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Increased level of tumor necrosis factor-alpha (TNF-α) on the skin of Japanese obese males: measured by quantitative skin blotting

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Abstract

OBJECTIVE A state of chronic inflammation, characterized by an increased level of tumor necrosis factor-alpha (TNF-α), is often found in the obese population. The negative effects of elevated TNF-α are not limited to systemic metabolism. It also reportedly affects skin integrity. Recently, the relationship between obesity and skin fragility was reported; however, there has been little insight into how the level of TNF-α in the skin *in situ* is related to the severity of obesity. In this study, we aimed to measure the level of TNF-α on the skin and to find the relationship between obesity and the level of TNF-α detected on the skin.

METHODS We used a novel, non-invasive method called quantitative skin blotting. Fifty-nine healthy (but some were classified as being overweight or obese) Japanese males were enrolled as subjects. The levels of TNF-α detected on the abdominal and thigh skin along with the body composition were measured, followed by a correlation analysis.

RESULTS Significant positive correlations were found between the levels of TNF-α detected on the skin and the severity of obesity such as body mass index (BMI), body fat weight, and visceral fat rating.

CONCLUSION We found that high levels of TNF-α were detected on the skin of Japanese obese males, which implied the higher TNF-α in the skin. The elevation of skin TNF-α may be one factor related to skin fragility that is often found in obese individuals.

1. Introduction

Obesity is a condition of excess body-fat accumulation under the skin and/or in the internal organs, leading to several health problems. Obesity is associated with hypertension, cardiovascular diseases, type 2 diabetes, sleep apnea, and several kinds of cancers [1]. The lifespan of obese individuals may decrease by up to 8–13 years, depending on the severity, due to conditions such as cardiovascular events, cancer, and diabetes [2, 3].

The mechanisms of metabolic deterioration in obesity have been extensively studied. A recent study showed that the metabolic impairment in obese individuals is caused by a vicious cycle between adipocytes and macrophages via inflammatory cytokines such as tumor necrosis factor (TNF)-α [4]. The serum level of TNF-α was found to be higher in obese individuals than in non-obese individuals [5, 6], which may be related to obesity-induced insulin resistance [7]. Given the high levels of systemic inflammatory cytokines, obese individuals are considered to be in a state of "chronic inflammation" [8, 9].

In addition to metabolic deterioration, obesity can also affect the physiology of the skin. A number of studies have pointed out the link between obesity and skin disorders. For example, obesity causes prolonged wound healing periods [10-12], higher prevalence of pressure ulcers [13], allergic disorders [14], and psoriasis [15]. Skin fragility is also related to obesity. Some studies have found higher oxidative stress and collagen-degrading enzymes in the skin [16-18] along with lower dermal integrity, which was assessed by dermal echogenicity [17], in obese populations.

Skin fragility may be linked to TNF-α elevation in obesity. During the wound healing process, TNF-α has a crucial role in re-epithelialization and neovascularization, and has a beneficial effect on histological repair of the skin [19, 20]. However, excess TNF-α impairs wound healing [21-23] TNF-α also upregulates or activates

collagen-degrading enzymes and matrix metalloproteinases (MMPs) in several kinds of cell lines or tissues, including skin [24-27] In an animal model of obesity, it was reported that the level of TNF-α in the skin is higher in obese mice than in lean mice models [28]. It is plausible, therefore, that higher levels of TNF-α might be observed in the skin of obese individuals, which may trigger skin fragility. Thus, it is important to assess the basal level of TNF-α in the skin before the appearance of obvious skin disorders.

It is quite easy to determine the amount of TNF-α in the skin, only if a biopsy is allowed. However, it is ethically quite difficult to obtain a biopsy from obese individuals just for measuring the level of TNF-α unless they have severe skin disorders, such as melanoma, that require a medical diagnosis. Therefore, non-invasive skin protein assessment tools should be employed. Recently, Minematsu et al. proposed a non-invasive skin assessment tool called skin blotting [29]. In this method, a small, wet nitrocellulose membrane is attached to the skin. The attached membrane captures soluble proteins, such as TNF-α, leaked from the skin, and the amount of the proteins is analyzed using an immunoblot technique. In that study they found no correlation between the body mass index (BMI) and the immunoreactivity of blotted TNF-α [29]; this might be because a normalization method, which eliminates individual differences due to the protein adsorption, attachment force, skin conditions, etc., was not used. For quantification of skin blotting, a normalization method was developed [30]. With a normalization method, quantitative skin blotting can be utilized for skin protein quantification. As a preliminary result of normalization experiment, the levels of TNF-α detected on the skin were significantly higher in the obese (BMI $\geq$ 30) subjects than in the normal (BMI < 25) subjects [30], which led us to conduct the detailed analyses between obesity and TNF-α on the skin.

In this study, we aimed to measure the basal level of TNF-α detectable on the

skin in Japanese males using a non-invasive, modified quantitative skin blotting technique, and we further attempt to show the correlation between the severity of obesity and the level of TNF-α detected on the skin.

2. Materials and Methods

2.1. Participants

All experiments involving human subjects were approved by the Medical Ethics Committee of Kanazawa University (approval number: 430) and were performed in accordance with the Declaration of Helsinki. In this study, we recruited male volunteers from the students and staff members of Kanazawa University as well as company workers and residents near the university. A written informed consent was obtained from all participants. We excluded individuals who had apparent skin disorders such as dry skin, edema, redness, and rash on the site of skin blotting as well as those with systemic diseases such as liver cirrhosis and renal insufficiency.

2.2. Body characteristics measurement

Body characteristics such as height, weight, waist circumference, and body composition were examined as previously described [17]. In brief, we first measured height and waist circumference using a tape measure. The body composition, including total body weight, body fat weight, body fat ratio, and visceral fat rating, was then measured using a body composition analyzer (MC-190; Tanita corp., Tokyo, Japan). Body mass index (BMI; in kg m^{-2}) was calculated as total body weight (kg) divided by the square of the height (m). Body fat ratio (%) was calculated as the ratio of body fat weight (kg) to total body weight (kg). The visceral fat rating is a rating system calculated by the MC-190, with scores 1–59 calculated on the basis of the bioelectrical impedance method [31], which is used as an index of risk for visceral fat accumulation [32-34]

2.3. Quantitative skin blotting

Quantitative skin blotting was performed to measure the level of TNF-α detectable on the skin, as described previously [29, 30]. In brief, a piece of nitrocellulose membrane (1 cm × 1 cm each; Bio-Rad Laboratories, Hercules, CA, USA) was wetted with 50 µl of sterile normal saline (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan). The pre-wetted nitrocellulose membrane was then attached to the skin with gentle pressure, followed by covering the membrane with a piece of non-permeable medical film to prevent drying. After 10 min, the membrane was removed and stored at 4 °C until analyses. The membrane was first analyzed using a total protein quantification [30]. In brief, the skin blotting membranes, along with the membranes with known concentrations of the bovine serum albumin (Nacalai Tesque, Kyoto, Japan) for a calibration curve, were stained with 3% Reactive Brown 10 (Sigma–Aldrich, MO, USA). The membranes were scanned with a document scanner (GT-S640, Seiko Epson Corp., Nagano, Japan) with no gamma correction. Next, the membranes were used for the immunoblot detection of TNF-α (primary antibody: goat anti-TNF-α, sc-1350, Santa Cruz Biotechnology, USA, 1:200 dilution; secondary antibody: horse radish peroxidase conjugated anti-goat IgG, sc-2020, Santa Cruz Biotechnology, 1:1000 dilution). Densitometric analyses with standard curves for total protein and TNF-α were performed using the ImageJ software (National Institute of Health, Bethesda, MD, USA). The linearity of standard curves for total protein and TNF-α was previously confirmed [30]. To normalize the difference of protein adsorption in each subject, the amount of TNF-α was divided (i.e., normalized) by that of total protein existing on the whole membrane [30]. The membranes that showed values below the lower detection limit [i.e., below the lower limit (256 pg) of the standard curve range] were excluded from further analyses. Two body sites were chosen for TNF-α quantification: abdomen

(2 cm to the left of the umbilicus) and thigh (posterior side of the left thigh at the midpoint between the trochanter major and the popliteal fossa), where the BMI-dependent changes of dermal structure were previously observed [17].

2.4. Western blotting

To test the molecular weight of TNF-α detectable on the skin, western blotting was performed against the lysate of the stratum corneum which could be considered as the closest sample to the skin blotting. In brief, the cells of the stratum corneum was collected by a tape stripping method [35] from a healthy volunteer with informed consent. The protein from the stratum corneum was separated by a 15% polyacrylamide gel followed by the transfer to a polyvinylidene difluoride membrane. The membrane was then analyzed by the immunodetection in the same procedure as the skin blotting, except that the Immunostar LD (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was used as a chemiluminescent substrate for more sensitive detection.

2.5. Statistical analysis

All statistical analyses were performed using Origin Pro (version 2015; OriginLab Corp., Northampton, MA, USA). The normality of variables was first tested by the Shapiro–Wilk test with alpha level set at 0.05. A simple correlation analysis was then performed between the levels of TNF-α detected on the skin and the variables of body characteristics, such as age, weight, and body fat ratio. According to the results of the normality test, the Pearson product-moment correlation coefficient (denoted by r; for normal distribution of both variables) or the Spearman's rank correlation coefficient (denoted by ρ; for non-normal distribution of either variables) was employed. A p-value of <0.05 was considered as statistically significant.

3. Results

3.1. Demographic data and body characteristics of the subjects

After applying the inclusion and exclusion criteria, 59 males were eligible for analysis. Their demographic and body composition data are shown in Table I.

3.2. Relationship between skin TNF-α and the variables of body characteristics

First, we have confirmed that the TNF-α antibody used in this study can indeed detect TNF-α protein specifically (Fig. S1). The representative images of TNF-α staining and total protein staining are shown in Fig. 1. The results of the correlation analysis are shown in Table II and Figs. 2 and 3. Among the 59 subjects, 11 membranes of the abdomen [5 from normal (BMI < 25), 5 from overweight (25 ≤ BMI < 30), and 1 from obese (BMI ≥ 30) subjects] and 14 membranes of the thigh (8 of normal, 6 of overweight subjects) were below the detection limit and thus omitted from further analyses. The level of TNF-α in the abdominal skin was significantly correlated with weight ($r = 0.293$, $p = 0.043$), BMI ($r = 0.407$, $p = 0.004$), body fat weight ($r = 0.295$, $p = 0.042$), and visceral fat rating ($\rho = 0.298$, $p = 0.040$) (Table II and Fig. 2). The level of TNF-α in the thigh skin was significantly correlated with BMI ($\rho = 0.327$, $p = 0.028$), body fat weight ($\rho = 0.310$, $p = 0.038$), waist circumference ($\rho = 0.325$, $p = 0.029$), and visceral fat rating ($\rho = 0.324$, $p = 0.030$) (Table II and Fig. 3). There was no significant correlation between the level of skin TNF-α and age, either in the abdomen ($\rho = 0.092$, $p = 0.533$) or the thigh ($\rho = 0.141$, $p = 0.356$) (Table II and Figs. 2 and 3). No significant correlations were found without total protein normalization (Figs. S1 and S2). The average levels of TNF-α detected on the skin, classified by the BMI, were as shown in Table III.

4. Discussion

The aim of this research was to show the relationship between the severity of obesity and the level of TNF-α on the skin in non-obese and obese Japanese males. To achieve this, we utilized a novel, non-invasive skin assessment tool called skin blotting with a normalization method. We then analyzed the correlation between the demographic and body composition data as well as the TNF-α levels detected on the skin. Here, we discuss the implications and limitations of this research.

4.1. The relationship between the level of TNF-α detected on the skin and the severity of obesity

In this study, we evaluated the level of TNF-α on the skin in non-obese and obese Japanese males using a non-invasive quantitative skin blotting technique. This study is the first to find significant positive correlations between the indices of obesity (BMI, body fat weight, and visceral fat rating) and the levels of TNF-α detected on the skin in the abdomen and the thigh (Tables II and III; Figs. 2 and 3). It is noteworthy that the elevation of TNF-α can be observed not only in the adipose tissue and serum [5, 6], but also through the skin *in situ*.

The mechanisms of metabolic syndromes in obese populations have been extensively studied, and low-grade chronic inflammation is proposed to be a key factor in obesity-induced systemic dysregulation [8, 9]. In 2003, Weisberg first reported macrophage accumulation in the adipose tissue of obese mice [36], which implied the involvement of the inflammatory process in obesity. Later, in 2005, Suganami proposed a vicious cycle between adipocytes and macrophages via secretion of TNF-α [4]. Obese individuals have a higher level of serum TNF-α than non-obese individuals [5, 6]. For these reasons, TNF-α has been recognized as a key factor of systemic metabolic syndrome. Along with the results of this study, it is speculated that the detection of the higher level of TNF-α on the skin might be a reflection of low-grade, systemic

inflammation according to the fat accumulation in the subcutaneous tissue.

Contrary to this study, Minematsu et al. found no correlation between the BMI classification (<20, 20–25, 25−30, and ≥30) and the intensity of TNF-α immunoreactivity [29]. However, the previous study did not utilize the total protein normalization [30]. The protein adsorption of skin blotting greatly varies among individual subjects due to several factors such as attachment pressure, skin barrier, humidity of membrane, and so forth. As it is difficult to control these factors, it is difficult, or even impossible, to use the values of immunoreactivity or protein amount *per se* for quantification, even if the area normalization (i.e., dividing raw values by the area of skin or membrane) is utilized. Indeed, no significant correlations were found if the total protein normalization was not implemented (data not shown). In the preliminary study, a significant difference of TNF-α detected on the skin between normal (BMI < 25) and obese (BMI ≥ 30) populations was observed only when the normalization method was applied [30]. In this study, we utilized a total protein normalization (i.e., dividing raw values by the amount of total protein on the same membrane) to make quantitative analyses possible.

During the analyses, 11 and 14 membranes that were attached to the abdomen and thigh, respectively, were not used because of the detection limit (<256 pg). However, these omitted membranes were derived mostly from the normal (BMI < 25) or overweight (25 ≤ BMI < 30) subjects, and just 1 membrane was from the abdomen of the obese (BMI ≥ 30) subject. We consider, therefore, that the correlation analyses in this study were not greatly biased by the data removal.

4.2. The relationship between the level of TNF-α detected on the skin and aging

Although we found a significant relationship between the severity of obesity and the level of TNF-α on the skin, we could not find a link between aging and TNF-α

(Table II and Figs. 2 and 3). Some studies have shown a positive correlation between aging and the level of serum TNF-α [37-40] However, the age-related upregulation of TNF-α seems lower in the younger to middle-aged populations (i.e., less than a 1.4-fold increase from the 30s to the 60s) [39]; it is mainly observed in the elderly population (>80 years old) [40], which was not included in this study (Table I). Therefore, the most obvious explanation is that the level of TNF-α is affected more by obesity than by aging in the study population, although further studies on the elderly would be required.

4.3. Elevation of TNF-α as a potential factor for skin fragility

The elevation of TNF-α was observed on the skin of obese individuals. The next question will be "How does TNF-α affect the skin?"

A number of studies have detected the adverse effects of TNF-α on skin function. For example: excessive TNF-α impairs the process of wound healing [21-23]; TNF-α damages the skin barrier via downregulation of filaggrin and loricrin [41]; high levels of TNF-α were observed in psoriatic patients [42] and the inhibition of TNF-α has a beneficial role against psoriasis [43]. Interestingly, some studies have revealed a link between TNF-α and collagen degradation. In these studies, the collagen-degrading enzymes MMP-1, -2, and -3 were upregulated or activated by TNF-α in the skin [24, 27]. The elevation of these MMPs is indeed related to skin collagen degradation [27]. There is another animal study that showed the relationship between MMP-2 upregulation and skin fragility due to the thinning of collagen fibers [16]. In a clinical study, Koyano et al. reported that higher levels of TNF-α and MMP-2 were observed in the skin of those who had skin tears [44]. Moreover, Matsumoto et al. observed lower echogenicity in the abdominal and thigh dermis in obese individuals, implying that the lower density of collagen content was caused by obesity [17]. From the results of these studies and our research, it is plausible that the higher level of TNF-α in the skin of

obese individuals might be one of the key factors related to skin fragility, which may lead to skin disorders or conditions in the future. Therefore, assessing the level of TNF-α through the skin might help in preventing skin disease in obese individuals.

4.4. Limitations of this study

That being said, some limitations of this study should be mentioned. First, only Japanese males were included in this study; the reason being that this study was performed in parallel with another study [17]. In the other study, hair matrix cells were used to test the oxidative stress of the skin in Japanese males because it is generally difficult to obtain hair samples from female subjects. Considering the fact that the susceptibility of the skin to various insults is different between males and females [45-47], the link between the level of skin TNF-α and the severity of obesity could be different in female subjects; this would require further research.

Second, we did not perform blood tests. Although considered safe, blood sampling is a relatively invasive procedure compared to ultrasonography, hair analysis, and skin blotting; thus, it was omitted from our study. Therefore, the metabolic status, including the levels of blood sugar, triglycerides, cholesterols, and TNF-α, of the subjects could not be determined. Although we showed the link between body fat composition and the level of TNF-α on the skin, it would also be interesting to investigate the link between such metabolic parameters and skin inflammation by utilizing a fingertip blood test kit [48].

Third, we did not assess the pattern of TNF-α signals on the skin blotting membrane. There are two parameters for the skin blotting membrane analysis: the intensity itself and the pattern of protein. In the previous study, a follicular pattern (dim background with strong spots, corresponding to the position of hair follicles) was more observed in the obese populations than the normal populations [29]. According to the

previous study, the background staining of TNF-α is derived through the stratum corneum from the living cells in the epidermis and/or dermis, whereas the follicular-pattern staining is derived from the sweat or sebum, which reflects the state of subcutaneous adipose tissue [29]. Our western blotting analysis also showed that the TNF-α detectable on the skin could be both from the epidermis and from the subcutaneous tissue via the follicular route, as shown by the difference of molecular weight (Fig. S1). However, the skin blotting is not able to distinguish such different molecular weight, because the size-separation process (e.g., gel electrophoresis) cannot be implemented in principle. In this study, therefore, the origin of TNF-α is still ambiguous. The combination of pattern and intensity analyses with a normalization method would increase the accuracy of skin blotting (e.g., origin of the protein) for the prediction of skin and subcutaneous status.

Finally, among a number of soluble inflammatory cytokines, we only tested TNF-α because the detection method is established [29]. Other cytokines related to obesity, such as interleukin-6, would also be eligible for the skin blot analysis to reveal the state of chronic inflammation in the skin of obese people.

Despite these shortcomings, this study provides a positive link between the level of TNF-α detected on the skin and obesity in human subjects. The findings of this study can help understand the skin fragility of obese individuals and may lead to insights into therapeutic strategies, including weight loss and skincare, for the prevention of skin deterioration caused by obesity.

5. Conclusion

In this study, we investigated the level of TNF-α on the skin in Japanese males by means of a novel, non-invasive, quantitative method of skin blotting. We found a significant positive correlation between the severity of obesity and the level of TNF-α

detected on the skin. The findings of this study suggest that there is a requirement of preventive skin care treatment, such as anti-inflammatory and anti-oxidative treatments in obese individuals, as well as treatment against metabolic deterioration.

Conflict of interest

The authors have declared that no competing interests exist.

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Figure legends

Figure 1 Representative images of the tumor necrosis factor (TNF)-α staining and the total protein staining among normal [body mass index (BMI) = 21.4], overweight (BMI = 29.4), and obese (BMI = 34.7) subjects.

Figure 2 Scatter plots of the relationships between the levels of normalized tumor necrosis factor (TNF)-α (*y*-axes) on the abdominal skin versus age, weight, body mass index (BMI), body fat ratio, body fat weight, waist circumference, and visceral fat rating (each *x*-axis). The dashed circle in each plot denotes the 95% confidence ellipse. $^{**}p < 0.01$, $^{*}p < 0.05$.

Figure 3 Scatter plots of the relationships between the levels of normalized tumor necrosis factor (TNF)-α (*y*-axes) on the thigh skin versus age, weight, body mass index (BMI), body fat ratio, body fat weight, waist circumference, and visceral fat rating (each *x*-axis). The dashed circle in each plot denotes the 95% confidence ellipse. $^{*}p < 0.05$.

Figure S1 Western blotting analysis of the skin-detectable TNF-α. The cells of the stratum corneum, which was considered to be the closest sample to the skin blotting, was analyzed by western blotting. As a result, a major band (apparent molecular weight was ~28 kDa, corresponding to the theoretical molecular weight of full TNF-α) with a fragment of smaller molecular weight (10–17 kDa, probably the fragment of TNF-α) was detected. Arrows denote the detected bands of TNF-α.

Table I Demographic data and body characteristics of the subjects

Items	**Values**[*]
Age (years)	[#]40 (21)
Weight (kg)	75.0 (10.7)
Body mass index (BMI) (kg m^{-2})	25.5 (3.5)
Body fat ratio (%)	20.9 (6.0)
Body fat weight (kg)	16.2 (6.3)
Waist circumference (cm)	90.2 (9.5)
Visceral fat rating[†]	[#]11 (6)

n = 59

[*]Mean (standard deviation) or [#]Median (interquartile range)

[†]Rating with score 1–59

Table II Correlation between the demographic and body composition data and the level of tumor necrosis factor (TNF)-α detected on the skin

	Abdominal TNF-α	**Thigh TNF-α**
n	48	45
Age	$\rho = 0.092$	$\rho = 0.141$
	$p = 0.533$	$p = 0.356$
Weight	$r = 0.293$	$\rho = 0.286$
	$^{*}p = 0.043$	$p = 0.057$
Body mass index (BMI)	$r = 0.407$	$\rho = 0.327$
	$^{**}p = 0.004$	$^{*}p = 0.028$
Body fat ratio	$r = 0.232$	$\rho = 0.286$
	$p = 0.113$	$p = 0.056$
Body fat weight	$r = 0.295$	$\rho = 0.310$
	$^{*}p = 0.042$	$^{*}p = 0.038$
Waist circumference	$r = 0.280$	$\rho = 0.325$
	$p = 0.054$	$^{*}p = 0.029$
Visceral fat rating	$\rho = 0.298$	$\rho = 0.324$
	$^{*}p = 0.040$	$^{*}p = 0.030$

(Upper rows) Pearson product-moment correlation coefficient (r) or Spearman's rank correlation coefficient (ρ)
(Lower rows) p-value of the respective correlation coefficients
$^{*}p < 0.05$, $^{**}p < 0.01$

Table III Normalized levels of TNF-α detected on the skin, classified by the body mass index (BMI)

Site	Normal (BMI < 25)	Overweight (25 ≤ BMI < 30)	Obese (BMI > 30)
Abdomen	8.4 (3.9)	9.4 (3.6)	15.5 (5.4)
n = 48	*n* = 20	*n* = 24	*n* = 4
Thigh	6.2 (3.4)	9.8 (5.3)	10.3 (9.7)
n = 45	*n* = 17	*n* = 23	*n* = 5

(Upper rows) Mean (standard deviation) with the unit pg ng^{-1}.

(Lower rows) The number of the subjects.

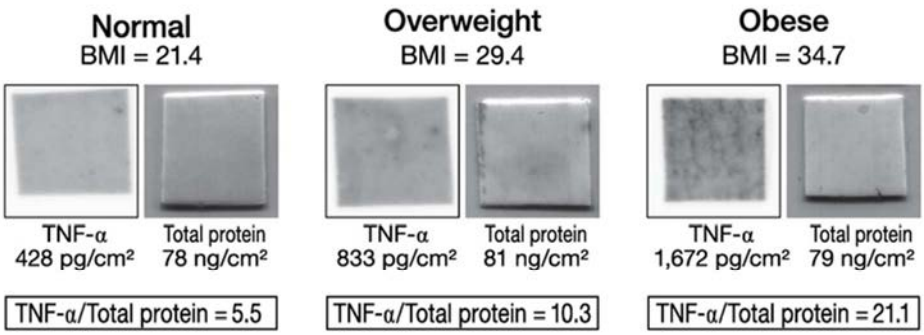


Figure 1 Representative images of the tumor necrosis factor (TNF)-α staining and the total protein staining among normal [body mass index (BMI) = 21.4], overweight (BMI = 29.4), and obese (BMI = 34.7) individuals.
62x23mm (300 x 300 DPI)

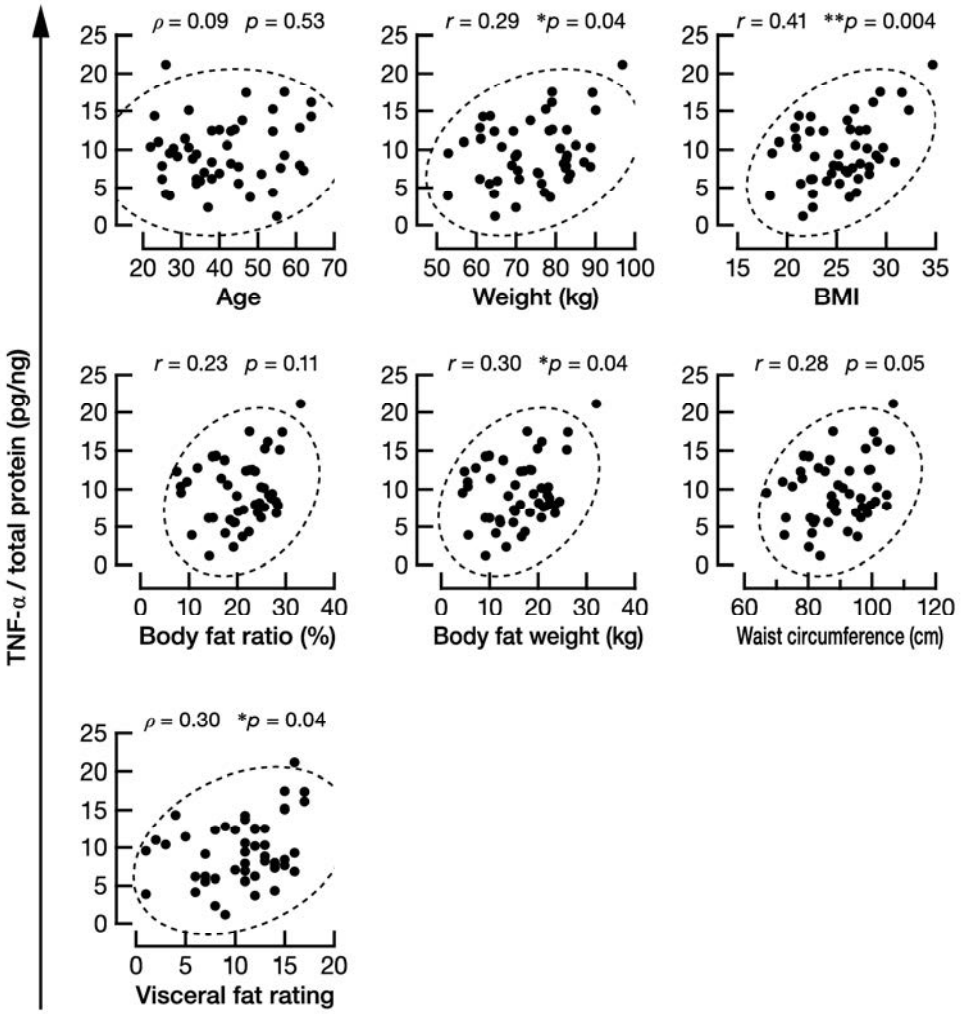


Figure 2 Scatter plots of the relationships between the levels of normalized tumor necrosis factor (TNF)-α (*y*-axes) on the abdominal skin versus age, weight, body mass index (BMI), body fat ratio, body fat weight, waist circumference, and visceral fat rating (each *x*-axis). The dashed circle in each plot denotes the 95% confidence ellipse. $^{**}p < 0.01$, $^{*}p < 0.05$.
191x202mm (300 x 300 DPI)

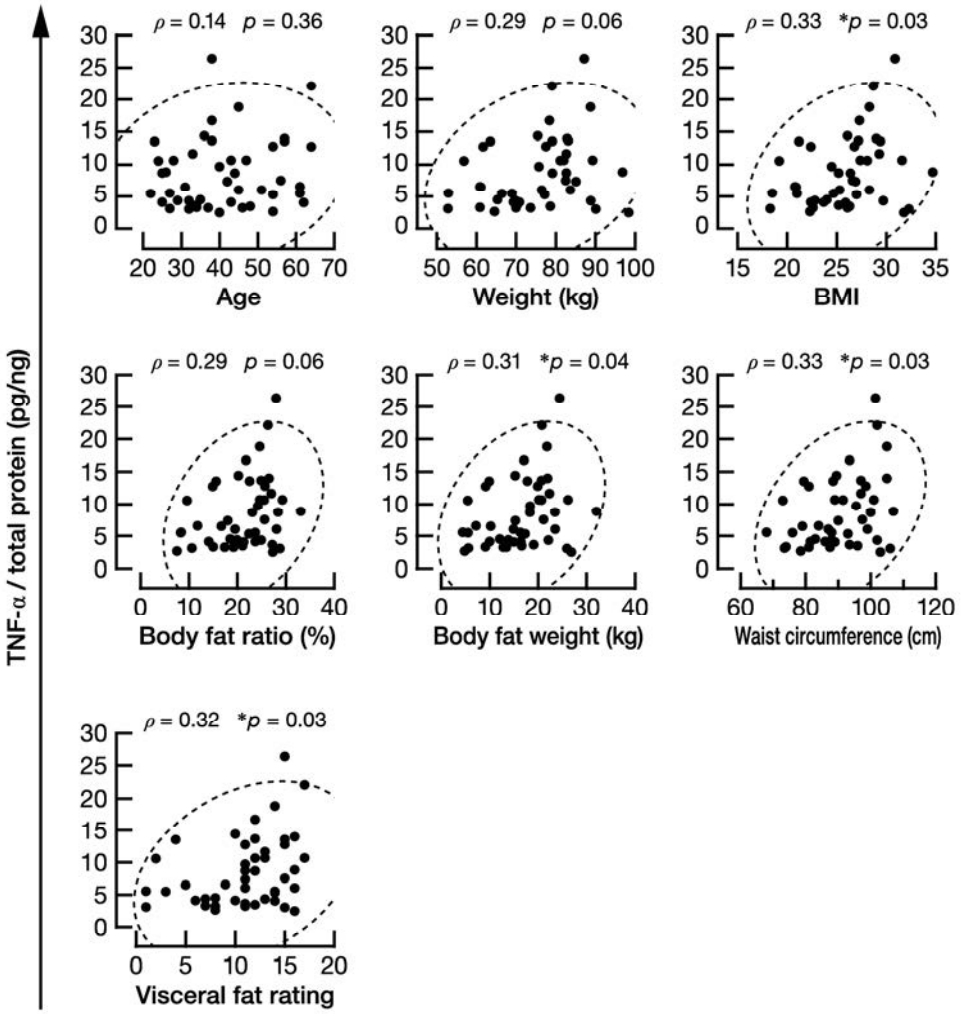
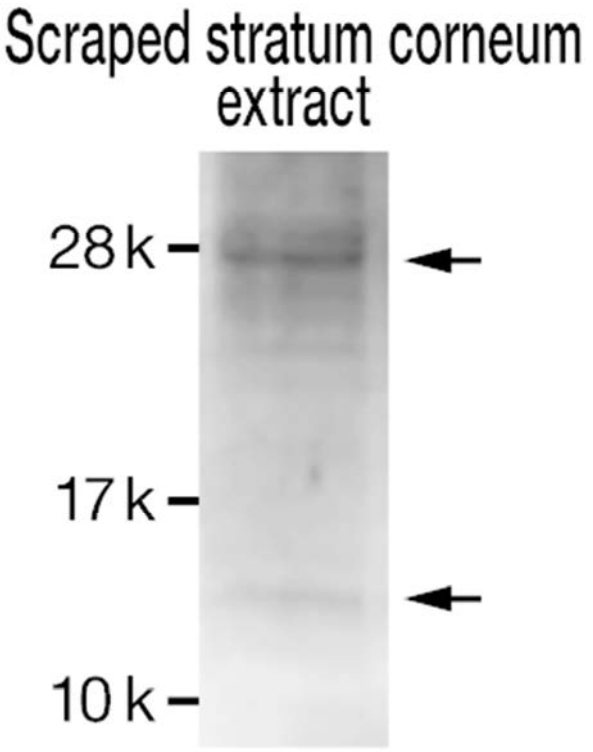


Figure 3 Scatter plots of the relationships between the levels of normalized tumor necrosis factor (TNF)-α (*y*-axes) on the thigh skin versus age, weight, body mass index (BMI), body fat ratio, body fat weight, waist circumference, and visceral fat rating (each *x*-axis). The dashed circle in each plot denotes the 95% confidence ellipse. *$p < 0.05$.
191x202mm (300 x 300 DPI)



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