Journal of the Oman Medical Association

MDPI

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Contribution of Neuromuscular Junction Degradation to Muscle Decline in Burn Patients

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Abstract: Burn injuries cause severe muscle wasting and weakness. However, the relative contribution of neuromuscular junction (NMJ) degradation remains elusive. We investigated the associations of plasma c-terminal agrin fragment-22 (CAF22), a marker of NMJ degradation, with muscle decline in burn patients. We recruited male patients with burns (n = 32, age = 32.3 ± 4.5 years, percent burn area = 15.2 ± 2.3) and age-matched controls to evaluate handgrip strength (HGS), skeletal muscle mass index (SMI), phase angle, and creatine kinase and plasma levels of CAF22, c-reactive protein, and 8-isoprostanes. We used an unpaired t-test and regression analysis for statistics. The burn patients had lower HGS, SMI, and phase angle than the controls (all $p < 0.05$). These patients also exhibited higher plasma CAF22, CRP, 8-isoprostanes, and creatine kinase than the controls (all $p < 0.05$), suggesting NMJ degradation and heightened inflammation and oxidative stress. Correlation analysis revealed significant correlations of plasma CAF22 with HGS and phase angle in the burn patients, suggesting the potential contributions of NMJ degradation to muscle weakness and atrophy (both $p < 0.05$). We also found correlations of plasma CRP with HGS and phase angle in these patients (both $p < 0.05$). Altogether, NMJ degradation appears to play a significant role in burn-induced muscle injury and may warrant further investigation for potential therapeutic interventions.

Keywords: burn; handgrip strength; CAF22; neuromuscular junction

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Academic Editor: Matteo Riccò

Received: 24 August 2024
Revised: 18 September 2024
Accepted: 21 January 2025
Published: 6 February 2025

Citation: Khan, I.M.; Javed, M.; Zuhra, H.; Qaisar, R. Contribution of Neuromuscular Junction Degradation to Muscle Decline in Burn Patients. *J. Oman Med. Assoc.* **2025**, *2*, 3. https://doi.org/10.3390/joma2010003

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1. Introduction

Skeletal muscle weakness and wasting are expected consequences after a burn injury [1]. Muscle wasting is defined as an unintentional loss of up to 10% of muscle mass. However, patients with severe burn injuries may lose up to 25% of muscle mass [1]. This rapid breakdown of muscle tissue is attributed to the diversion of essential nutrients from skeletal muscle to vital organs and healing wounds during the initial stages of recovery [2]. In addition, several catabolic factors also play a role in muscle weakness and wasting following a burn injury [3]. This muscle loss is detrimental and strongly linked to increased mortality rates. For example, patients losing 40% of their lean body mass have a 90% chance of death [4].

Several potential mechanisms trigger and/or exacerbate muscle weakness and wasting in burn patients [2]. Specifically, the relevant role of elevated systemic stress is thoroughly characterized [1]. It is recognized that stress hormones, including glucocorticoids and catecholamines, are released in circulation following a burn injury [1]. These hormones harm skeletal muscle by blunting anabolic pathways and activating catabolic pathways, leading to rapid muscle mass loss [5]. However, the subcellular effects of stress on the skeletal muscle of burn patients are not well defined.

It is generally known that the neuromuscular junction (NMJ) plays a critical role in regulating skeletal muscle health [6]. This is supported by experimental studies reporting a rapid loss of skeletal muscle mass and strength following NMJ disruption [6,7]. In clinical studies, taking a muscle biopsy to investigate NMJ morphology may not be convenient in most settings. In this context, plasma levels of c-terminal agrin fragment-22 (CAF22) may be a valuable tool to reflect the health of the NMJ and skeletal muscle [7]. Plasma CAF22 is a by-product of agrin found at the NMJ [7]. Catabolic conditions cause a breakdown of agrin and the release of CAF22 into blood, which can be measured using simple ELISA tests [7]. Several studies show the biomarker potential of plasma CAF22 in evaluating skeletal muscle mass and strength in patients with muscle-atrophying conditions [7,8]. However, to the best of our knowledge, relevant studies on burn patients do not exist. The indirect evidence suggests a potential role for NMJ disruption in muscle wasting following burn injury. For example, NMJ is vulnerable to breakdown in chronic stress conditions [6,9]. Similarly, patients with prolonged inflammation and oxidative stress, which are expected consequences of stress, exhibit NMJ loss [9]. Thus, it may be possible that NMJ loss is a contributory factor to muscle wasting in burn patients.

The measurement of handgrip strength (HGS) is a simple assessment technique to measure muscle strength [10]. It requires a simple dynamometer, is cost-effective, and can be performed with minimal technical expertise. Several studies show that HGS can be a reliable representation of the state of health of whole-body skeletal muscles [11]. In addition, a robust coupling is suggested between HGS and muscle mass, as patients with low HGS may also exhibit significant muscle wasting. Previous reports also indicate substantial inverse associations between elevated plasma CAF22 and low HGS in patients with muscle diseases [6,7,11]. However, relevant data from burn patients remain elusive.

In this study, we attempt to fill this gap in the literature by investigating plasma CAF22 levels in burn patients. We also investigate the associations of plasma CAF22 with HGS and markers of muscle loss. We hypothesize that burn injuries are associated with elevated plasma CAF22 levels, negatively correlating with HGS and muscle mass.

2. Materials and Methods

2.1. Study Design and Participants

We recruited burn patients and age-matched controls at the Burns and Plastic Surgery Center of Hayatabad Medical Complex, Peshawar, after obtaining ethics approval from the Clinical Research Ethics Committee of the hospital. The recruitment was carried out from 24 February to 15 April 2024. The controls were matched for age, gender, ethnicity, and demographic profile, including socioeconomics. We used a sample size calculator to estimate the sample size for this study. Briefly, assuming mean CAF22 levels of 200 $\pm$ 40 pmol and an expected increase of 10% of CAF22 levels in burn patients and keeping alpha levels at 0.05 and study power at 80%, we required a sample size of 31 per group to obtain statistically significant results. Thus, a sample size of 32–35 participants per group seems adequate for our study design. We obtained written informed consent from all study participants before collecting data. The inclusion criteria were the provision of written informed consent, and the presence of 11–19% burns not involving the genitals, head,

and face areas. The exclusion criteria were the presence of a burn on the dominant arm and/or hand, which could interfere with the measurement of HGS. Further, participants with musculoskeletal disorders and comorbidities were also excluded. The burn patients were evaluated 5–8 days after the burn incident. All participants were Caucasian men of Pakhtoon ethnicity. This study was conducted according to the Declaration of Helsinki.

2.2. HGS Measurements

The procedure for HGS measurement was briefly explained to the participants. The HGS dynamometer (CAMRY, South El Monte, CA, USA) was thoroughly cleaned and calibrated, and the participants were asked to sit in a chair with their feet flat on the floor. The arm being tested was hanging at the side of the body, with the elbow flexed at 90 degrees. The handle of the HGS dynamometer was adjusted comfortably to fit the participant's hand. In addition, it was ensured that the handle of the HGS dynamometer rested on the palm's base (heel of the hand) while the middle three fingers encircled the handle. Next, the participants were asked to squeeze the dynamometer as hard as possible. Three measurements were taken for the dominant hand with a short rest (15–30 s) between measurements. The highest value of HGS was recorded for further analysis [12].

2.3. Body Composition Measurements

We measured body composition using a bioelectrical impedance analysis (BIA) scale (RENPHO, Dubai, United Arab Emirates). The procedure was explained to the participants, who were asked to remove any metal objects, such as jewelry and watches, that might interfere with the electrical current. The participants were also asked to stand barefoot on the BIA device in a relaxed manner after ensuring that their feet were dry of sweat and water. The BIA device measured several parameters of body composition, including skeletal muscle mass index (SMI), phase angle, lean mass, and fat content, as described previously [13].

2.4. Measurement of Circulating Biomarkers

Blood samples were obtained from the cubital veins of all participants between 09:00 and 10:00 AM. Blood was drawn into anticoagulant tubes and centrifuged at 5000 revolutions per minute for 15 min to isolate plasma for storage at −80 °C, as previously described by us [12]. We used ELISA assays to measure the plasma levels of CAF22 with an ELISA kit (NTCAF, ELISA, Neurotune, Schlieren-Zurich, Switzerland), 8-isoprostanes (Cayman Chemical, Ann Arbor, MI, USA), c-reactive proteins (CRP) (R&D Systems, Minneapolis, MN, USA), and creatine kinase (Cat # ab155901, Abcam, Abu Dhabi, United Arab Emirates), as described elsewhere [8,12]. Data on complete blood count, plasma electrolytes, liver function tests, renal function tests, and other blood investigations were obtained from clinical records.

2.5. Statistical Analysis

We used unpaired *t*-tests to compare the controls with the burn patients. The associations of HGS, SMI, phase angle, and fat mass with CAF22, CRP, 8-isoprostanes, and CK levels were analyzed using regression analysis. The associations of percent burn areas with CAF22 levels were also investigated using regression analysis. Data are presented as the mean and standard deviation, and the p-value < 0.05 was taken as statistically significant. We used GraphPad Prism 8 (Graph Stats Technologies Private Limited, Bangalore, India) for data analysis.

3. Results

3.1. Characteristics of the Participants

Table 1 exhibits the baseline characteristics of the study population. Age, education, and smoking status were similar between the controls and burn patients. The body areas of burns in the burn patients were trunk (59.3%), left upper limb (15.6%), and lower limbs (25%). The percentage burn areas for various body regions were 18.1 ± 2.3 for the trunk, 5.6 ± 0.9 for the left upper limb, and 14.2 ± 1.6 for lower limbs. The burn patients exhibited higher levels of platelets (367.5 ± 42.2 vs. 163.4 ± 13.3 × 10^3 per μL) and white blood cells (11.62 ± 3.1 vs. 8.32 ± 2.1 × 10^3 per μL) than the controls (both $p < 0.05$) (Table 1). These patients also exhibited lower potassium (3.4 ± 0.3 vs. 4.3 ± 0.2 mmol/L) and higher AST (62.1 ± 6.3 vs. 27.1 ± 3.9 IU/L) and ALT (75.1 ± 9.1 vs. 21.4 ± 6.2 IU/L) levels than in the controls (all $p < 0.05$). Among the markers of renal function, the plasma levels of creatinine were higher in the burn patients than in the controls (0.94 ± 0.16 vs. 0.73 ± 0.11 mg/dL) ($p < 0.05$). Lastly, the burn patients also exhibited lower levels of plasma total proteins (5.6 ± 0.2 vs. 7.2 ± 0.2 g/dL) and albumin (3.2 ± 0.1 vs. 4.1 ± 0.2 g/dL) than the controls (both $p < 0.05$) (Table 1).

Table 1. Baseline characteristics of control and burn patients.

	Controls (*n* = 35)	**Burn Patients (*n* = 32)**
Age (years and months)	28.4 ± 4.2	32.3 ± 4.5
Education status, *n* (%)		
Illiterate	5 (14.3)	8 (25)
Primary	10 (28.6)	10 (31.3)
Secondary	11 (31.4)	8 (25)
Tertiary	9 (25.7)	6 (18.8)
Characteristics of burn		
Percent area		15.2 ± 2.3
Location of burn, *n* (%)		
Trunk		19 (59.3)
(Percent burn area)		18.1 ± 2.3
Left upper limb		5 (15.6)
(Percent burn area)		5.6 ± 0.9
Lower limbs		8 (25)
(Percent burn area)		14.2 ± 1.6
Scald burn, *n* (%)		9 (28.1)
Fire burn, *n* (%)		23 (71.9)
Smoking status, *n* (%)		
Non-smoker	28 (80)	25 (78.1)
Ex-smoker	5 (14.3)	6 (18.8)
Current smoker	2 (5.7)	1 (3.1)
Complete blood count		
WBC × 103/μL	8.32 ± 2.1	11.62 ± 3.1 *
Platelets × 103/μL	163.4 ± 13.3	367.55 ± 42.2 *
Hemoglobin (g/dL)	12.41 ± 2.2	12.92 ± 2.2
Plasma electrolytes		
Chloride (mmol/L)	101.2 ± 4.5	104.4 ± 5.2
Potassium (mmol/L)	4.3 ± 0.2	3.4 ± 0.3 *
Sodium (mmol/L)	133.5 ± 6.3	145.3 ± 5.9

Table 1. *Cont.*

	Controls (n = 35)	Burn Patients (n = 32)
Liver function tests		
AST (IU/L)	27.15 ± 3.94	62.11 ± 6.32 *
ALT (IU/L)	21.44 ± 5.12	75.11 ± 9.15 *
Renal function tests		
BUN (mg/dL)	14.6 ± 1.2	16.2 ± 1.3
Creatinine (mg/dL)	0.73 ± 0.11	0.94 ± 0.16 *
Miscellaneous blood tests		
Total proteins (g/dL)	7.2 ± 0.2	5.6 ± 0.2 *
Albumin (g/dL)	4.1 ± 0.2	3.2 ± 0.1 *
HbA1c %	5.4 ± 0.2	5.5 ± 0.2
RBS (mg/dL)	121.5 ± 24.3	133.8 ± 31.5

* $p < 0.05$ vs. controls using unpaired t-test (WBC: white blood cells, AST: aspartate transaminase, ALT: alanine transaminase, BUN: blood urea nitrogen, HbA1c: glycosylated hemoglobin, RBS: random blood sugar).

3.2. Body Composition, HGS, and Phase Angle

We next investigated the body composition of the study groups. The BMI of the two groups was not different (Figure 1A). However, the burn patients exhibited lower lean mass and fat mass than the controls (both $p < 0.05$) (Figure 1B and C, respectively).

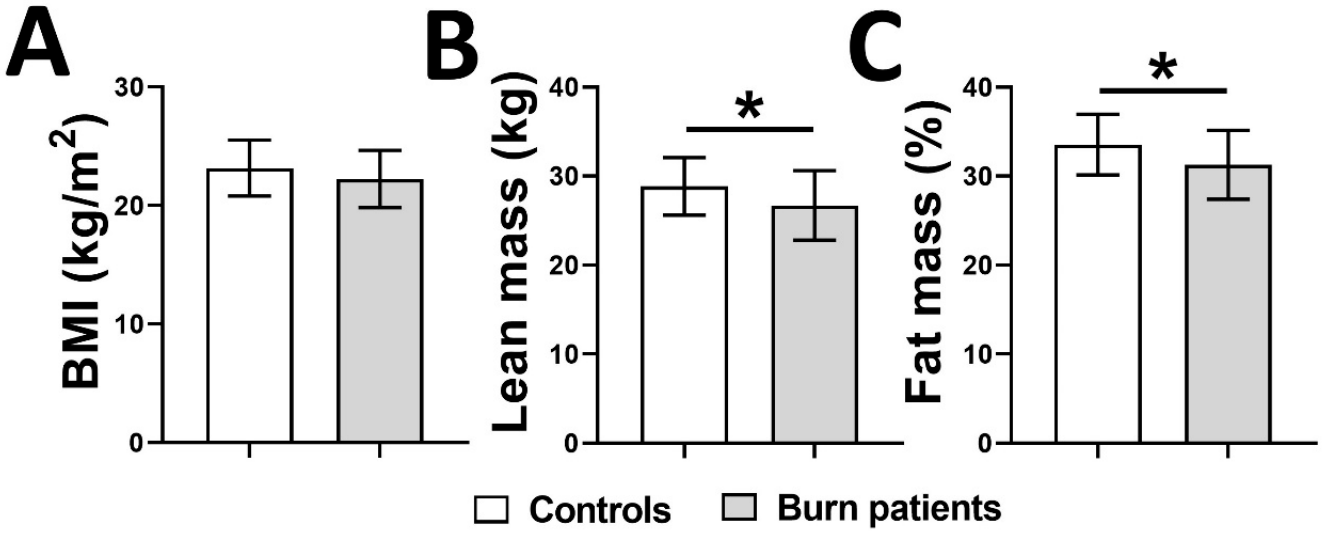


Figure 1. BMI (**A**), lean mass (**B**), and fat mass (**C**) in controls (n = 35) and burn patients (n = 32). * $p < 0.05$ vs. controls using unpaired t-test (BMI: body mass index).

We also found significantly lower HGS in the burn patients than in the age-matched controls ($p < 0.05$) (Figure 2A). SMI and phase angle are considered the established markers of muscle mass. We found that the burn patients exhibited lower SMI and phase angle than the controls, suggesting muscle wasting (both $p < 0.05$) (Figure 2B and C, respectively).

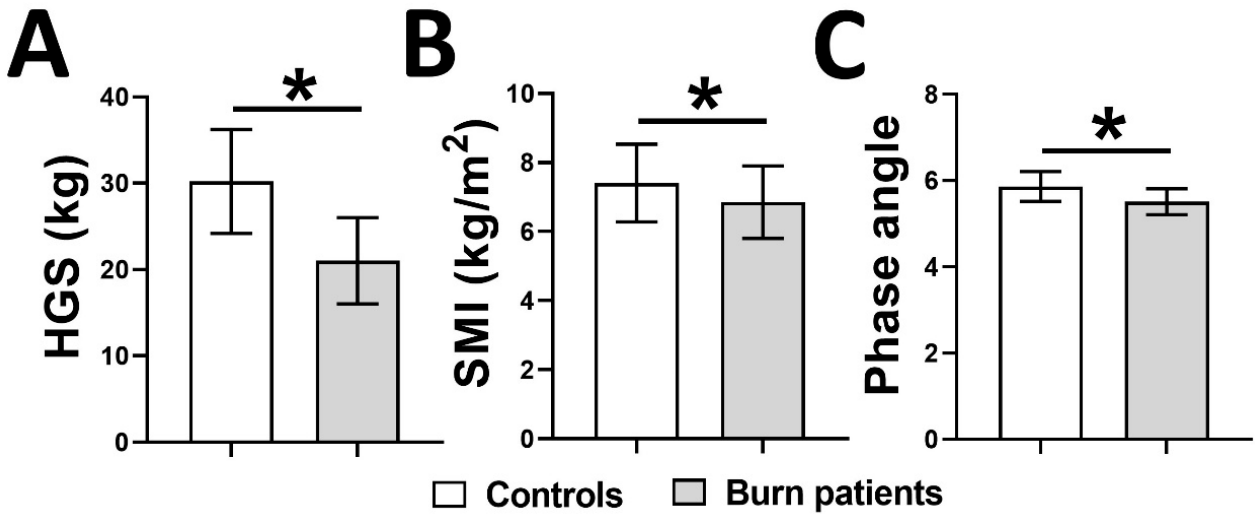


Figure 2. HGS (**A**), SMI (**B**), and phase angle (**C**) in controls (n = 35) and burn patients (n = 32). * $p < 0.05$ vs. controls using unpaired t-test (HGS: handgrip strength, SMI: skeletal muscle index).

3.3. Levels of Plasma Biomarkers

We next investigated the plasma levels of various biomarkers relevant to muscle mass and strength. We found significantly higher levels of plasma CAF22 in the burn patients than in the controls, suggesting NMJ degradation ($p < 0.05$) (Figure 3A). We also found higher levels of plasma CRP and 8-isoprostanes in the burn patients, suggesting elevated systemic inflammation and oxidative stress (both $p < 0.05$) (Figure 3B and C, respectively). Lastly, the expression of creatine kinase, a marker of muscle damage, was also upregulated in the burn patients when compared to the controls ($p < 0.05$) (Figure 3D).

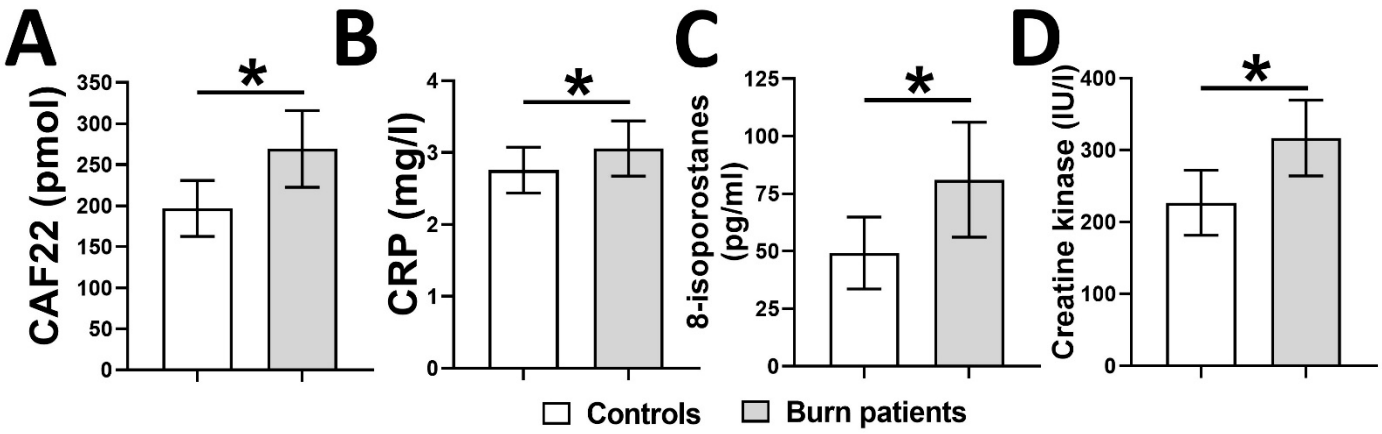


Figure 3. Plasma levels of CAF22 (**A**), CRP (**B**), 8-isoprostanes (**C**), and creatine kinase (**D**) in controls (n = 35) and burn patients (n = 32). * $p < 0.05$ vs. controls using unpaired t-test (CAF22: c-terminal agrin fragment-22, CRP: c-reactive proteins).

3.4. Associations of Plasma Biomarkers with HGS and Body Composition

Next, we investigated the associations of plasma biomarkers with HGS and various measures of body composition, including SMI, phase angle, and fat mass in the controls and burn patients. We observed a significant correlation of plasma CAF22 with HGS in the control group ($r^2 = 0.157$, $p < 0.05$) (Figure 4A). We also found a significant correlation of plasma CRP with fat mass in the control group ($r^2 = 0.114$, $p < 0.05$) (Figure 4A). On the other hand, the burn patients exhibited significant correlations of plasma CAF22 with HGS ($r^2 = 0.255$, $p < 0.05$) (Figure 4B) and phase angle ($r^2 = 0.185$, $p < 0.05$) (Figure 4B). These patients also exhibited significant correlations of plasma CRP with HGS ($r^2 = 0.125$,

$p < 0.05$) (Figure 4B) and phase angle ($r^2 = 0.133$, $p < 0.05$) (Figure 4B). Lastly, we also found a significant correlation of plasma CK levels with phase angle in the burn patients ($r^2 = 0.126$, $p < 0.05$) (Figure 4B).

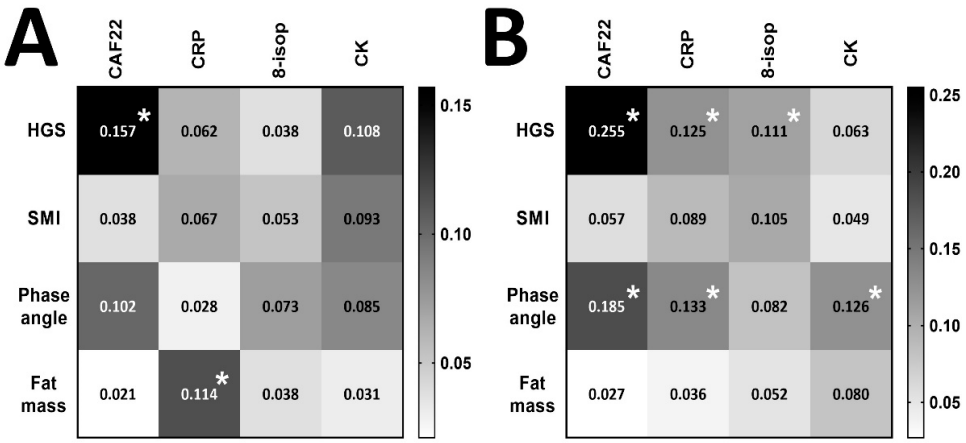


Figure 4. Heat maps showing the coefficients of determination of plasma CAF22, CRP, 8-isoprostanes, and CK with HGS, SMI, phase angle, and fat mass in controls ($n = 35$) (**A**) and burn patients ($n = 32$) (**B**). * $p < 0.05$ (CAF22: c-terminal agrin fragment-22, CRP: c-reactive proteins, 8-isop: 8-isoprostanes, HGS: handgrip strength, SMI: skeletal muscle mass index).

3.5. Associations of Plasma CAF22 with Percent Burn Area

Lastly, we investigated the associations of plasma CAF22 with percent burn area in the burn patients. We found a significant positive correlation between CAF22 levels and percent burn area in the cumulative cohort of 32 burn patients ($r^2 = 0.162$, $p < 0.05$) (Figure 5A). However, when the burn patients were categorized according to the body region, we did not find significant correlations of CAF22 with percent burn area despite a robust trend towards a positive correlation between the two variables in patients with burns of the left upper limb and lower limbs (Figure 5B).

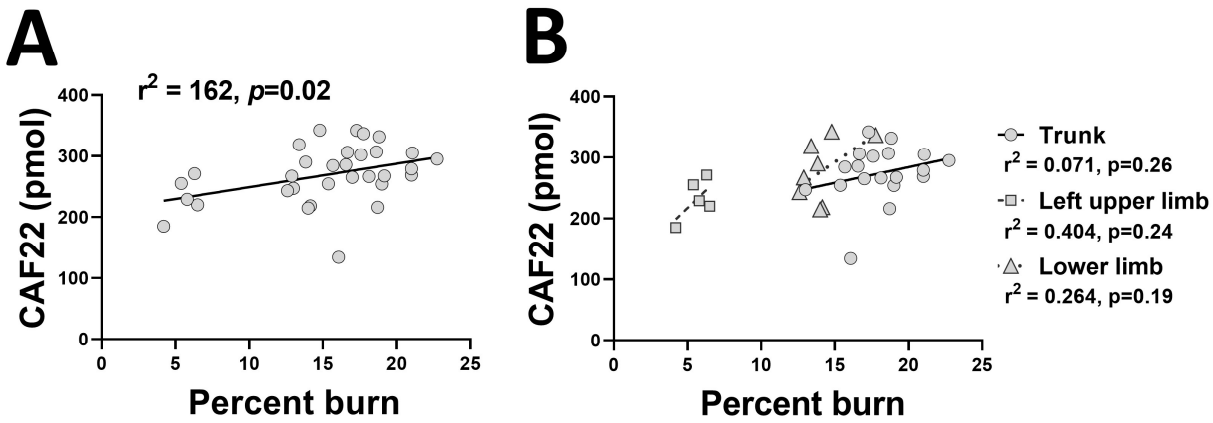


Figure 5. The correlations of plasma CAF22 levels with percent burn area in the cumulative cohort (**A**) and according to burn region (**B**) in burn patients ($n = 32$). (CAF22: c-terminal agrin fragment-22.).

4. Discussion

Our novel findings suggest a potential role for NMJ disintegration in burn-induced muscle weakness and atrophy. We found lower HGS, lean mass, SMI, and phase angle in the burn patients than the controls, suggesting muscle wasting and weakness due to burns. We also found higher levels of plasma CAF22 in these patients, suggesting NMJ degradation. The significant correlations of plasma CAF22 with HGS and phase angle indicate a potential contribution of NMJ degradation to muscle wasting and weakness

in these patients. Lastly, we also observed higher plasma CRP, 8-isoprostanes, and CK levels in the burn patients, indicating systemic inflammation, oxidative stress, and muscle damage, respectively.

No previous study has investigated NMJ loss and its contribution to muscle decline in burn patients. However, experimental data from mice show that burn injury is associated with the degeneration of motor neurons, which is partly due to microglia activation [14]. We previously reported the pivotal role of motor neurons in maintaining NMJ integrity [8]. Specifically, motor neuron dysfunction leads to an accelerated degradation of NMJ in experimental mice [15]. These reports are consistent with our findings of NMJ degradation in burn patients. Further, we also found significantly lower HGS in these patients than in the controls. The mechanistic role of NMJ degradation in muscle weakness is previously described in clinical and experimental animal studies [6,9]. Our observation of a robust association between CAF22 levels and HGS in burn patients validates and further extends those reports. The onset of muscle weakness occurs earlier than atrophy in various muscle-wasting conditions [16]. However, a burn injury is associated with a severe muscle phenotype and frequently results in accelerated muscle atrophy [2]. We found lower SMI and phase angle in the burn patients than the controls, validating muscle atrophy in these patients. SMI is a standard measure of muscle mass, and a low SMI is used as a diagnostic criterion of age-related muscle atrophy [10]. Similarly, the phase angle is considered a valuable bioelectrical marker of muscle wasting in human patients [17]. Together, the reduction in SMI and phase angle suggests significant muscle wasting in burn patients, which is consistent with previous reports [1,2,18].

Burn injuries are also associated with systemic inflammation and oxidative stress [19]. Consistent with similar findings, we observed higher expressions of plasma CRP and 8-isoprostane levels in these patients. Previous reports show the detrimental effects of heightened inflammation on NMJ components in burn patients. For example, burn injury causes the activation of microglia cells, the resident macrophages of the nervous system, suggesting heightened inflammation [14]. Further, the activated microglia cells are directly implicated in the degeneration of motor neurons, which contribute to NMJ damage [14]. In addition, inflammation also activates several molecular pathways associated with NMJ damage [2,20].

Elevated oxidative stress is a common occurrence in burn injuries [19]. Experimental data from porcine models also show that oxidative stress can be a potential therapeutic target for alleviating the negative consequences of burns on body tissues [19]. We found higher 8-isoprostanes levels in the burn patients than the controls, validating higher oxidative stress in these patients. The detrimental effects of oxidative stress on skeletal muscle and NMJ have previously been recognized [21,22]. Specifically, elevated oxidative stress damages muscle contractile machinery, leading to muscle weakness and reduced HGS [23]. In support of these findings, an inverse association has been recognized between elevated plasma 8-isoprostanes and low HGS in patients with muscle diseases [12]. Consistent with these reports, we found a significant positive correlation of plasma 8-isoprostane levels with HGS in the burn patients. Thus, our data suggest that oxidative stress may also contribute to muscle weakness in burn patients.

We also found higher CK levels in the burn patients, validating muscle wasting. CK is used as an indirect marker of muscle damage in various conditions. Higher plasma CK also exhibits correlations with the extent of muscle damage following an electrical burn [24]. We observed significant positive correlations of plasma CK levels with phase angle, consistent with muscle decline in these patients.

Lastly, we found a significant positive correlation between plasma CAF22 and percent burn area, which suggests higher NMJ damage with a higher severity of burn. This finding

suggests a dose-dependent detrimental effect of burns on NMJ and skeletal muscle. It is possible that this correlation is partly region dependent. For example, we found a trend towards a positive correlation between CAF22 levels and burn area in patients with burns of the left upper limb and lower limbs. However, relevant trends were not found in patients with burn injuries to the trunk region. The clinical relevance and validation of these findings require a larger sample size and mechanistic studies involving experimental animal models.

The systemic consequences of a burn injury were evident in higher platelets and white blood cells, as well as platelet count and disruption of renal and liver function tests. These effects may have indirectly contributed to muscle decline in these patients. However, they are outside the scope of the potential contribution of NMJ damage to muscle detriment in burn injury.

We did not investigate the psychological consequences of burn injury in our patients. It is recognized that poor psychological health can contribute to muscle decline and frailty in a gender-specific manner [25]. A vicious cycle may be proposed so that poor psychological health in burn patients may contribute to accelerated physical decline and vice versa. Such an interface may occur independent of NMJ involvement and remains to be investigated.

This study offers several advantages. The simple ELISA test for plasma CAF22 and the non-invasive nature of HGS measurements make them practical for clinical use. However, due to the HGS limitation, we could not assess patients with hand burns. The single-center design of this study also limits the generalization and wider application of our findings to diverse populations.

Nevertheless, our findings suggest that NMJ degradation plays a significant role in burn-induced muscle injury and may warrant further investigation for potential therapeutic interventions.

Author Contributions: Conceptualization, I.M.K., M.J., H.Z. and R.Q.; methodology, I.M.K., M.J., H.Z. and R.Q.; software, N/A; validation, I.M.K., M.J., H.Z. and R.Q.; formal analysis, R.Q.; investigation, I.M.K., M.J., H.Z. and R.Q.; resources, I.M.K., M.J., H.Z. and R.Q.; data curation, R.Q.; writing—original draft preparation, I.M.K., M.J., H.Z. and R.Q.; writing—review and editing, R.Q.; visualization, N/A; supervision, I.M.K., M.J., H.Z. and R.Q.; project administration, I.M.K., M.J., H.Z. and R.Q.; funding acquisition, R.Q. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by a competitive grant (24010901166) from the University of Sharjah to Rizwan Qaisar.

Institutional Review Board Statement: This study was conducted according to the Declaration of Helsinki [18] and approved by the Clinical Research Ethics Committee of the Hayatabad Medical Complex, Peshawar (Approval Number: HMC-REC-22-05-03, Dated: 15 August 2023).

Informed Consent Statement: A written informed consent was obtained from all participants involved in this study.

Data Availability Statement: The data are available from the corresponding author on request due to the privacy of the patients.

Conflicts of Interest: The authors declare no conflicts of interest.

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