The Influence of Psychological Stress on the Levels of the Skin's Natural Moisturizing Factor in Croatian Medical Students

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ABSTRACT

Psychological stress may affect skin barrier homeostasis and slow down its recovery. Therefore, we aimed to examine the association between psychological stress levels and natural moisturizing factor (NMF) levels in the *stratum corneum* (SC).

NMF levels were determined in the SC of 25 third-year and 25 sixth-year medical students in two periods (stressful and non-stressful) using the tape stripping method, high-performance liquid chromatography, and spectrophotometry. Additionally, students completed the Perceived Stress Scale questionnaire.

Most students had medium stress levels. During the non-stressful period, third-year students were under higher stress than sixth-year students ($P<0.001$). However, there was no significant difference between the two examined groups in the stressful period. Only among the sixth-year students were significantly higher stress levels found in the stressful period compared with the non-stressful period. In both groups, the levels of NMF did not differ significantly between the examined periods. Regarding the non-stressful period, there was a clear trend of increasing NMF levels among third-year students compared with sixth-year students ($P=0.0547$), consistent with the higher stress levels among third-year students during this period. Additionally, sixth-year female students had significantly lower NMF levels during the non-stressful period than third-year female students.

In conclusion, third-year students were exposed to longer-term stress, while the stress of sixth-year students was associated with the exam-writing period. Additionally, we observed a trend of increasing NMF levels with increasing stress. Further research is needed to determine the mechanism by which stress, as an independent factor, influences NMF levels in the SC.

KEY WORDS: psychological stress, natural moisturizing factor, medical students

INTRODUCTION

During their medical studies, students prepare for their future high-responsibility careers, where they will provide appropriate patient care and promote health. Therefore, the education of future physicians is demanding and associated with a heavy workload, often including sleep deprivation, especially during

exam periods. Psychological stress adversely affects skin barrier homeostasis and impairs the skin's immune system, the wound healing process, skin protective function, and resistance to infection (1-6).

One of the first studies to confirm the adverse effects of psychological stress on skin barrier permeability was conducted in mice exposed to stress through immobilization. Immobilization resulted in the delayed recovery of the skin barrier, which was proportional to the duration of stress (7). Later, human studies showed that acute psychological stress could slow down skin barrier recovery, with a positive correlation between the intensity of stress and recovery duration (8,9).

In maintaining the protective barrier of the skin, a significant role is played by the natural moisturizing factor (NMF), a pool of hygroscopic compounds, primarily free amino acids and their derivatives such as pyrrolidone-5-carboxylic acid and urocanic acid, sugars, and ions. Free amino acids are formed by the proteolysis of the filaggrin protein (10). Pyrrolidone-5-carboxylic acid is highly hygroscopic and the most abundant individual component of the NMF, and is derived from glutamine and glutamic acid, while formation of urocanic acid is mediated by urocanic acid from histidine. Urocanic acid also acts as a major chromophore in the SC. NMF is assumed to play a role in maintaining the acidic pH of the skin as well as SC hydration and water retention (11). The only known study that investigated the association between stress and NMF levels in the skin was conducted on an animal model, and there are no studies conducted in humans that investigated the association between these two parameters (12).

This study aimed to determine the subjective perception of stress levels in third- and sixth-year medical students during stressful and non-stressful periods and to examine the association between the level of psychological stress and the level of NMF in the SC. Third- and sixth-year medical students were included because of the generally accepted belief among the students at our School of Medicine that the third year of medical school is one of the most demanding in terms of course load, while the sixth-year medical students will have have developed the mechanisms to cope with the stress.

MATERIALS AND METHODS

Study population

Through a public call, we recruited 25 third-year and 25 sixth-year students of the School of Medicine, University of Zagreb, from March to May 2022. The exclusion criteria were acute and chronic skin diseases, acute systemic disorders during the research period, and the use of any systemic or topical immunosuppressants or immunomodulators.

Participants were informed about the details and procedures of the research and provided informed consent prior to their inclusion in the study. The research was conducted in accordance with the Helsinki Declaration and was approved by the Ethics Committee of the School of Medicine, University of Zagreb, Croatia (Reference Number: 380-59-10106-22-111/21, Class: 641-01/22-02/01).

Perceived Stress Scale Questionnaire

The Perceived Stress Scale (PSS) (13,14) was used to assess the students' perception of stress. The PSS consists of 10 items, and participant responses are scored on a Likert-type scale with five levels (0 – never; 1 – almost never; 2 – sometimes; 3 – fairly often; 4 – very often). The sum of the responses yields a total score, with higher scores indicating higher levels of perceived stress.

The participants filled out the questionnaire immediately before skin sampling, and the sum of total points is referred to as the "stress index".

Sampling of the stratum corneum

The SC was sampled on the inner side of the forearm by pressing adhesive tapes (with a surface area

Table 1. Social and demographic characteristics of participants

	Third-year students	Sixth-year students
Total number of participants, *n*	25	25
Age (in years)		
Median age ± SD (range)	21,9 ± 0,8 (21,1 – 23,6)	24,7 ± 1,2 (23,7 – 29,8)
Gender		
Female, *n* (%)	15 (60%)	12 (48%)
Male, *n* (%)	10 (40%)	13 (52%)

SD: standard deviation

of 3.8 cm^2) onto the skin for 10 seconds (15,16). A total of 10 adhesive tapes were taken from the same location and stored individually at -20 °C until analysis.

The participants underwent skin sampling on two occasions: during an exam-free period (non-stressful period) and during a period of 7 days before or after the exams (stressful period). The interval between the two samplings was greater than four weeks to minimize the possibility of exam-related stress affecting the participants' responses during the non-stressful period.

Determination of NMF components

NMF was defined as the sum of the concentrations of histidine, pyroglutamic acid, and trans- and cis-isomers of urocanic acid. The fifth adhesive tape was used to measure the NMF levels in the SC according to a slightly adapted method described elsewhere (15). Briefly, NMF components were extracted with 600 mL of ultra-pure water and subsequently analysed by high-performance liquid chromatography (HPLC) (15).

Since the amount of SC sampled by each adhesive tape varies, it was necessary to normalize the concentration of the NMF components for the total amount of the SC proteins on the tape strip. Due to incomplete extraction of SC proteins with water, the second extraction was performed by adding 1.0 mol/L KOH solution (15). The total amount of proteins from both extractions was determined using the Pierce Micro BCA protein assay kit (Thermo Fischer Scientific, Rockford, IL, USA). The level of NMF in the SC was expressed as mmol NMF/g protein.

Statistical analysis

Prism 9.4 software (GraphPad, La Jolla, CA, USA) was used to analyze analytical and statistical data. Data distribution was tested using the Shapiro-Wilk test. Differences in levels of NMF and the stress index between the two groups of participants were determined using a two-tailed Student's t-test or Mann-Whitney U test in case of skewed data distribution. Differences in NMF levels and stress index within the same group of participants during stress and non-stress periods were determined using a two-tailed Wilcoxon signed-rank test or paired t-test. The Mann-Whitney U test was used to determine differences in NMF levels and stress index between male and female participants within the same groups. The association between stress index and NMF levels was examined using a two-tailed Spearman's rank correlation test. The significance level (P) was set at $P<0.05$.

RESULTS

Demographic characteristics of participants

Twenty-five third-year and twenty-five sixth-year students of the School of Medicine of the University of Zagreb participated in this study. Their demographic characteristics are presented in Table 1.

The age of third-year students ranged from 21.1 to 23.6 years, while the age of sixth-year students ranged from 23.7 to 29.8 years. There was gender parity in both student groups; 40% of third-year and 52% of sixth-year students were men.

Stress index

Participants were divided into three groups based on their scores on the Perceived Stress Questionnaire: a low stress level group (0 to 13 points), a moderate stress level group (14 to 26 points), and a high stress level group (27 to 40 points) (7). Based on the total scores from the questionnaire responses, most participants were classified into the moderate-stress level group for both groups (Table 2).

Given the small number of participants classified into the high-stress group, all participants were included in further statistical data analysis regardless of the grouping based on stress levels shown in Table 2.

Table 2. Stress index among third-year and sixth-year students

	Stress index					
	Non-stressful period (NS)			Stressful period (S)		
	Low level	Medium level	High level	Low level	Medium level	High level
3rd year students						
n (M)	-	21 (9)	4 (1)	-	21 (8)	4 (2)
6th year students						
n (M)	-	23 (13)	-	-	23 (12)	2 (1)

M: male students

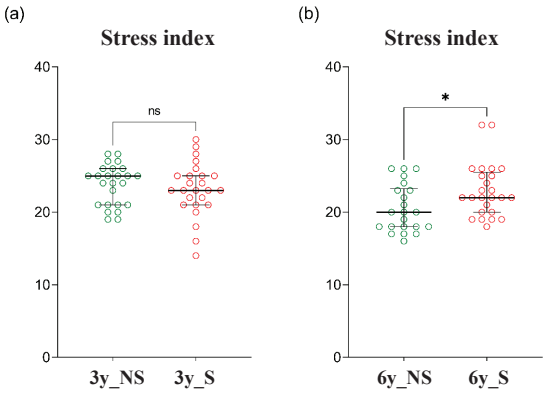

Figure 1. Stress index (a) among students in the third year during non-stressful and stressful periods, and (b) among students in the sixth year during non-stressful and stressful periods. Values are presented as median and quartiles. (*P<0.05; ns: not significant). The difference in stress index levels between the non-stressful and stressful periods among students was determined using Wilcoxon's test.
Abbreviations: 3y_NS: third-year students during the non-stressful period; 3y_S: third-year students during the stressful period; 6y_NS: sixth-year students during the non-stressful period; 6y_S: sixth-year students during the stressful period.

The stress index among third-year students did not significantly differ between the stressful and non-stressful periods. However, the stress index among sixth-year students was significantly higher during the stressful period compared with the non-stressful period (Figure 1).

The comparison of third-year and sixth-year participants showed that third-year students experienced higher stress levels during the non-stressful period than sixth-year students (Figure 2, a). However, the stress index level did not significantly differ between the two groups during the stressful period (Figure 2, b). As a result, there was a greater relative change in the stress index between the non-stressful and stressful periods among sixth-year students compared with third-year students (Figure 2, c).

The stress index level did not differ between male and female participants, regardless of the student year (Figure 3, a, b, c). Similarly, there was no difference between female and male participants among the third- and sixth-year students during the non-stressful and stressful periods (Figure 3, d, e, f).

NMF levels in the *stratum corneum*

NMF levels among third-year students did not significantly differ between the non-stressful and stressful periods (Figure 4, a). Similarly, the level of NMF did not differ significantly between the non-stressful and stressful periods among the sixth-year students, unlike the stress index level, which was significantly higher in the stressful period (Figure 4, b).

There was no significant difference in the level of NMF in the skin of third- and sixth-year students during the non-stressful period (Figure 5, a). However, a trend of lower NMF levels among sixth-year students could be observed (P=0.0547). During the stressful

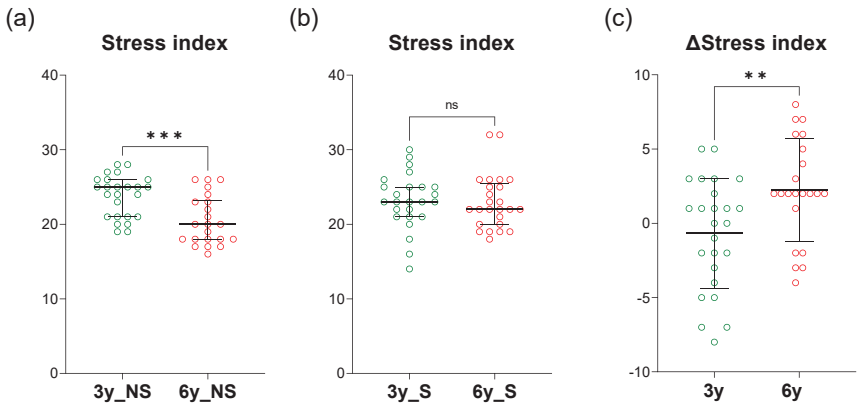

Figure 2. Stress index of students in the third and sixth year of the study: (a) during the non-stressful period, (b) during the stressful period, and (c) the relative change in the stress index between the stressful and non-stressful periods. Values are shown as median and quartiles in (a) and (b), and as mean ± SD in (c) (**P<0.01, ***P<0.001; ns: not significant). The relative change in the stress index represents the difference between the total number of points in the stressful and non-stressful periods. The difference in the level of the stress index between students in the third year and the sixth year during the non-stressful and stressful period was determined using the Mann-Whitney test (a and b) and Student's t-test (c).
Abbreviations: 3y_NS: third-year students during the non-stressful period; 3y_S: third-year students during the stressful period; 6y_NS: sixth-year students during the non-stressful period; 6y_S: sixth-year students during the stressful period; SD: standard deviation.

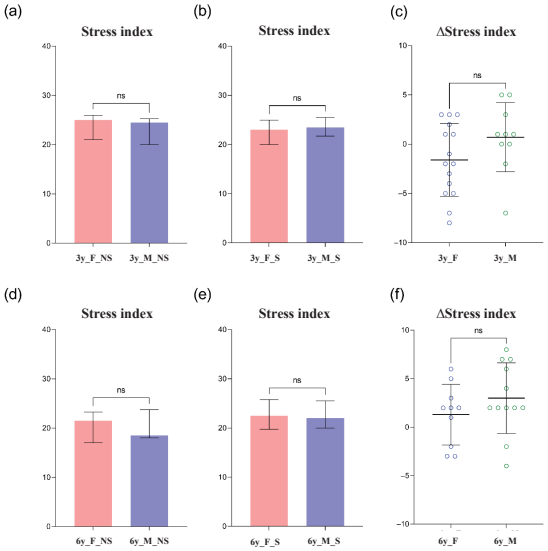

Figure 3. The level of stress index in female and male participants in the third year: (a) during the non-stressful period, (b) during the stressful period, and (c) the relative change in stress index. The level of stress index in female and male participants in the sixth year: (d) during the non-stressful period, (e) during the stressful period, and (f) the relative change in stress index. Values are presented as median and quartiles in (a), (b), (d), and (e), and as mean ± standard deviation in (c) and (f) (ns: not significant). The relative change in stress index represents the difference between the total score in the stressful period and the score in the non-stressful period. The difference in stress index levels between male and female participants in the non-stressful and stressful periods was determined using the Mann-Whitney U test (in a, b, d, and e), and the Student's t-test (under c and f).
Abbreviations: 3y_NS: third-year students during the non-stressful period; 3y_S: third-year students during the stressful period; 6y_NS: sixth-year students during the non-stressful period; 6y_S: sixth-year students during the stressful period; M: male students; F: female students; SD: standard deviation.

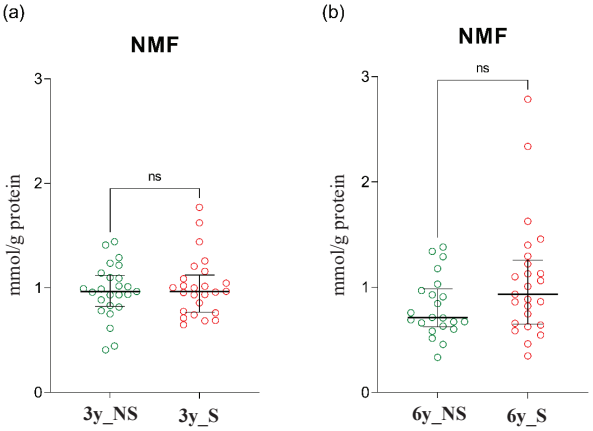

Figure 4. The level of NMF in the stratum corneum of (a) participants in the third year during the non-stressful and stressful periods, and (b) participants in the sixth year during the non-stressful and stressful periods. Values are presented as median and quartiles (ns, not significant). The difference in NMF levels among participants between non-stressful and stressful periods was determined using Wilcoxon's test.
Abbreviations: 3y_NS: third-year students during the non-stressful period; 3y_S: third-year students during the stressful period; 6y_NS: sixth-year students during the non-stressful period; 6y_S: sixth- sixth-year students during the stressful period; NMF: natural moisturizing factor.

period, there was no significant difference in NMF level between the two group**s** (Figure 5, b). The relative change in NMF level was significantly higher between the non-stressful and stressful periods in sixth-year students than in third-year students (Figure 5, c).

The NMF values of subjects stratified by gender are shown in Figure 6 and Figure 7. There were no significant differences in NMF values between male and female participants of the same student year during both the non-stressful and stressful periods (Figure 6).

Sixth-year female students had significantly lower NMF levels than third-year female students during the non-stressful period (Figure 7, a), resulting in a higher relative change in NMF levels (Figure 7, c). There was no significant difference in NMF levels between third- and sixth-year male students (Figure 7, d, e, f).

In addition, Spearman's correlation analysis was performed to assess the association between the level of NMF and the stress index. No significant association was found between these two variables.

DISCUSSION

To date, psychological stress in medical students has been examined mostly in single-center, cross-sectional studies. However, in the United States and Canada, it has been shown that medical students are exposed to higher psychological stress than the general population and the population of the same age group (17).

The results of our study indicated that third-year students were exposed to chronic stress, while the stress of sixth-year students was associated with the exam-writing period with a relaxation period between exams. This statement is supported by the fact that the change in stress index levels between the non-stressful and stressful periods was greater in sixth-year students than in third-year students.

Our results align with the findings of Yusoff *et al.*, who have also shown that what year the students were in was a significant factor influencing the stress

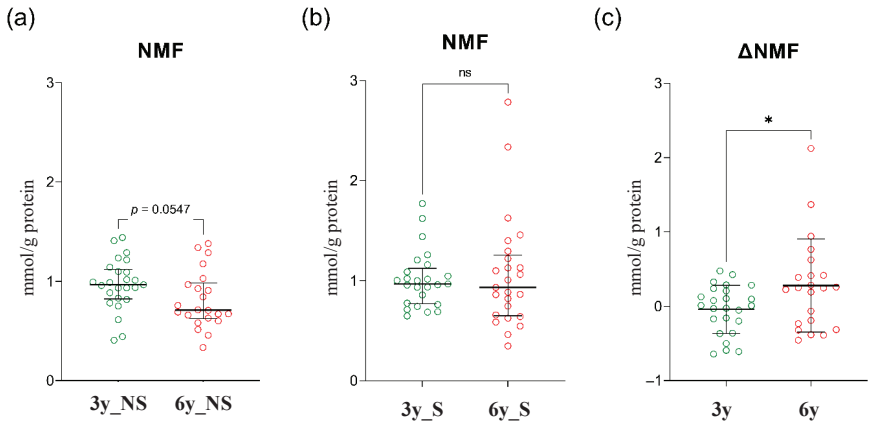

Figure 5. The level of NMF in the skin of students in the third year and the sixth year: (a) during the non-stressful period, (b) during the stressful period, and (c) the relative change in the NMF level between the stressful and non-stressful periods. Values are shown as median and quartiles in (a) and (b), and as mean ± SD in (c) (*P<0.05; ns: not significant). The relative change in the NMF level represents the difference between the total number of points in the stressful and non-stressful periods. The difference in the level of NMF between students in the third year and the sixth year in the non-stressful and stressful period was determined using the Mann-Whitney test (in a and b) and Student's t-test (in c).
Abbreviations: 3y_NS: third-year students during the non-stressful period; 3y_S: third-year students during the stressful period; 6y_NS: sixth-year students during the non-stressful period; 6y_S: sixth-year students during the stressful period; NMF: natural moisturizing factor; SD: standard deviation.

level among medical students. Final-year students had the lowest stress level, while significantly higher stress levels were observed in second- and fourth-year students (18). The low prevalence of stress among final-year students may be associated with the fact that they have acquired skills during their studies that enable them to cope with stress more effectively than younger students. On the other hand, the authors hypothesized that, considering the study program at the examined center, second- and fourth-year students were in a transitional period in which they needed to adapt to the demands of the new phase of their studies (18).

We also investigated if there were differences between female and male students, and no association between gender and stress level was found, whereas the data in the literature are contradictory. Namely, many cross-sectional and longitudinal studies have not found an influence of gender on the mental health of medical students. In contrast, some studies indicate higher anxiety levels in women than in men (17).

Previous research has revealed a negative impact of psychological stress on the function and recovery period of the skin barrier after mechanical damage (10,11,19,20). Assessment of skin function is based on the determination of transepidermal water loss (TEWL).

For instance, Garg *et al.* included 27 medical, pharmacy, and dentistry students in their study. They found that increased stress levels were associated with disrupted homeostasis of skin barrier permeability (increased TEWL) and a longer recovery period of the skin barrier (9). Similarly to the aforementioned study, Fukuda *et al.* conducted a study on the impact of stress on skin barrier permeability and repair kinetics involving 16 female students. It was found that psychological stress was associated with increased TEWL values and delayed recovery of the skin barrier after mechanical damage (19).

However, the study by Muizzuddin *et al.*, which included 28 women exposed to stress due to divorce, failed to confirm the influence of stress on skin barrier integrity but showed delayed recovery of the skin barrier after mechanical irritation compared with the control group (20). Altemus *et al.* demonstrated that acute psychosocial stress and sleep deprivation stress were associated with impaired recovery of the skin's protective barrier, which could be related to elevated levels of IL-1 beta, IL-10, and TNF-alpha (8).

TEWL, which was assessed in the studies mentioned above, assesses the maintenance of homeostasis of skin barrier permeability, which is influenced by three factors: lamellar lipids of the SC, preserved skin pH, and NMF, which constitute the skin's hydration. Dysfunction of any of these three factors, including NMF, can impair the skin barrier function (21).

Since, to the best of our knowledge, no studies have been conducted on humans regarding the impact of stress on NMF levels, our research aimed to investigate the association between these two variables in young and healthy individuals.

The level of NMF in both groups of participants did not differ significantly between the non-stressful

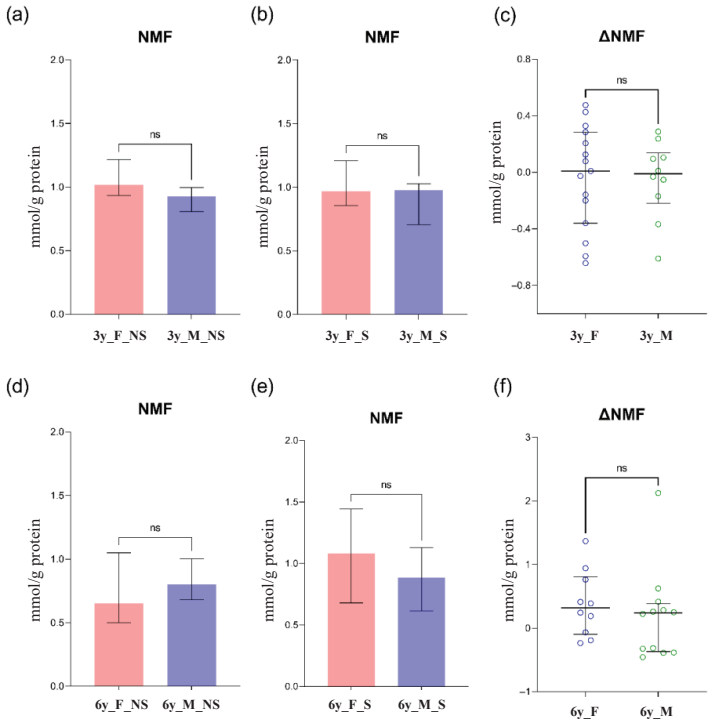

Figure 6. The level of NMF in female and male students in the third year: (a) during the non-stressful period, (b) during the stressful period, and (c) the relative change in the level of NMF. The level of NMF in female and male students in the sixth year: (d) during the non-stressful period, (e) during the stressful period, and (f) the relative change in the level of NMF. Values are presented as median and quartiles in (a), (b), (d), and (e), and as mean ± SD in (c) and (f) (ns: not significant). The relative change in the NMF level represents the difference between the total number of points in the stressful and non-stressful periods. The difference in the level of NMF between male and female students in the non-stressful and stressful period was determined using the Mann-Whitney test (a, b, d, and e) and Student's t-test (c and f).
Abbreviations: 3y_NS: third-year students during the non-stressful period; 3y_S: third-year students during the stressful period; 6y_NS: sixth-year students during the non-stressful period; 6y_S: sixth-year students during the stressful period; F: female students; M: male students; NMF: natural moisturizing factor; SD: standard deviation.

and stressful periods. However, among sixth-year students, the stress index was significantly higher during the stress period. When comparing third- and sixth-year students, a similar trend was observed in NMF level as in stress level. During the stressful period, there was no difference in NMF levels between the two groups of participants. However, during the non-stressful period, a clear trend of higher NMF levels was observed in third-year students compared with sixth-year students (P=0.0547). Furthermore, the relative change in NMF level was significantly higher among sixth-year students when compared with third-year students.

We also examined the association between gender and NMF values. In both periods, NMF values did not differ between male and female participants regardless of the student year. However, when comparing female participants in the third and sixth year, it was shown that sixth-year female students had lower levels of NMF than third-year female students during the non-stressful period. Consequently, the relative change in NMF level was higher in sixth-year female students. There was no significant difference in NMF levels between third- and sixth-year male students.

Therefore, our study observed an increase in NMF values with an increase in stress levels. Similarly, in a study conducted on mice exposed to psychological stress due to insomnia, Choi *et al.* demonstrated that stress could induce the destruction of filaggrin and other proteins associated with epidermal differentiation, including involucrin and loricrin. Although these proteins have a long half-life, immunohistochemical analysis showed reduced expression of these proteins in the epidermis of mice after stress (22).

It is expected that the reduction of filaggrin, as a precursor of amino acids and peptides that constitute NMF, would lead to a decrease in NMF levels. However, only one study has been conducted on the association between filaggrin and NMF. The study enrolled patients with atopic dermatitis and found that the reduction of filaggrin leads to a decrease in NMF levels. However, it should be taken into consideration that this study included a selected population, namely

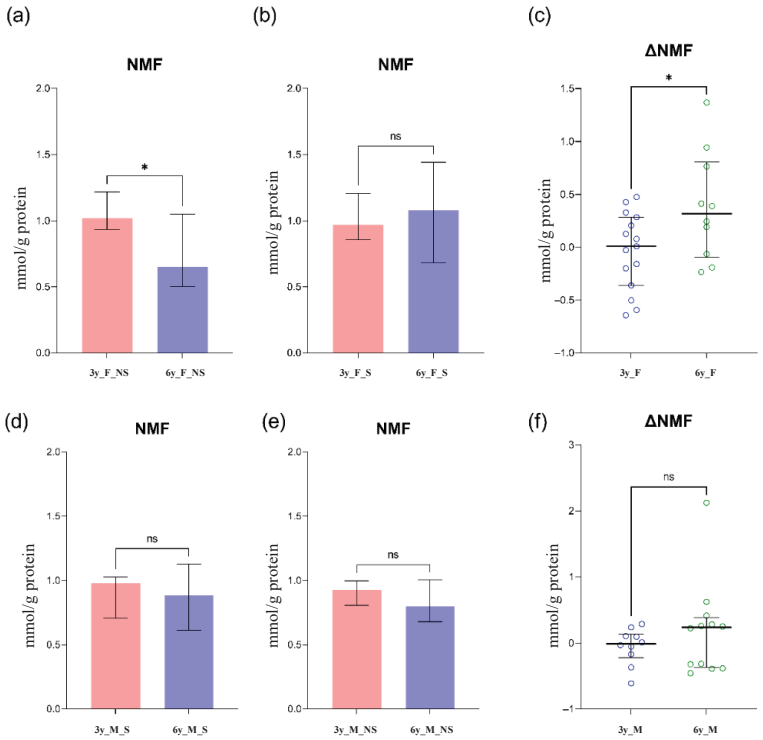

Figure 7. The level of NMF in female students in the third year: (a) during the non-stressful period, (b) during the stressful period, and (c) the relative change in the level of NMF. The level of NMF in male students in the sixth- year: (d) during the non-stressful period, (e) during the stressful period, and (f) the relative change in the level of NMF. Values are presented as median and quartiles in (a), (b), (d), and (e), and as mean ± SD in (c) and (f) (ns: not significant). The relative change in the NMF level represents the difference between the total number of points in the stressful and non-stressful periods. The difference in the level of the stress index between students in the third year and sixth year according to gender in the non-stressful and stressful period was determined using the Mann-Whitney test (a, b, d and e) and Student's t-test (c and f).
Abbreviations: 3y_NS: third-year students during the non-stressful period; 3y_S: third-year students during the stressful period; 6y_NS: sixth-year students during the non-stressful period; 6y_S: sixth-year students during the stressful period; F: female students; M: male students; NMF: natural moisturizing factor; SD: standard deviation.

patients who had null mutations in the gene for FLG (loss-of-function mutations), which is very rare (23).

Based on the above-mentioned studies, it would be expected that psychological stress leads to a decrease in NMF levels in the skin, mediated by a reduction in filaggrin levels. However, our study found an opposite trend: an increase in NMF levels with an increase in psychological stress levels. Therefore, our results could be explained by a compensatory mechanism either at the step of filaggrin synthesis, where increased production of filaggrin from its precursor profilaggrin may occur, or at the stage of increased degradation of filaggrin into amino acids and their metabolic products that are components of NMF.

Furthermore, our results align with the findings of Kitagawa *et al.*, who conducted a study on ICR (Institute of Cancer Research) mice to investigate the effects of repeated stress over eight days on the composition of fatty acids and NMF levels in the SC of the skin. Although a decrease in NMF levels was observed after 4 and 7 days of repeated stress, initial stress (on the second day of the study) resulted in higher NMF levels in the stress-exposed group compared with the control group (12). Therefore, it can be assumed that the high NMF value during the stressful period in our study may result from the fact that the sampling of the SC was done within a few days before or after the exams, i.e., during the early phase of the stress period. Additional measurements of NMF over the following days could reveal the dynamics of NMF in a human model, i.e., whether NMF levels change after the initial increase. In addition, massive filaggrin degradation may occur during the early stress response, resulting in transient accumulation of filaggrin degradation products, which are components of NMF.

CONCLUSION

Our study supports our hypothesis that psychological stress influences the levels of NMF in SC. Moreover, this is the first study on humans to investigate the association between psychological stress and NMF levels in the skin. The limitations of this study

include a relatively small sample size, representation of only one center, and the lack of evaluation of environmental factors that could further influence NMF levels in the skin. However, considering these limitations and the fact that compensatory mechanisms leading to increased NMF in the skin of stress-exposed participants have yet to be investigated, further research is needed to determine the impact of stress, as an independent factor, on NMF levels.

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