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Development of an improved method for quantitative analysis of skin blotting: Increasing reliability and applicability for skin assessment

著者	Ogai Kazuhiro, Matsumoto Masaru, Minematsu T., Kitamura K., Kobayashi M., Sugama Junko, Sanada H.
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Abstract

OBJECTIVE: A novel skin assessment tool named "skin blotting" has been recently developed, which can easily predict the skin status to avoid its deterioration. The aim of this study is to propose a normalization method for skin blotting to compensate for individual differences that can hamper the quantitative comparisons and clinical applications.

METHODS: To normalize individual differences, we utilized a total protein as a "normalizer" with calibration curves. For evaluation, we performed a simple simulation experiment, in which the same concentration of a protein of interest [tumor necrosis factor (TNF)-α] was applied at different volumes as a virtual individual difference. Moreover, to demonstrate the applicability of this normalization, male volunteers were recruited for skin blotting followed by the estimation of TNF-α with normalization.

RESULTS: We obtained good calibration curves for total protein (R^2 = 0.995) and TNF-α (R^2 = 0.997), both of which were necessary for an exact quantification. In the simulation experiment, we estimated the exact concentration of TNF-α regardless of the applied volume, demonstrating the applicability of this normalization method in skin blotting. Further, skin blotting on human subjects showed a wide range of variation in the total protein content, although the normalization was thought to reduce such individual variations.

CONCLUSION: This study has proposed total protein normalization for skin blotting with calibration curves. This method may strengthen the quantitative performance of skin blotting, which may expand the applicability of this method as a skin assessment tool in broader fields, such as nursing and cosmetology.

Key words: Biotechnology/Fermentation; Safety testing; Skin blotting; Skin physiology/structure; Normalization

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Introduction

The skin is the largest organ of our body, serving as a barrier against several external invasions, such as mechanical, chemical, and bacterial insults. In addition, the skin participates in water retention, sensory acquisition, immune reaction, and the appearance of our body [1, 2]. In contrast, apparent skin disorders, such as wounds, burns, pressure ulcers [3], psoriasis [4], dermatitis [5], and infections can interfere with the function of the skin, cause secondary dysfunctions (e.g., pain, discomfort, and psychological uncertainty), and may ultimately deteriorate the quality of life [6-9]. Beside these drawbacks, the skin can also be fragile, and several conditions, such as obesity and aging can adversely affect skin integrity without any apparent symptoms. For example, a mouse model for obesity has shown severe difficulties, involving wound healing [10, 11] and the same has been equally true for human subjects [12]; obese subjects may have a higher prevalence of pressure ulcers [13] and a number of studies have reported the association between obesity and psoriasis [14]. Furthermore, it has been reported that the skin of obese people is continuously exposed to oxidative stress, collagen-degrading enzymes [15], and inflammatory cytokines, e.g., tumor necrosis factor-α (TNF-α), all of which may be linked to skin weakness [16-20]. In another instance, skin maceration caused by incontinence leads to incontinence-associated dermatitis, which can be prevented by assessing the reduction of skin barrier function using a simple skin assessment tool [21]. From these findings, it may be important to assess the property of skin in a biochemical manner before the appearance of obvious drawbacks such as wound development that may affect the quality of life.

Under these circumstances, several types of non-invasive skin assessment tools have been thus developed using laser analysis [22], skin fluorescence [23, 24], trans-epidermal water loss [25], confocal microscopy [23], and ultrasonography [19, 20]. However, all these assessment tools require specialized equipment and training; thus, they are difficult to manage as daily assessment tools.

Minematsu et al. have recently developed a novel, non-invasive, and easy-to-handle skin assessment method called “skin blotting” [26]. Soluble proteins (e.g., TNF-α, nerve growth

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factor-β, and matrix metalloproteinase-2) in the dermis and epidermis can leak by diffusion through the epidermis and follicles when the skin is slightly macerated [18, 27]. In this method, a prewetted nitrocellulose membrane is patched to the region of interest on the skin, which creates a slightly macerated state. Under such a condition, the soluble proteins that leak from the epidermis and dermis can be captured by the membrane. The proteins on the membrane can then be analyzed using immunoblot techniques. This method is similar to the tape stripping method [28]. However, the tape stripping method can only capture the proteins from the stratum corneum, the very superficial layer of the skin; with this method, the real-time assessment of deeper skin layers may be more difficult. In addition, the tape stripping may be relatively more invasive than skin blotting, particularly for the elderly, obese people, and infants because it peels the stratum corneum off the skin, which may reduce the barrier function.

Considering the principle behind skin blotting, the quantitative analysis of skin blotting largely relies on the equality of the permeability of the proteins, the skin barrier function, and protein adsorption into the membrane. However, such equalities can be hardly achieved because skin conditions can greatly vary between individuals [29, 30]. Therefore, a "normalizer," such as a housekeeping protein should be employed to eliminate these individual differences as much as possible. However, it is impossible to select specific proteins (e.g., β-actin, broadly used in Western blotting) because these proteins generally do not leak from the cells unless the cells are disrupted. Considering such restrictive conditions, normalization could be achieved by quantifying the total protein content on the membrane, as performed in the tape stripping [28] and Sebutape methods [31]. Moreover, it is important to express the content of the protein of interest as an absolute quantity instead of using an arbitrary unit (i.e., intensity itself) because the intensity of immunodetection can vary according to the experimental conditions and examiners, thereby making it difficult to compare results among individuals and experiments unless performed by the same examiner at the same time.

In this study, therefore, we attempt to facilitate a normalization method of skin blotting by quantifying the total protein content on the membrane with a recent low-background protein staining technique [32]; we also attempt to express the protein content as an absolute quantity

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using calibration curves.

Methods

Skin blotting with human subjects

To evaluate normalization using the total protein content on the membrane, skin blotting on human subjects was performed as previously described [26] with the approval of the Medical Ethics Committee of Kanazawa University (#430); we also followed the Declaration of Helsinki. Nineteen healthy males (age: 43.7 ± 14.0) with a body mass index (BMI) of <25 (range: 20.8–24.7) and five obese males (age: 36.6 ± 7.99) with a BMI of >30 (range: 30.9–34.7) were recruited, in which a distinct difference in the level of chronic inflammation (i.e., TNF-α) was expected. A written informed consent was obtained from each participant before surveillance. After the completion of a verbal questionnaire about age and sex and the measurement of height and body weight (for a BMI calculation), a nitrocellulose membrane (1 cm × 1 cm; Bio-Rad Laboratories, CA, USA) prewetted with 10 µl saline was attached to the skin at 2 cm left to the navel, where the differences in dermal structure and level of oxidative stress were observed [20], for 10 min. The membranes were stored at 4°C until further analyses.

Calibration curve

For the quantitative analysis of the total protein content and TNF-α, calibration curves were prepared for each item. In brief, bovine serum albumin (BSA; Nacalai Tesque, Kyoto, Japan) and TNF-α peptide (Santa Cruz Biotechnology, CA, USA) were used as the total and TNF-α protein standards, respectively. Serial dilutions of BSA (800, 320, and 128 ng μl^{-1}) and TNF-α (1600, 640, and 256 pg μl^{-1}) were prepared, and 1 µl of each solution was dropped onto separate membranes. These membranes were then analyzed by the method described below.

Membrane analysis with total protein staining

Membrane analysis was performed as previously described [26] with a modification for the total protein staining. In brief, the membranes (1 cm × 1 cm) after skin blotting were first stained

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with 3% (*w*/*v*) Reactive Brown 10 [32] (RB10; Sigma–Aldrich, MO, USA) in water for 2 min. They were then washed with water for several times until the background staining was bleached. For densitometry of the total protein content, scanned images of the membranes was obtained with a graphics scanner (GT-S640, Seiko Epson Corp., Nagano, Japan) with no gamma correction. Next, the membranes were washed with 0.1% (*v*/*v*) Tween-20 in Tris buffered saline (pH = 7.4) for 5 min, followed by blocking with Blocking One (Nacalai Tesque) for 30 min and incubation with anti-TNF-α antibody (1:200; Santa Cruz Biotechnology) at 4°C overnight. On the next day, the membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibody (1:1000; Santa Cruz Biotechnology) at room temperature for 60 min. Following repeated washing, the signals were detected with Clarity Western ECL Substrate (Bio-Rad Laboratories) and the ChemiDoc MP system (Bio-Rad Laboratories). The total protein and TNF-α images were densitometrically analyzed using ImageJ software (National Institutes of Health, MD, USA). The cumulative signal intensity of total protein or TNF-α was calculated over the entire surface except the edge of each membrane with the same area, which enables the direct comparison of spotted (e.g., membranes for a calibration curve and the punctate signals of TNF-α) and diffuse (e.g., background staining) signals.

Evaluation for total protein normalization

To evaluate the effectiveness of total protein normalization, we performed a simple simulation experiment as follows. We prepared a mixture of BSA and TNF-α as a simulant skin protein. Two different concentrations of TNF-α (700 pg μl^{-1} as “high” and 350 pg μl^{-1} as “low”) in the same concentration of BSA (300 ng μl^{-1}) were prepared to simulate the physiological variations of TNF-α. For the simulation of protein adsorption to the membrane, different volumes (1, 1.5, or 2 µl) of the mixture were dropped onto the separate membranes and analyzed as described above. Total protein normalization was then performed by simply dividing the amount of TNF-α by that of BSA.

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Statistical analysis

We used GraphPad Prism software (version 6.0f; GraphPad Software, Inc., CA, USA) to calculate the calibration curves and to compare the two groups in simulation experiments by two-way analysis of variance followed by Tukey's *post hoc* test. In skin blotting analyses for human subjects, Student's *t*-test or Mann–Whitney *U* test was used for comparison between healthy and obese groups after the *F*-test of equality of variances. For logarithmic approximation, we used a two-parameter formula $Y = a–b\cdot\log X$. A p value of <0.05 was considered statistically significant.

Results

Calibration curves for total protein (BSA) and TNF-α

First, we serially diluted BSA and TNF-α and prepared calibration curves with a fixed amount of BSA or TNF-α (x-axis) versus the signal intensity calculated by ImageJ software (y-axis). For total protein staining, we obtained visible spots whose intensity correlated to the amount of BSA (Fig. 1A; RB10), whereas no positive staining was observed on the TNF-α-spotted membranes (Fig. 1B; RB10). Further, we obtained a good (adjusted coefficient of determination $R^2 = 0.995$, $n = 3$) calibration curve with a logarithmic approximation (Fig. 1A). In the same manner, a calibration curve of TNF-α was obtained (Fig. 1B; adjusted $R^2 = 0.997$, $n = 3$). Positive signals of TNF-α immunoblotting were detected only on TNF-α-spotted membranes (Fig. 1B; TNF-α) but not on the membranes with a drop of BSA solution (Fig. 1A; TNF-α). The following experiments were performed with the *ad hoc* calibration curves of BSA and TNF-α.

Efficacy of total protein normalization

To evaluate the effectiveness of total protein normalization, a simple model was prepared (Fig. 2A, B). We prepared two different concentrations of TNF-α (700 pg μl^{-1} as "high" and 350 pg μl^{-1} as "low") in a fixed concentration of BSA (300 ng μl^{-1}) to simulate the physiological variation of TNF-α. These mixtures were dropped onto separate membranes with different volumes (1, 1.5, or 2 µl), which reflected the protein adsorption difference to the membrane (Fig.

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2B). As a result of this quantification, the calculated amount of TNF-α was proportional to the droplet volume (Fig. 2C). The quantification of BSA also showed a similar result in which the amount of BSA reflected the applied volume of mixture to the membrane (Fig. 2D). After total protein normalization, the normalized amount of TNF-α showed comparable values regardless of the droplet volume (Fig. 2E; n.s., $p = 0.70$), whereas high and low concentrations of TNF-α could be distinguished (Fig. 2E; $p < 0.0001$ between "high" and "low" TNF-α). This simple simulation showed that total protein normalization could eliminate the effect of the droplet volume (i.e., differences in protein adsorption to the membrane).

Total protein normalization after skin blotting

We wanted to demonstrate the applicability of total protein normalization in practical skin blotting among human subjects. As a result of skin blotting on 19 healthy and 5 obese male volunteers, we confirmed a wide range of variation in the total protein content on the membrane (Fig. 3A, B). After total protein staining, we successfully detected signals of TNF-α by immunoblotting (Fig. 3C) except for six undetected samples (i.e., under the detection limit). The quantification of TNF-α without normalization did not show the significant difference between healthy and obese people (Fig. 3D; $p = 0.69$, Mann–Whitney *U* test). After normalization, however, the difference became significant (Fig. 3E; $p = 0.006$, Student's *t*-test).

Discussion

The aim of this study was to improve the quantitative performance of skin blotting [26], which is required for its general application as a skin assessment tool. To achieve this, we employed a novel, sensitive, and selective dye to stain the total protein on the membrane, which was then used as a normalizer for the protein of interest. We then evaluated the applicability of normalization through simulation and clinical experiments.

Why should total protein normalization be employed for skin blotting?

Skin blotting is a non-invasive and easy-to-handle method, which can evaluate the soluble

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protein content in and on the skin [26] and now has been used in several studies to show the properties of skin inflammation [26, 27], soluble skin components, and skin remodeling-related enzymes [18]. It has been reported that skin frailty is closely related to the degree of oxidative stress [16, 19] and skin inflammation with cytokine secretion [11, 18, 28, 33], and that such deteriorations were caused by obesity [16, 20, 34] and aging [18], both of which are now increasing worldwide. Considering these situations, it will be of importance to assess skin properties on a molecular biological basis (i.e., cytokines and gene expression) before the skin shows apparent symptoms, such as delayed wound healing [35, 36], skin tears [18, 37], or pressure ulcers [38]. For convenience and an ease of use, skin blotting may be an appropriate method to easily assess the properties of skin before the skin deteriorates.

The principle of skin blotting is based on the mild, temporal disruption of the skin barrier by local maceration, which allows protein to leak from the epidermis and dermis. However, the skin status, such as the skin barrier function and skin thickness can greatly vary among individuals; for example, the number of wrinkles is associated with age, sex, and sun exposure [29]; the water content of skin differs according to age and the places lived [30]; and the thickness of the dermis is greater in overweight ($25 \leq$ BMI < 30) people compared with non-overweight (BMI < 25) people [20]. Therefore, individual differences in skin properties should be considered when comparing the content of the protein of interest (e.g., cytokines) among individuals. In addition, protein adsorption to the membrane may be profoundly affected by the nature of the membrane (e.g., moistness and temperature) and handling by the operator (e.g., the force of membrane attachment). Considering such fluidity, it is difficult to compare the results of different individuals unless a normalizer is employed. Some of the quantitative methods for skin assessment involve normalization by the total protein content [28, 31]. Therefore, in this study, we attempted to employ the total protein content on the membrane as a normalizer (Fig. 2A) as utilized in previous studies.

RB10 as a potential total protein staining in skin blotting

There are a number of staining methods for total protein, including Coomassie brilliant blue

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(CBB) R-250 [39], Ponceau S [40], colloidal gold [41], and SYPRO Ruby [42]. Of these methods, we first tested the CBB method for total protein staining on the membrane; however, the background staining was too high to distinguish a small amount of protein (i.e., CBB staining was apparent even on the membrane without protein). Colloidal gold and SYPRO Ruby staining are highly sensitive methods to detect total protein. However, the former takes at least one hour and generally up to a day for protein staining, and the latter requires special imaging equipment for signal detection. A novel method for total protein staining utilizing a fabric dye RB10 has been recently proposed [32]. In this method, total protein can be stained under neutral pH conditions, a milder environment than CBB (acidic); thus, the staining may not affect the subsequent immunodetection process. The immunodetection of TNF-α after RB10 staining was indeed successfully achieved (Fig. 3C). In addition, RB10 can easily be bleached by washing with water where proteins do not exist. The sensitivity of RB10 may be considerably higher than CBB as we could see a specific staining on the skin blotting membrane with RB10, whereas CBB staining yielded such a high background pattern that we were unable to tell whether the staining was due to proteins or the remaining dye. Moreover, the RB10 signal can be scanned by a commercially available document scanner unless gamma correction is not applied. Most importantly, the calibration curve showed a good linearity (Fig. 1), enabling the reliable quantification of total protein on the membrane. Considering these advances, we propose RB10 as a total protein staining method for individual normalization.

Utilizing calibration curve for estimating protein of interest in skin blotting

In the original method of skin blotting, the amount of the protein of interest detected by immunoblotting was evaluated by its intensity. In this study, to ensure accuracy and to enable inter-experimental comparison, which may be difficult using intensity quantification, we attempted to express the content of the protein of interest as an absolute quantity (i.e., using grams rather than an arbitrary unit). This could be achieved, like a total protein normalization, by a calibration curve for the protein of interest (TNF-α in this study) that showed a good linearity (Fig. 1B). This method may enable an easier comparison of the protein content in a

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different experimental setup.

Applicability of total protein normalization in skin blotting

To evaluate the effectiveness of this normalization method, we performed a simple simulation experiment (Fig. 2B). In this method, we prepared two different concentrations of TNF-α (700 pg μl^{-1} as a "high" and 350 pg μl^{-1} as a "low" samples) with the fixed concentration of BSA (300 ng μl^{-1}). These mixtures were considered human samples with different TNF-α concentrations. Next, we prepared three membranes and 1, 1.5, or 2 µl of the mixture was dropped onto each membrane. The difference in the applied volume was expected to reflect individual differences in protein adsorption to the membrane. In this manner, the different amount of TNF-α would be detected as the volume differed: 700, 1050, or 1400 pg (in a "high" sample) and 350, 525, or 700 pg (in a "low" sample) of TNF-α on the 1-, 1.5-, or 2-µl membrane, respectively, although the sample was the same and thus those membranes should have shown the same results. The different amount of TNF-α was indeed detected in the same sample but with a different droplet volume (Fig. 2C). However, this apparent and false difference could be eliminated (Fig. 2E) by a total protein content that reflected the protein adsorption (Fig. 2D) proposed in this study (Fig. 2A). We should note that the different concentrations of TNF-α ("high" and "low") were falsely reported to be the same amount if the applied volume was different (Fig. 2C; denoted by §). Such false quantification could be avoided after total protein normalization was applied (Fig. 2E). From these results, it is plausible that total protein normalization in skin blotting can increase the quantitative performance by reducing individual instability caused by the difference in protein adsorption.

Next, we wanted to know the applicability of this normalization method to practical skin blotting in human participants. Therefore, we recruited healthy and obese male volunteers for the quantification of TNF-α with total protein normalization, because they reportedly show a distinct difference in skin property such as dermal structure and the level of oxidative stress in the abdominal skin [19, 20], and thus the different level of TNF-α was likewise considered plausible. As a result of skin blotting, we could observe a wide variation of total protein content

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on the skin blotting membrane (Fig. 3A, B). These results implied the requirement of normalization. For example, the amount of TNF-α in Figs. 3C1 and 3C2 were almost the same (1239 and 1046 pg per membrane, respectively); however, the total protein content of the corresponding membranes (Fig. 3B1 and 3B2) were considerably different (97 and 239 ng per membrane, respectively). As a result of normalization, these membranes were determined to have a different concentration of TNF-α [12.7 and 4.4 (pg ng^{-1}) per membrane, respectively], which could not have been achieved without total protein staining. In contrast, the membrane shown in Fig. 3C2 had a higher level of TNF-α than that shown in Fig. 3C4 (1082 and 600 pg per membrane, respectively). However, the total protein content (Fig. 3B2 and 3B4) were 239 and 134 ng per membrane, respectively; after normalization, the concentrations of TNF-α were similar at 4.4 and 4.5 (pg ng^{-1}) per membrane. Therefore, it is plausible that there would be an erroneous quantification of TNF-α if normalization was not employed.

Because obesity can increase the level of TNF-α [[43-45] and unpublished data], it is feasible that the amount of TNF-α in the skin blotting is greater in the obese people (BMI >30) than healthy people (BMI <25). However, we observed no difference in TNF-α amount itself between healthy and obese groups (Fig. 3D). After normalization, the higher level of normalized TNF-α was observed in obese males compared to healthy ones (Fig. 3E), which is consistent with the previous findings. Although the target of this experiment was limited to males and the number of obese males was very small (n = 5), and thus it requires careful interpretation as a clinical outcome, at least plausible results were obtained to show the applicability of total protein normalization.

From these results, it can be theorized that total protein normalization would be beneficial for a more precise quantification of skin blotting, in that the individual differences in protein adsorption to the membrane would become negligible.

Limitations of this study

As the protein of interest was also included in the “total protein”, it was possible that total protein normalization may underestimate the content of the protein of interest. At least in this

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study, this was not the case because the total protein staining of TNF-α was below the detection limit (Fig. 1B, RB10; note no signals in TNF-α-spotted membranes). It may be necessary to make sure that the maximum concentration of the protein of interest is below the detection limit of total protein staining.

Another limitation of this study was that we could not verify this method in humans, as we did not know the exact amount of TNF-α on human skin unless we employed skin biopsies. An animal experiment in which the skin can be directly analyzed by histochemical assays may be further required to assure the accuracy of this method. Nevertheless, this normalization method appears to be one of the best candidates for the quantification of skin blotting (Figs. 2 and 3), in terms of reproducibility and manageability.

Conclusion

In conclusion, this study has proposed an easy and reproducible normalization method for quantitative skin blotting by analyzing the total protein content on the membrane with a recent protein staining dye. We believe that the addition of this method can strengthen the quantitative performance of skin blotting. We also believe that this method will make skin blotting easier and more usable in a broader field, such as nursing and cosmetology, for predicting the physiological state of skin and preventing skin disorders before they worsen.

Conflict of interest

The authors have declared that no competing interests exist.

References

1. Chuong, C.M., Nickoloff, B.J., Elias, P.M., Goldsmith, L.A., Macher, E., Maderson, P.A., et al. What is the 'true' function of skin? *Exp Dermatol.* 11(2):159-87 (2002).

2. Madison, K.C. Barrier function of the skin: "la raison d'etre" of the epidermis. *J Invest Dermatol.* 121(2):231-41 (2003).

3. Iizaka, S., Sanada, H., Nakagami, G., Sekine, R., Koyanagi, H., Konya, C., et al.

For Peer Review

Estimation of protein loss from wound fluid in older patients with severe pressure ulcers. *Nutrition.* 26(9):890-5 (2010).

4. Motta, S., Monti, M., Sesana, S., Mellesi, L., Ghidoni, R. and Caputo, R. Abnormality of water barrier function in psoriasis. Role of ceramide fractions. *Arch Dermatol.* 130(4):452-6 (1994).

5. Stalder, J.F., Tennstedt, D., Deleuran, M., Fabbrocini, G., de Lucas, R., Haftek, M., et al. Fragility of epidermis and its consequence in dermatology. *J Eur Acad Dermatol Venereol.* 28 Suppl 4:1-18 (2014).

6. Finlay, A.Y. and Coles, E.C. The effect of severe psoriasis on the quality of life of 369 patients. *Br J Dermatol.* 132(2):236-44 (1995).

7. Lasek, R.J. and Chren, M.M. Acne vulgaris and the quality of life of adult dermatology patients. *Arch Dermatol.* 134(4):454-8 (1998).

8. O'Donnell, B.F., Lawlor, F., Simpson, J., Morgan, M. and Greaves, M.W. The impact of chronic urticaria on the quality of life. *Br J Dermatol.* 136(2):197-201 (1997).

9. Wolkenstein, P., Loundou, A., Barrau, K., Auquier, P., Revuz, J. and Quality of Life Group of the French Society of, D. Quality of life impairment in hidradenitis suppurativa: a study of 61 cases. *J Am Acad Dermatol.* 56(4):621-3 (2007).

10. Otranto, M., Nascimento, A.P. and Monte-Alto-Costa, A. Insulin resistance impairs cutaneous wound healing in mice. *Wound Repair Regen.* 21(3):464-72 (2013).

11. Frank, S., Stallmeyer, B., Kampfer, H., Kolb, N. and Pfeilschifter, J. Leptin enhances wound re-epithelialization and constitutes a direct function of leptin in skin repair. *J Clin Invest.* 106(4):501-9 (2000).

12. Coon, D., Gusenoff, J.A., Kannan, N., El Khoudary, S.R., Naghshineh, N. and Rubin, J.P. Body mass and surgical complications in the postbariatric reconstructive patient: analysis of 511 cases. *Ann Surg.* 249(3):397-401 (2009).

13. VanGilder, C., MacFarlane, G., Meyer, S. and Lachenbruch, C. Body mass index, weight, and pressure ulcer prevalence: an analysis of the 2006-2007 International Pressure Ulcer Prevalence Surveys. *J Nurs Care Qual.* 24(2):127-35 (2009).

For Peer Review

14. Bremmer, S., Van Voorhees, A.S., Hsu, S., Korman, N.J., Lebwohl, M.G., Young, M., et al. Obesity and psoriasis: from the Medical Board of the National Psoriasis Foundation. *J Am Acad Dermatol.* 63(6):1058-69 (2010).

15. Minematsu, T., Huang, L., Ibuki, A., Nakagami, G., Akase, T., Sugama, J., et al. Altered expression of matrix metalloproteinases and their tissue inhibitors in matured rat adipocytes in vitro. *Biol Res Nurs.* 14(3):242-9 (2012).

16. Ibuki, A., Akase, T., Nagase, T., Minematsu, T., Nakagami, G., Horii, M., et al. Skin fragility in obese diabetic mice: possible involvement of elevated oxidative stress and upregulation of matrix metalloproteinases. *Exp Dermatol.* 21(3):178-83 (2012).

17. So, T., Ito, A., Sato, T., Mori, Y. and Hirakawa, S. Tumor necrosis factor-alpha stimulates the biosynthesis of matrix metalloproteinases and plasminogen activator in cultured human chorionic cells. *Biol Reprod.* 46(5):772-8 (1992).

18. Koyano, Y., Nakagami, G., Iizaka, S., Minematsu, T., Noguchi, H., Tamai, N., et al. Exploring the prevalence of skin tears and skin properties related to skin tears in elderly patients at a long-term medical facility in Japan. *Int Wound J.* (2014).

19. Horii, M., Sugama, J., Oe, M., Minematsu, T., Nagase, T., Yabunaka, K., et al. Ultrasonography reveals structural changes in the dermis and subcutaneous tissue of obese males: investigation of the association with oxidative stress. *Journal of Japanese Society of Wound, Ostomy and Continence Management.* 17(1):23-32 (2013).

20. Matsumoto, M., Ibuki, A., Minematsu, T., Sugama, J., Horii, M., Ogai, K., et al. Structural changes in dermal collagen and oxidative stress levels in the skin of Japanese overweight males. *Int J Cosmet Sci.* 36(5):477-84 (2014).

21. Mugita, Y., Minematsu, T., Huang, L., Sugama, J., Ichikawa, Y., Nakagami, G., et al. A convenient and highly sensitive method for quantification of ceramide in tape-stripping samples by dot-blot analysis as a skin barrier assessment technique in murine macerated skin. *Journal of Japanese Society of Wound, Ostomy and Continence Management.* 17(3):201-12 (2013).

22. Puissant, C., Abraham, P., Durand, S., Humeau-Heurtier, A., Faure, S., Leftheriotis, G., et al. Reproducibility of non-invasive assessment of skin endothelial function using laser

For Peer Review

Doppler flowmetry and laser speckle contrast imaging. *PLoS One.* 8(4):e61320 (2013).

23. Bargo, P.R., Walston, S.T., Chu, M., Seo, I. and Kollias, N. Non-invasive assessment of tryptophan fluorescence and confocal microscopy provide information on skin barrier repair dynamics beyond TEWL. *Exp Dermatol.* 22(1):18-23 (2013).

24. Meerwaldt, R., Graaff, R., Oomen, P.H., Links, T.P., Jager, J.J., Alderson, N.L., et al. Simple non-invasive assessment of advanced glycation endproduct accumulation. *Diabetologia.* 47(7):1324-30 (2004).

25. Angelova-Fischer, I., Mannheimer, A.C., Hinder, A., Ruether, A., Franke, A., Neubert, R.H., et al. Distinct barrier integrity phenotypes in filaggrin-related atopic eczema following sequential tape stripping and lipid profiling. *Exp Dermatol.* 20(4):351-6 (2011).

26. Minematsu, T., Horii, M., Oe, M., Sugama, J., Mugita, Y., Huang, L., et al. Skin blotting: a noninvasive technique for evaluating physiological skin status. *Adv Skin Wound Care.* 27(6):272-9 (2014).

27. Kishi, C., Minematsu, T., Huang, L., Mugita, Y., Kitamura, A., Nakagami, G., et al. Hypoosmotic Shock-Induced Subclinical Inflammation of Skin in a Rat Model of Disrupted Skin Barrier Function. *Biol Res Nurs.*in press (2014).

28. de Jongh, C.M., Verberk, M.M., Spiekstra, S.W., Gibbs, S. and Kezic, S. Cytokines at different stratum corneum levels in normal and sodium lauryl sulphate-irritated skin. *Skin Res Technol.* 13(4):390-8 (2007).

29. Akiba, S., Shinkura, R., Miyamoto, K., Hillebrand, G., Yamaguchi, N. and Ichihashi, M. Influence of chronic UV exposure and lifestyle on facial skin photo-aging--results from a pilot study. *J Epidemiol.* 9(6 Suppl):S136-42 (1999).

30. Hillebrand, G.G., Miyamoto, K., Schnell, B., Ichihashi, M., Shinkura, R. and Akiba, S. Quantitative evaluation of skin condition in an epidemiological survey of females living in northern versus southern Japan. *J Dermatol Sci.* 27 Suppl 1:S42-52 (2001).

31. Perkins, M.A., Osterhues, M.A., Farage, M.A. and Robinson, M.K. A noninvasive method to assess skin irritation and compromised skin conditions using simple tape adsorption of molecular markers of inflammation. *Skin Res Technol.* 7(4):227-37 (2001).

For Peer Review

32. Yonan, C.R., Duong, P.T. and Chang, F.N. High-efficiency staining of proteins on different blot membranes. *Anal Biochem.* 338(1):159-61 (2005).

33. Omori-Miyake, M., Yamashita, M., Tsunemi, Y., Kawashima, M. and Yagi, J. In vitro assessment of IL-4- or IL-13-mediated changes in the structural components of keratinocytes in mice and humans. *J Invest Dermatol.* 134(5):1342-50 (2014).

34. Hotamisligil, G.S., Arner, P., Caro, J.F., Atkinson, R.L. and Spiegelman, B.M. Increased adipose tissue expression of tumor necrosis factor-alpha in human obesity and insulin resistance. *J Clin Invest.* 95(5):2409-15 (1995).

35. Pence, B.D. and Woods, J.A. Exercise, Obesity, and Cutaneous Wound Healing: Evidence from Rodent and Human Studies. *Adv Wound Care (New Rochelle).* 3(1):71-9 (2014).

36. Pierpont, Y.N., Dinh, T.P., Salas, R.E., Johnson, E.L., Wright, T.G., Robson, M.C., et al. Obesity and surgical wound healing: a current review. *ISRN Obes.* 2014:638936 (2014).

37. Holmes, R.F., Davidson, M.W., Thompson, B.J. and Kelechi, T.J. Skin tears: care and management of the older adult at home. *Home Healthc Nurse.* 31(2):90-101 (2013).

38. Stojadinovic, O., Minkiewicz, J., Sawaya, A., Bourne, J.W., Torzilli, P., de Rivero Vaccari, J.P., et al. Deep tissue injury in development of pressure ulcers: a decrease of inflammasome activation and changes in human skin morphology in response to aging and mechanical load. *PLoS One.* 8(8):e69223 (2013).

39. Burnette, W.N. "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate--polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal Biochem.* 112(2):195-203 (1981).

40. Nakamura, K., Tanaka, T., Kuwahara, A. and Takeo, K. Microassay for proteins on nitrocellulose filter using protein dye-staining procedure. *Anal Biochem.* 148(2):311-9 (1985).

41. Moeremans, M., Daneels, G. and De Mey, J. Sensitive colloidal metal (gold or silver) staining of protein blots on nitrocellulose membranes. *Anal Biochem.* 145(2):315-21 (1985).

42. Berggren, K., Steinberg, T.H., Lauber, W.M., Carroll, J.A., Lopez, M.F., Chernokalskaya, E., et al. A luminescent ruthenium complex for ultrasensitive detection of proteins immobilized on membrane supports. *Anal Biochem.* 276(2):129-43 (1999).

For Peer Review

43. Tzanavari, T., Giannogonas, P. and Karalis, K.P. TNF-alpha and obesity. *Curr Dir Autoimmun.* 11:145-56 (2010).

44. Prasai, M.J., Mughal, R.S., Wheatcroft, S.B., Kearney, M.T., Grant, P.J. and Scott, E.M. Diurnal variation in vascular and metabolic function in diet-induced obesity: divergence of insulin resistance and loss of clock rhythm. *Diabetes.* 62(6):1981-9 (2013).

45. Akase, T., Nagase, T., Huang, L., Ibuki, A., Minematsu, T., Nakagami, G., et al. Aging-like skin changes induced by ultraviolet irradiation in an animal model of metabolic syndrome. *Biol Res Nurs.* 14(2):180-7 (2012).

Figure legends

Figure 1. Calibration curves for total protein and TNF-α. (A) A calibration curve of BSA for total protein and (B) for TNF-α. The experiment was repeated three times and the average was used for calculations. The data are shown as means ± the standard error of the mean (SEM). The unit a.u. stands for arbitrary unit.

Figure 2. Simulation experiment of total protein normalization. (A) Principle of the simulation experiment. In this simulation, difference in the TNF-α concentration reflects the physiological TNF-α amount on the skin, and the droplet volume reflects the difference in protein adsorption to the membrane, which can be estimated by the amount of BSA (i.e., total protein). (B) Experimental paradigm. In this experiment, different (1, 1.5, or 2 μl) volumes of the same concentration of BSA (300 ng μl^{-1}) with different concentrations of a TNF-α [700 (high) or 350 (low) pg μl^{-1}] mixture were applied to separate membranes and analyzed according to the calibration curves. (C) As expected, the detected amount of TNF-α was proportional to the droplet volume, although the concentration of TNF-α was the same. Note that the different concentrations of TNF-α were falsely detected as the same amounts due to the droplet volume (denoted by §). (D) The amount of BSA (total protein) showed similar results, corresponding to the applied volume of the mixture. (E) As a result of total protein normalization, high and low concentrations of TNF-α could be distinguished regardless of the applied volume (repeated

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three times; *$p < 0.0001$). n.s.: not significant. The experiment was repeated three times and the average was used for calculations. The data are shown as means ± SEM.

Figure 3. Total protein normalization of skin blotting on human subjects. (A) Variation of total protein amounts on the membrane. In boxplot, each border in the box shows the 25, 50, and 75 percentile from the bottom, with a min–max whisker. (B, C) Examples of total protein staining (B) and TNF-α immunoblotting (C). The same number indicates the same membrane. (D, E) Plots of TNF-α amount without (D) or with (E) normalization in healthy (BMI < 25; $n = 13$) and obese (BMI > 30; $n = 5$) males. In (E) the data are shown as means ± standard deviation. Note that the significant difference in the level of TNF-α between healthy and obese people was observed only when the normalization was applied [D, $p = 0.69$ (Mann–Whitney U test; n.s.: not significant) without normalization; E, $p = 0.006$ (Student's t-test) after normalization].

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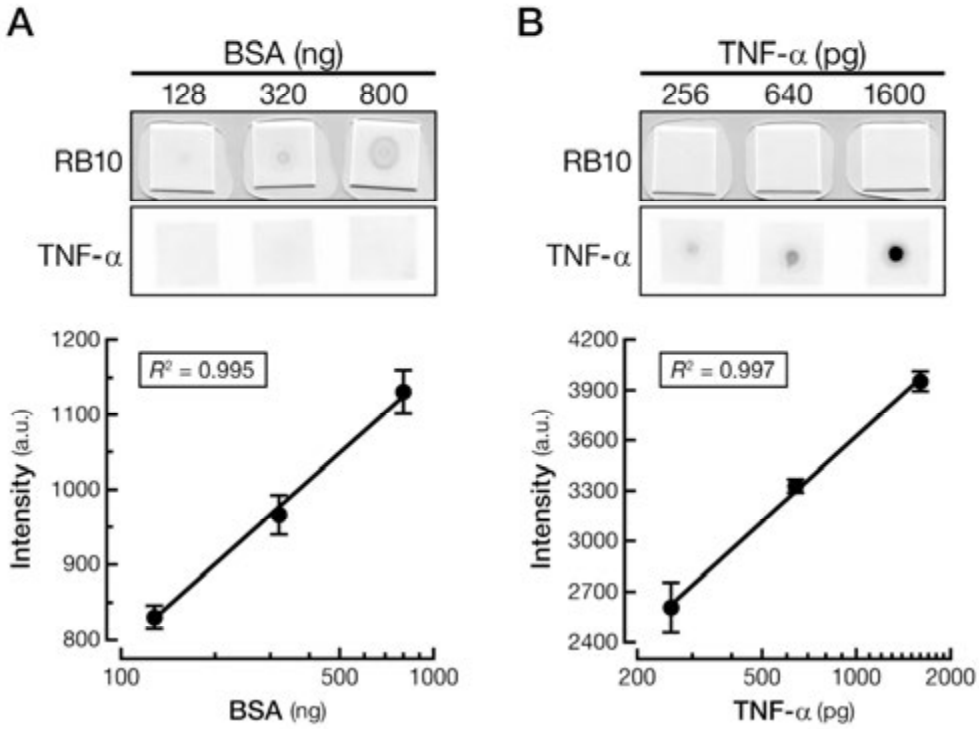

Calibration curves for total protein and TNF-α. (A) A calibration curve of BSA for total protein and (B) for TNF-α. The experiment was repeated three times and the average was used for calculations. The data are shown as means ± the standard error of the mean (SEM). The unit a.u. stands for arbitrary unit.
157x116mm (300 x 300 DPI)

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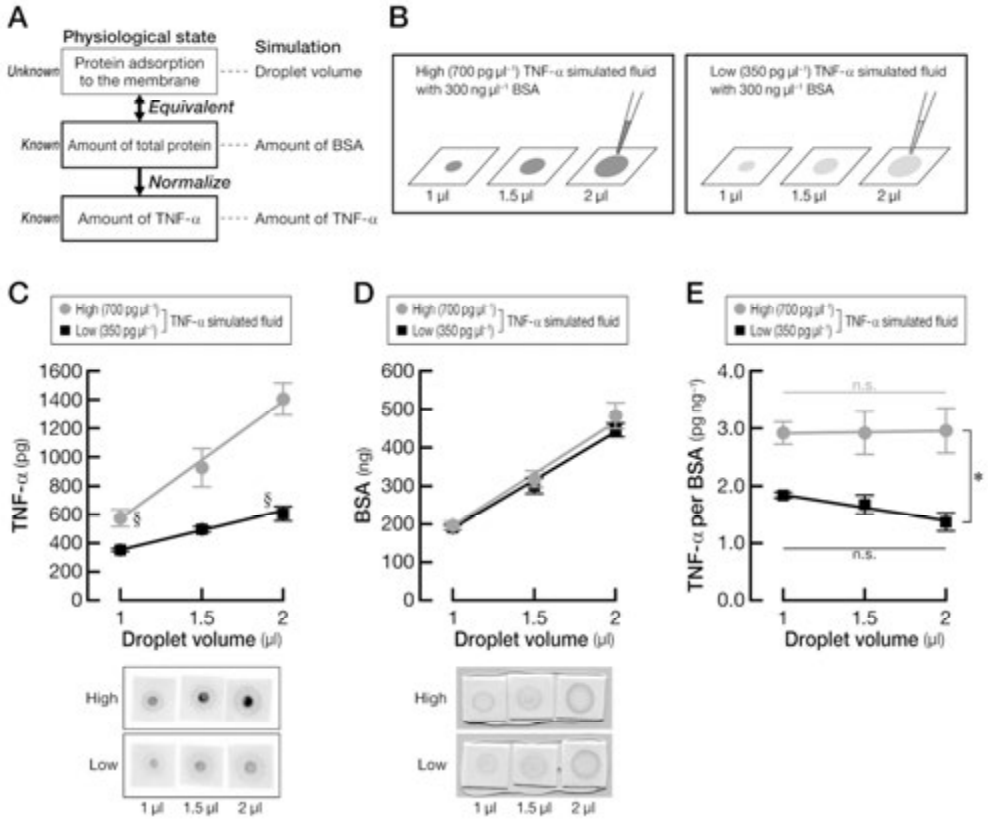

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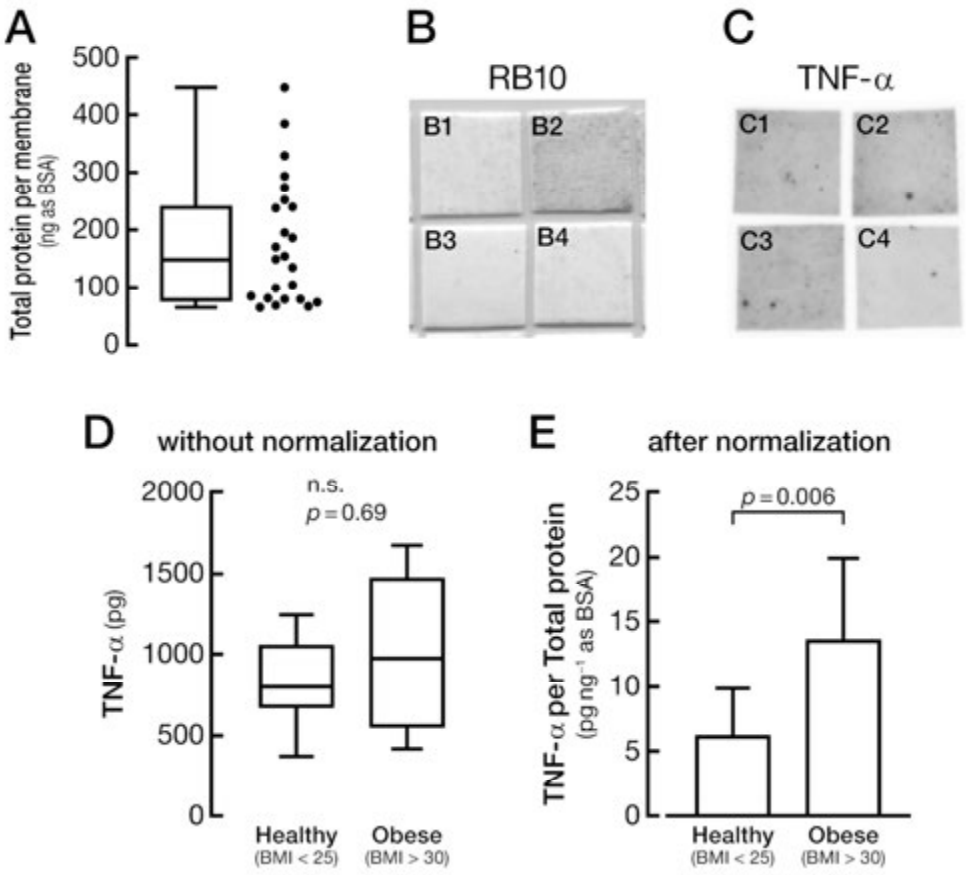
185x153mm (300 x 300 DPI)



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137x120mm (300 x 300 DPI)