa prefecture, Japan. The study period was from August to October 2017.

### 2.2. Participants

This study was conducted among patients aged > 65 years who had Category I PUs and whose degree of independence was ranked B or C using the criteria for evaluation of the degree of independence during daily living [28]. We also recruited patients who matched age, sex, body mass index, and Braden Scale with patients having Category I PUs. These criteria of the degree of independence are proposed in the long-term care insurance system for disabled elderly individuals by the Ministry of Health and Welfare, Japan. This scale includes four ranks, J, A, B, and C. The definitions of the ranks are as follows: rank J: Although a patient has some disability, he/she can spend daily life almost independently and can go out without any assistance from others, rank A: A patient can spend indoor life independently, but he/she needs some assistance to go out, rank B: Although a patient can maintain a sitting position by him/herself, he/she spends most of the day in bed and requires some assistance in indoor life, and rank C: A patient spends all day in bed and requires assistance to urinate/defecate, to have a meal, and to change his/her clothes. The exclusion criteria were patients with Category I PUs on their fingers, toes, soles, and heels; recurrent Category I PUs; lymphoid disease; topical skin injuries or inflammatory diseases on the PU site; and difficulty in joining the study as judged by the medical staff.

### 2.3. Identification of a Category I PU

In this study, Category I PU was operationally defined as redness of a localized area over a bony prominence or its periphery for > 24 h after its discovery. The researcher avoided pressure at the site of the Category I PU for > 60 min before investigation. The researcher also performed the glass plate compression test for the redness. Incontinence-associated dermatitis, skin tear, and deep tissue injury are difficult to distinguish from Category I PUs. A wound, ostomy, and continence nurse with extensive experience in the research and management of PUs confirmed that incontinence-associated dermatitis and skin tear were not included in Category I PUs by observation of photographs. Ultrasonographic images of the PU site were obtained using an ultrasonographic machine (M-Turbo, Sonosite, Bothwell, WA) with high-frequency linear transducer (L25x/13-6 MHz, Sonosite), which automatically determines the most appropriate frequency (from 6 to 13 MHz) and focus. An experienced PU research sonographer distinguished deep tissue injuries from Category I PUs [29].

### 2.4. Measurements

#### 2.4.1. Characteristics of the participants

Information on age, sex, hospital length of stay, disease, body mass index (BMI), degree of independence, and mattress type was collected from medical records. The Braden Scale [30] was scored by the researcher. The presence or absence of contracture was observed during the investigation. Subcutaneous fat thickness was measured at the midpoint between the acromion process and olecranon on the upper arm by a caliper (Adipometer, Abbott Japan Co. Ltd., Japan).

#### 2.4.2. Evaluation of the prognosis of Category I PU

The prognosis of Category I PU was evaluated and divided into two groups, the normal and impaired healing groups, according to the morphological changes of the PUs one week after investigation. Sato et al. reported the time-course change of morphologies of healing and worsening of Category I PUs [12]. Pigmentation appearance around the Category I PU edge or at the double erythema and change from a non-blanchable to a blanchable erythema, which were judged using glass plate compression, were signs of healing. PUs showing these changes were categorized into the normal healing group. PUs without these changes or with the appearance of erosion, blisters and partial purpura were categorized into the impaired healing group.

#### 2.4.3. Predictive parameters

Predictive parameters were the detection of biomarkers on skin blotting, DESIGN-R score, and skin surface temperature.

The process of staining and evaluation in skin blotting were blinded. Skin blotting using nitrocellulose membranes (1-cm square) was performed on the Category I PUs in accordance with the method described in a previous report [19]. The vacuum-driven immunodetection system (SNAP i.d. 2.0, Merck Millipore, Billerica, MA) was used as the staining procedure. For candidate biomarker staining, the nitrocellulose membranes were divided into two pieces, one for double staining for PAI1 (dilution 1:200; Abcam, Cambridge, UK) and VEGF-C (dilution 1:100; Cell Signaling Technology, Danvers, MA), and the other for HSP90α (dilution 1:100; Abcam) and IL-1α (dilution 1:200; Proteinteck, Rosemont, IL) with appropriate secondary antibodies labeled with alkaline phosphatase or peroxidase (dilution 1:1000; Jackson ImmunoResearch Laboratories, West Grove, PA). Some pieces of skin blotting samples were stained with alkaline phosphatase labeled antibody for human albumin (dilution 1:50, American Qualex, San Clemente, CA). Dot blot samples of the recombinant peptide of candidate biomarkers (PAI1: Merck Millipore, Billerica, MA; VEGF-C: BioLegend, San Diego, CA; HSP90α: StressMarq, British Columbia, Canada; IL-1α: Sigma-Aldrich, Tokyo, Japan) and pooled normal plasma (George King Bio-Medical Inc., Overland Park, KS) were used as positive control. Immunoreactivities were visualized using chemiluminescent substrates for alkaline phosphatase (BioFX Chemiluminescent AP Microwell/Membrane Substrate, Ultra Sensitive, SurModics, Eden Prairie, MN) and peroxidase (Luminata Forte, Merck Millipore), followed by recording using a chemiluminescence imaging device (LumiCube Liponics, Tokyo, Japan). The presence or absence of signals was judged by banalization using threshold intensity, which was (mean + 2 × standard deviation) of the signal intensity of the negative control sample without any blotting measured by an image analysis software, Image J version 1.51s (National Institutes of Health, Bethesda, MD).

DESIGN-R was scored by the researcher.

The skin surface temperatures at the center of the Category I PU area and the five points around the Category I PU were measured using infrared thermography (Thermo Shot F30, Nippon Avionics, Tokyo, Japan). The five points were selected at regular intervals in the normal skin area, pictures of which were taken parallel to the infrared camera and the distance of which was 2 cm from the Category I PU edge (Fig. 1). Increased temperature in the PU area was calculated by subtracting the average temperature of the surrounding area from that of the Category I PU area [12].

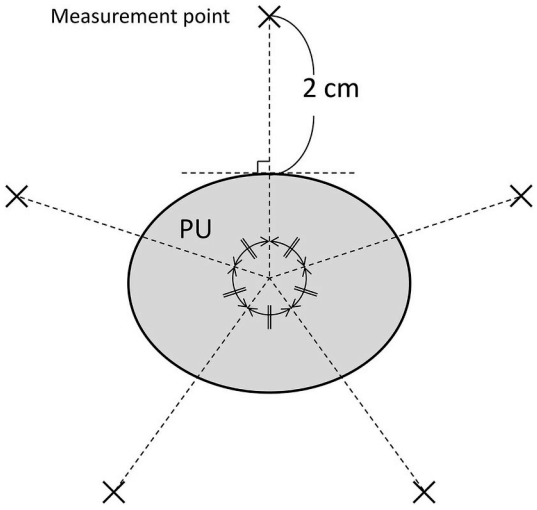


**Fig. 1.** Measurement points of skin surface temperatures around the Category I PU.

### 2.5. Statistical analysis

The differences in characteristics of participants and Category I PUs between two groups were tested using the Mann-Whitney *U* test or Fisher's exact probability test. The association between the detection of biomarkers by skin blotting and the prognosis of Category I PUs was tested using the Fisher's exact probability test. Subsequently, the associations of DESIGN-R score and increased skin surface temperature with the prognosis of Category I PUs were tested using the Mann-Whitney *U* test or Fisher's exact probability test. The sample size was calculated considering 80% power at a significance level of 0.05. The data are shown as median and range, and a $p$ value of $< 0.05$ was regarded as statistically significant. All statistical analyses were performed using JMP Pro version 13.0.0 (SAS Institute, Cary, NC).

### 2.6. Ethical considerations

The study protocol was approved by the Research Ethics Committee of the Graduate School of Medicine, The University of Tokyo (No. 11591) and by the ethics committee of the subject facility (No. 117). The participants provided written informed consent. If the participants could not decide for themselves owing to their low consciousness, their legal representatives provided informed consent.

## 3. Results

### 3.1. Feasibility of skin blotting

In order to show the feasibility of skin blotting for the morbid bony prominence, skin blotting samples were collected from three sites. Fig. 2-A and B show the nitrocellulose membrane attached to the bony prominences. The tape around the nitrocellulose membrane attached on the bony prominence was crinkled. In one out of three samples, albumin was not detected, whereas albumin was detected on the entire membrane of the other samples (Fig. 2-C).

### 3.2. Prognosis prediction of Category I PUs

Nineteen patients with Category I PUs were recruited for the prognosis prediction of PUs by skin blotting, DESIGN-R score and skin surface temperature. No PU was determined to be a deep tissue injury on ultrasonography. The median age and BMI of the participants were

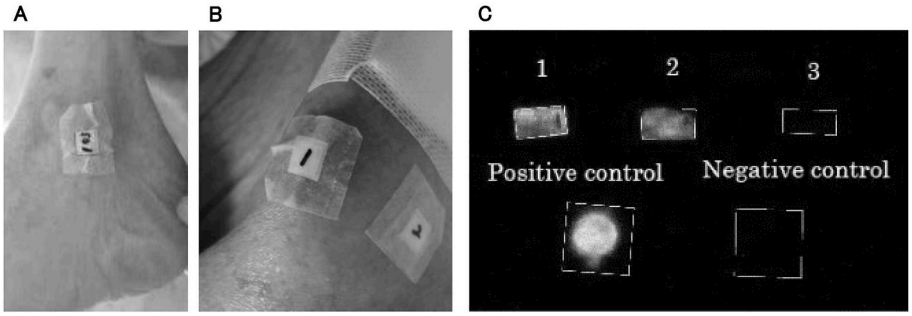


**Fig. 2.** Feasibility of skin blotting.

**Table 1**
Characteristics of the participants.

| | Baseline | Follow-up | | |
|---|---|---|---|---|
| | | Normal healing group | Impaired healing group | *p* |
| | (n = 19) | (n = 5) | (n = 6) | |
| Age (years) | 84 (67–101) | 82 (81–98) | 88 (80–96) | 0.783 |
| Sex | | | | 0.242 |
| Male | 10 (47.4) | 4 (80.0) | 2 (33.3) | |
| Female | 9 (52.6) | 1 (20.0) | 4 (66.7) | |
| Length of stay (days) | 165 (10–2902) | 245 (22–472) | 448 (10–2902) | 0.584 |
| Disease | | | | |
| Cerebrovascular disease | 11 (57.9) | 4 (80.0) | 2 (33.3) | 0.242 |
| Cardiovascular disease | 11 (57.9) | 3 (50.0) | 3 (50.0) | 1.000 |
| Respiratory disease | 11 (57.9) | 4 (80.0) | 3 (50.0) | 0.546 |
| Digestive disease | 2 (10.5) | 0 (0.0) | 1 (16.7) | 1.000 |
| Orthopedic disease | 2 (10.5) | 0 (0.0) | 1 (16.7) | 1.000 |
| Urological disease | 5 (26.3) | 1 (20.0) | 1 (16.7) | 1.000 |
| Dementia | 8 (42.1) | 2 (40.0) | 2 (33.3) | 1.000 |
| Body mass index (kg/m$^2$) | 15.2 (10.1–18.8) | 15.6 (10.1–18.8) | 15.0 (14.0–16.3) | 0.807 |
| Subcutaneous fat thickness (mm) | 5.0 (2.0–12.7) | 6.0 (3.3–12.7) | 6.5 (2.0–8.7) | 0.713 |
| Braden scale score | 11 (9–13) | 12 (11–13) | 11 (11–12) | 0.480 |
| Degree of independence[a] | | | | 1.000 |
| Rank J | 0 (0.0) | 0 (0.0) | 0 (0.0) | |
| Rank A | 0 (0.0) | 0 (0.0) | 0 (0.0) | |
| Rank B | 5 (26.3) | 2 (40.0) | 2 (33.3) | |
| Rank C | 14 (73.7) | 3 (60.0) | 4 (66.7) | |
| Contracture | | | | 1.000 |
| Presence | 12 (63.2) | 3 (60.0) | 4 (66.7) | |
| Absence | 7 (36.8) | 2 (40.0) | 2 (33.3) | |
| Mattress type | | | | 0.455 |
| Air mattress | 14 (73.7) | 4 (80.0) | 4 (66.7) | |
| Urethane foam mattress | 3 (15.8) | 0 (0.0) | 2 (33.3) | |
| Standard mattress | 2 (10.5) | 1 (20.0) | 0 (0.0) | |

Median (range)/n (%).

[a] Degree of independence, rank J: Although a patient has some disability, he/she can spend daily life almost independently and can go out without any assistance from others, rank A: A patient can spend indoor life independently, but he/she needs some assistance to go out, rank B: Although a patient can maintain a sitting position by him/herself, he/she spends most of the day in bed and requires some assistance in indoor life, and rank C: A patient spends all day in bed and requires assistance to urinate/defecate, to have a meal, and to change his/her clothes.

84 years and 15.2 kg/m$^2$, respectively. The median Braden Scale score was 11 points (Table 1). The median area of Category I PUs was 2.16 cm$^2$. Category I PUs mostly occurred at the greater trochanter, followed by the iliac crest, upper rear iliac spine, and the malleolus (Table 2).

Table 3 shows the detection of four biomarkers by skin blotting of Category I PUs. Among 19 Category I PUs, PAI1 was detected in 13, IL-1α and VEGF-C was detected in five, and HSP90α was detected in four. VEGF-C was detected only in Category I PUs without purpura and HSP90α was detected only in PUs with nonblanchable redness by glass plate compression.

Among nineteen participants, eleven were followed up after one week (Fig. 3). Five out of 11 participants were categorized into the normal healing group and six participants were into the impaired healing group. Two PUs in the normal healing group were completely healed, and a PU worsened to a Category II in the impaired healing group at follow-up. PAI1 signals were detected in four of the five PUs in the normal healing group, and five of the six PUs in the impaired healing group. IL-1α signals were detected in one PU in the normal healing group and in two PUs in the impaired healing group. VEGF-C signals were detected in three PUs in the normal healing group, and one PU in the impaired healing group. HSP90α signals were detected in two PUs only in the impaired healing group (Table 4). There were no differences in the detection of any candidate biomarkers by skin blotting between groups. Further analysis of the marker combinations showed that VEGF-C-positive and HSP90α-negative tests tended to be associated with the prognosis of Category I PUs ($p = 0.061$, Table 5).

The median DESIGN-R score was 6 in both groups, without significant difference between the groups ($p = 0.838$). The median increase in skin surface temperature in the Category I PUs was −0.05 °C in the healed group and 0.39 °C in the impaired healing group, without a significant difference between the groups ($p = 0.850$; Table 6).

### 3.3. Sample size estimation

The sample size was estimated as 16 subjects with Category I PUs for the study on the predictive validity of the combination of VEGF-C and HSP90α for prognosis assessment of Category I PUs in Japanese elderly patients.

## 4. Discussion

This is the first study to apply the skin blotting method using PAI1, IL-1α, VEGF-C, and HSP90α to Category I PUs of patients. The feasibility of skin blotting was confirmed, and these proteins could be detected from the morbid bony prominence. The combination of VEGF-C and HSP90α in skin blotting showed the tendency for association with the prognosis of Category I PUs, whereas DESIGN-R score and infrared thermography showed no difference between groups. The required sample size was estimated as 16 for the next study on prediction of Category I PU prognosis by skin blotting.

In the present study, the associations of DESIGN-R score and skin surface temperature with the prognosis of Category I PUs were analyzed. However, the results did not agree with those of previous studies [12,17]. This inconsistency is considered because the previous studies included cases of deep tissue injuries. The DESIGN-R score could not predict the prognosis of Category I PUs because it is mostly decided by the size of the wound in Category I PU, but the healing of Category I

**Table 2**
Characteristics of pressure ulcers.

| | Baseline | Follow-up | | |
|---|---|---|---|---|
| | | Normal healing group | Impaired healing group | *p* |
| | (n = 19) | (n = 5) | (n = 6) | |
| Locations | | | | 1.000 |
| Spinal column | 1 (5.3) | 1 (20.0) | 0 (0.0) | |
| Rib | 1 (5.3) | 0 (0.0) | 1 (16.7) | |
| Iliac crest | 3 (15.8) | 0 (0.0) | 1 (16.7) | |
| Greater trochanter | 5 (26.3) | 2 (40.0) | 0 (0.0) | |
| Upper rear iliac spine | 3 (15.8) | 0 (0.0) | 1 (16.7) | |
| Sacrum | 1 (5.3) | 0 (0.0) | 1 (16.7) | |
| Medial condyle | 1 (5.3) | 1 (20.0) | 0 (0.0) | |
| Malleolus | 3 (15.8) | 0 (0.0) | 2 (33.3) | |
| Fifth metatarsal head | 1 (5.3) | 1 (20.0) | 0 (0.0) | |
| Size (baseline) ($cm^2$) | 2.16 (0.60–20.25) | 5.94 (0.96–20.25) | 6.09 (2.16–9.00) | 1.000 |
| Size (follow-up) ($cm^2$) | – | 0.90 (0.00–12.60) | 2.46 (0.28–11.00) | 0.464 |
| Redness remaining during glass plate compression | 12 (63.2) | 2 (40.0) | 3 (50.0) | 1.000 |
| Double erythema | 11 (57.9) | 1 (20.0) | 4 (66.7) | 0.242 |
| Purpura | 6 (31.2) | 1 (20.0) | 1 (16.7) | 1.000 |
| Pigmentation | 3 (15.8) | 2 (40.0) | 0 (0.0) | 0.182 |

Median (range)/n (%).

**Table 3**
Visual inspection and skin blotting detection of candidate biomarkers in Category I pressure ulcers.

| Patient No. | Visual inspection | | | | Skin blotting detection | | | |
|---|---|---|---|---|---|---|---|---|
| | Glass plate compression[a] | Double erythema | Purpura | Pigmentation | PAI1 | IL-1α | VEGF-C | HSP90α |
| 1 | – | – | – | + | + | – | + | – |
| 2 | – | – | – | + | + | – | + | – |
| 3 | – | + | – | + | + | + | – | – |
| 4 | – | – | – | – | – | – | – | – |
| 5 | – | – | – | – | + | + | – | – |
| 6 | – | – | – | – | + | + | + | – |
| 7 | – | + | – | – | + | – | – | – |
| 8 | + | – | – | – | + | – | – | + |
| 9 | + | – | – | – | – | – | – | – |
| 10 | + | – | – | – | + | – | + | – |
| 11 | + | + | – | – | + | + | – | – |
| 12 | + | + | – | – | – | – | – | + |
| 13 | + | + | – | – | + | – | + | + |
| 14 | + | + | + | – | – | – | – | – |
| 15 | + | + | + | – | + | – | – | – |
| 16 | + | + | + | – | + | – | – | + |
| 17 | + | + | + | – | + | – | – | – |
| 18 | + | + | + | – | – | + | – | – |
| 19 | + | + | + | – | – | – | – | – |
| Total | 12 | 11 | 6 | 3 | 13 | 5 | 5 | 4 |

[a] Category I pressure ulcers were examined as to whether the redness was blanchable (−) or nonblanchable (+) by glass plate compression.

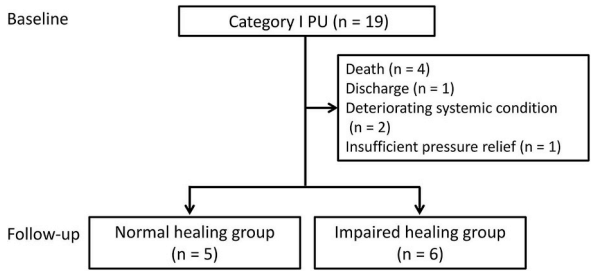


**Fig. 3.** Flow diagram of participants.

PUs is not necessarily accompanied by reduction in wound size. In fact, our result that the wound sizes were not significantly different between groups, showed the wound size of Category I PUs did not reflect the progress of healing. Comprehensive evaluation including the depth of tissue damage is required. Especially in Category I PUs, the evaluation

**Table 4**
The detection of four biomarkers in the normal healing group and impaired healing group.

| | Normal healing group | Impaired healing group | *p* |
|---|---|---|---|
| | (n = 5) | (n = 6) | |
| PAI1 | | | 1.000 |
| Positive | 4 (80.0) | 5 (83.3) | |
| Negative | 1 (20.0) | 1 (16.7) | |
| IL-1α | | | 1.000 |
| Positive | 1 (20.0) | 2 (33.3) | |
| Negative | 4 (80.0) | 4 (66.7) | |
| VEGF-C | | | 0.242 |
| Positive | 3 (60.0) | 1 (16.7) | |
| Negative | 2 (40.0) | 5 (83.3) | |
| HSP90α | | | 0.454 |
| Positive | 0 (0.0) | 2 (33.3) | |
| Negative | 5 (100.0) | 4 (66.7) | |

n (%).

**Table 5**
Prediction of prognosis in Category I pressure ulcers by the combination of VEGF-C and HSP90α.

| | Normal healing group | Impaired healing group | *p* |
|---|---|---|---|
| VEGF-C-positive and HSP90α-negative | 3 (60.0) | 0 (0.0) | 0.061 |
| Others[a] | 2 (40.0) | 6 (100.0) | |

n (%).
[a] Others include VEGF-C and HSP90α positive, VEGF-C negative and HSP90α positive, and VEGF-C and HSP90α negative.

**Table 6**
Association of DESIGN-R score and skin surface temperature with the prognosis of Category I pressure ulcers.

| | Normal healing group | Impaired healing group | *p* |
|---|---|---|---|
| | (n = 5) | (n = 6) | |
| DESIGN-R score | 6 (3–8) | 6 (3–6) | 0.838 |
| Increase in skin surface temperature (°C) | −0.05 (−0.46 to 1.07) | 0.39 (−0.85 to 0.73) | 0.855 |

Median (range).

of damage of the internal tissue is more difficult than those in Category II or severer PUs, because the tissue damage was fully covered with intact skin in Category I PUs. This indicates that the assessment tool which reflects the invisible tissue damage or reaction such as skin blotting may be suitable for the prediction of healing.

In skin blotting, albumin could not be detected in one of three skin blotting samples collected from the morbid bony prominence. This result indicates that the nitrocellulose membrane, which is an inflexible material, should be carefully attached and its close attachment should be confirmed during skin blotting. In order to improve this problem, we made an incision in the medical tape around the nitrocellulose membrane to ease the attachment to the bony prominence, and compressed it down during blotting. The use of a smaller piece of nitrocellulose membrane can make the attachment closer. Furthermore, the elimination of the analyzing area of nitrocellulose membrane to the albumin-positive area is required.

In the present study, PAI1, VEGF-C, IL-1α, and HSP90α were detected in Category I PUs. VEGF-C was not detected in Category I PU with purpura and HSP90α was not detected when redness disappeared during glass plate compression. These results suggested that the skin blotting examination can reflect the pathology of PUs. The presence of purpura indicates that the blood vessels are seriously injured. Blanchable redness by glass plate compression shows the vasodilation due to inflammatory reaction. Therefore, the stratified investigation according to the pathology of PUs and the combined examination of biomarkers, which reflect the different pathophysiologies might be a possible strategy for prognosis prediction by the biomarkers.

In this study, Category I PU was operationally defined as redness of a localized area over a bony prominence or its periphery for > 24 h after its discovery. Thus, the elapsed time from PU occurrence varied. This problem probably affected the detection of biomarkers, because the expression/secretion of biomarkers can be altered accompanied with the progression of healing or deterioration of PUs. Therefore, the patients have to be recruited before the occurrence of Category I PU and the PU occurrence should be identified precisely by the daily observation of the entire body of participants. Sample size estimation showed that 16 subjects with Category I PUs are required for the study on the prognostic prediction of Category I PUs in Japanese elderly patients. Because the incidence of PUs is 1.9% in Japanese long-term-care hospitals [4], more than 842 elderly patients should be observed daily for the prospective cohort study.

PAI1 is an ischemic marker; its expression/secretion is increased by hypoxia-inducible factor 1 activated by vascular occlusion and formation of a thrombus due to vascular injury caused by an external force [23]. IL-1α is a cytokine involved in inflammasome formation by ischemia-reperfusion injury [24]. Expression and secretion of HSP90α reflects the mechanical stress of fibroblasts [31]. Thus, the detection of these proteins is speculated to indicate a further load of an external force and to be associated with worsening Category I PU, and the detection of these proteins may relate to which injury was occurred. VEGF-C is a specific growth factor that promotes lymphangiogenesis [25] and can trigger the healing of Category I PUs. In Category I PU, the vascular system in the dermal layer is injured by an external force and the injuries remain or expand by the accumulation of such anaerobic metabolites and reactive oxygen species [32,33]. In the inflammatory reaction, macrophages secrete several cytokines, including VEGF-C. After lymphangiogenesis, the damaged tissue due to anaerobic metabolites and oxidative stress recovers, and PU healing progresses. Thus, the detection of VEGF-C expression most likely identified the PUs in which lymphangiogenesis was initiated, and VEGF-C may predict the prognosis of Category I PU. The combination of HSP90α with VEGF-C may yield a larger detection power than the use of a single biomarker. However, several systemic factors such as nutritional status and aging, as well as local factors such as pressure and edema, can impair the healing of Category I PUs [31,34]. Therefore, the evaluation of a single biomarker was thought to be insufficient, and combining biomarkers was the probable strategy to predict the prognosis of Category I PUs.

## 5. Conclusion

Although the attachment of nitrocellulose membrane needed to be modified, this pilot study showed that PAI1, IL-1α, VEGF-C, and HSP90α were detectable from the morbid bony prominence with Category I PU of patients. VEGF-C and HSP90α is a possible combination in the prediction of the prognosis of Category I PUs using skin blotting, whereas DESIGN-R score and infrared thermography could not be associated with the prognosis of Category I PUs. In a future study, we will investigate 842 patients to determine whether skin blotting using these biomarkers can predict the prognosis of Category I PU.

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