Improved Classification of Acute Physical Fatigue Using Salivary Proteomic Biomarkers: An Exploratory Study

Bryndan Lindsey[1,2], Kenneth Bowden[2], Yosef Shaul[3], Emanuel Petricoin[4], Shane V. Caswell[3], Rayan Alhammad[4,5], Anissa N. Elayadi[2], Brandon Roberts[6], and Joel Martin[3,✉]

[1]State Key Laboratory of Mechanical System and Vibration, School of Mechanical Engineering, Shanghai Jiao Tong University, Shanghai, China
[2]Research and Exploratory Development Department, Johns Hopkins Applied Physics Laboratory, Laurel, Maryland, USA
[3]Sports Medicine Assessment Research & Testing (SMART) Laboratory, George Mason University, Virginia, USA
[4]Center for Applied Proteomics and Molecular Medicine, School of Systems Biology, George Mason University, Manassas, Virginia, USA
[5]Clinical Laboratory Department, College of Applied Medical Sciences, King Saud bin Abdulaziz University for Health Sciences, Al Ahsa, Saudi Arabia
[6]Experimental Therapeutics, Walter Reed Army Institute of Research, Silver Spring, Maryland, USA

Introduction: Physical fatigue is a key determinant of operational readiness in the physically demanding occupations of tactical athletes. Specific hormonal, immune, and enzymatic biomarkers have been proposed for fatigue assessment, but their reliability can be affected by external factors. This study aimed to explore the predictive accuracy of targeted stress-related small molecules to proteins identified via untargeted salivary proteomics to classify physical fatigue. **Methods:** Ten recreationally active adults (6M, 4F) completed a fatiguing protocol designed to simulate common operational movements and intensities. Saliva samples were collected pre- and post-protocol and analyzed for targeted biomarkers using commercial immunoassays and for untargeted proteins via liquid chromatography-mass spectrometry. Machine learning models were trained to classify pre- vs post-exercise state using these biomarkers, with performance assessed through sensitivity, specificity, and area under the receiver operating characteristic curve (AUC). **Results:** Targeted small molecules achieved an overall model accuracy of 86%, with immunoglobulin A and uric acid demonstrating the highest predictive power. However, a proteomic panel of four proteins (ATP1B1, STOML2, PGLYRP2, FH) exhibited superior performance, with 95% classification accuracy and improved sensitivity. Pathway analysis revealed that these proteins were involved in mitochondrial function, immune regulation, and metabolic adaptation, suggesting their role in fatigue-associated physiological changes. **Conclusions:** Salivary proteomics identified biomarkers with greater sensitivity and specificity for detecting physical fatigue than targeted stress-related molecules within this sample. These findings support the potential for non-invasive proteomic monitoring of fatigue in operational and athletic settings. Future studies should validate these findings in larger, more diverse populations and assess their applicability to chronic fatigue monitoring.

Physical Fatigue | Salivary Proteomics | Biomarkers | Occupational Performance

Correspondence: *jmarti38@gmu.edu*

DRAFT

Introduction

Operational readiness, the ability of military forces to fight and meet the demands of the assigned missions, is shaped by various factors (1, 2). One important factor is fatigue, which is characterized by a measurable decline in muscular performance or an increase in perceived exertion (3) that can impair functional capacity, elevate injury risk, and compromise mission success (4). Following sustained occupational activities, fatigue is frequently elevated; however, capturing this in a rapid, objective manner remains challenging, especially in tactical populations.

Physical exertion-induces coordinated physiological stress responses that are reflected in measurable changes in circulating biomarkers (5). Specifically, prolonged physical exertion causes changes in the autonomic nervous system (ANS), influencing the activation of hormones like cortisol and testosterone amongst other compounds (6). Biomarkers such as cortisol and testosterone (7), immunoglobin-A (IgA) (8), alpha-amylase (AA) (9), interleukin-6 (IL-6) (10), and uric acid (UA) (11) have all been shown to change as a result of military related stressors, and therefore, have been proposed as potential biomarkers of physical readiness, but to date have mostly been measured via blood samples which is invasive, requires trained personnel, and presents compliance and logistical challenges for repeated sampling in operational settings.

Saliva sampling offers a novel, non-invasive, easily collected medium for assessing biomarkers rapidly and in field environments. Recently, untargeted proteomic approaches with saliva have identified proteins related to immunoregulation, inflammation, and metabolism that predict fatigue status in civilian (12, 13) and military settings (14). These proteins have been hypothesized to be more specific markers of physical fatigue as opposed to hormones like cortisol and testosterone which are influenced by diurnal rhythm and psychological stressors (15). However, the comparative performance of proteomic biomarkers relative to established stress-related salivary markers has not been evaluated in an exploratory, within-cohort comparison. Therefore, the objective of this study aimed to compare the performance of salivary biomarkers identified via untargeted liquid chromatography-mass spectrometry (LC-MS) with established stress-related salivary biomarkers for classifying physical fatigue following a controlled fatiguing protocol.

Methods

Study Participants

Ten recreationally active adults (6 males, 4 females; age 32.4 ± 9.1 years) were recruited from April 1, 2023 to August 30, 2023. Inclusion criteria included ages 18-55, absence of injury or chronic disease, and ability to complete a weighted 2+ mile ruck. Participants engaged in $\geq$30 minutes of daily

physical activity and reported no limitations in performing standard exercises. All provided written informed consent that was witnessed by the study principal investigator. The study was approved by the university's IRB (IRB #2004820) and conducted in accordance with the ethical standards set by the Helsinki Declaration.

Data Collection Protocol

Saliva samples (∼5mL per collection) were collected at 7 timepoints over a 3-day span (Figure 1) using the mLife True oral fluid collection device (mLife, TX, USA). The protocol spanned three days: baseline (day 1), fatigue (day 2), and recovery (day 3). On the baseline and recovery days, samples were taken at approximately 0800 and 1300 hours, and participants were instructed not to perform any strenuous exercise 24 hours before day 1 or during the protocol outside the prescribed fatiguing protocol on day 2. On the second day, participants performed an hour-long physical fatiguing protocol, donning a wearable electrocardiogram (ECG) monitor (Zephyr Bioharness 3.0, Zephyr Technology Corporation, Annapolis, MD, USA) which they wore approximately 1-hour before, during, and 1-hour after the protocol.

The fatigue protocol involved participants performing one hour of continuous circuits of a 400m run, 30.5m standing power throw (4.5kg), 30.5m sprint, and a 6m single arm farmer's carry (M = 22.7kg, F = 15.9kg) while wearing a weighted vest (9.1kg). This circuit was developed to simulate common occupational tasks and movements in operational settings. Directly before and after the fatiguing protocol, participants performed a series of physical performance tests including Queen's step test (16), maximum standing vertical leap (CMJ) and isometric mid-thigh pull (IMTP) on force plate (ForceDecks, Vald Performance, Brisbane, Queensland, Australia), maximum hand-release pushups (HRPU) and plank hold, and average anaerobic power (watts) over 60s max sprint on cycle ergometer (max power; Rogue Echo Bike, Rogue, Columbus, Ohio, USA). On the fatigue day, saliva samples were collected at approximately 0800, 1200 (directly after completion of the post-fatigue protocol performance assessment) and 1300 (one hour after post- fatigue protocol performance assessment). Samples were stored on dry-ice until cessation of each data collection day after which they were preserved at $-80°$C at the Center for Proteomics and Molecular Medicine (CAPMM) until analysis.

Targeted Analysis

Aliquots of saliva (∼0.5 mL) were analyzed for cortisol, testosterone, IgA, alpha-amylase (AA), IL-6, and uric acid (UA) via immunoassays at a commercial lab (Salimetrics, Carlsbad, CA). Before testing, samples were thawed to room temperature, vortexed, and centrifuged at approximately 3,000 RPM (1,500 × g) for 15 minutes. High-sensitivity enzyme immunoassays were used to quantify cortisol, testosterone, and IgA, while kinetic enzyme immunoassays were employed for AA and UA. IL-6 was measured using the Salimetrics Cytokine Panel and assayed in duplicate at the Salimetrics SalivaLab using a proprietary electrochemiluminescence method developed and validated for saliva.

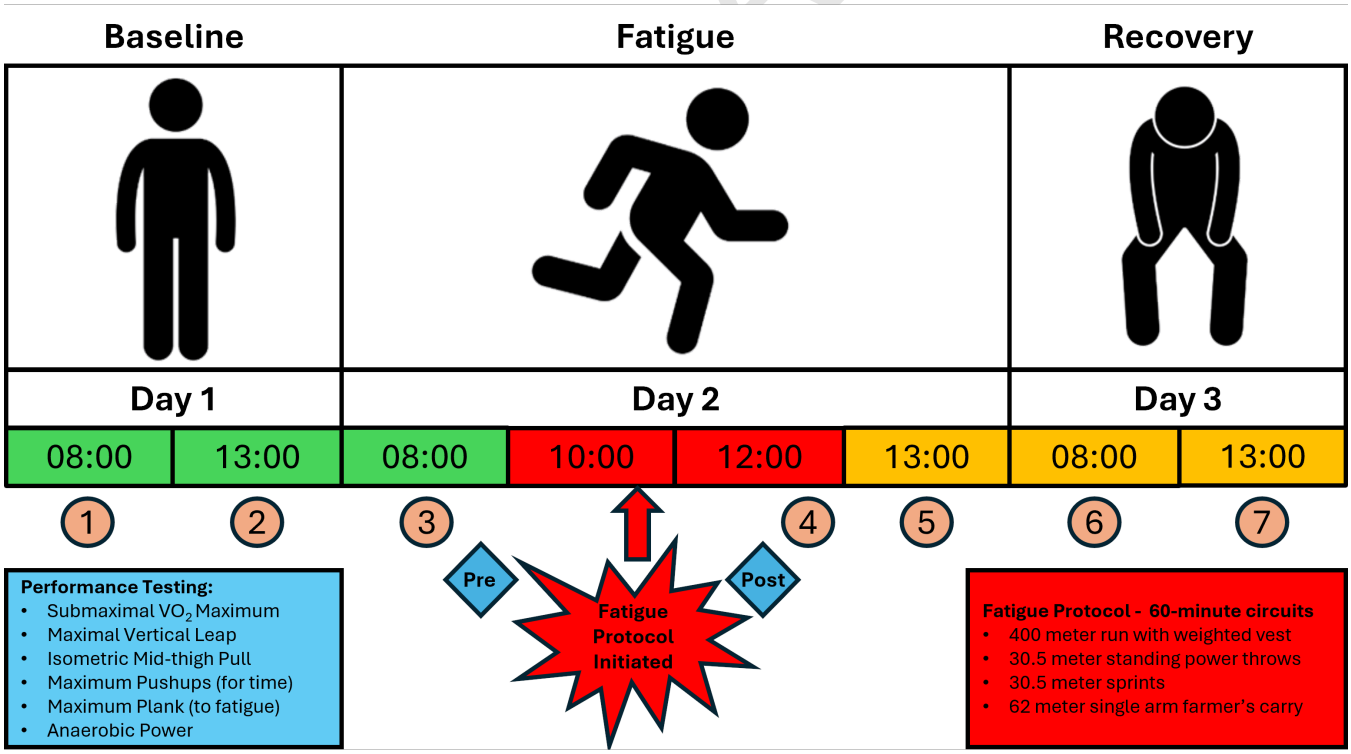

Fig. 1. Overview of the three-day experimental protocol and saliva sampling timeline. Baseline measurements were collected on Day 1, followed by a fatigue induction protocol on Day 2 and recovery monitoring on Day 3. Saliva samples were obtained at standardized time points (08:00 and 13:00) during baseline and recovery, as well as before (sample 3), immediately after (sample 4), and one hour following (sample 5) the fatigue protocol on Day 2. The fatigue protocol consisted of a 60-minute circuit of running, sprinting, power throws, and loaded carries, followed by a battery of physical performance tests assessing aerobic, neuromuscular, and anaerobic function. Numbered circles denote the order of saliva sample collection.

Proteomic Analysis

The remaining 4.5 mL from each saliva sample was analyzed at the Center for Applied Proteomics and Molecular Medicine (CAPMM) at George Mason University to identify and quantify salivary proteins. Low abundance proteins were enriched by selective capture using core-shell hydrogel nanoparticle based technology as previously described (16, 17). After multiple washes to remove unbound proteins, the protein-nanoparticle complexes were eluted by incubation with 4% sodium dodecyl sulfate (SDS) in 50 mM ammonium bicarbonate at room temperature. The SDS was then removed using detergent removal columns according to manufacturer instructions.

Eluted proteins were digested with trypsin at 37°C for 6 hours overnight, followed by quenching with the addition of 2 μL of Trifluoroacetic Acid (TFA). Trypsinized peptides were purified using C18 spin columns with 80% acetonitrile/0.1% formic acid solution. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was performed using an Exploris 480 mass spectrometer (ThermoFisher Scientific, Waltham, MA, USA) equipped with a nanospray EASY-nLC 1200 HPLC system. Peptides, resuspended in 0.1% formic acid were separated using a reversed-phase PepMap RSLC C18 LC column (ThermoFisher Scientific) using a mobile phase of 0.1% aqueous formic acid (A) and 0.1% formic acid in 80% acetonitrile (B).

Data acquisition was performed in data-dependent mode, with one full MS scan (300–1500 m/z, 60,000 resolving power) followed by MS/MS scans of the most abundant molecular ions, dynamically selected and fragmented by higher-energy collisional dissociation (HCD) at a collision energy of 27%. Tandem mass spectra were searched against the NCBI human database using Proteome Discover v 2.4 (Thermo Fisher Scientific). Database searches employed the SEQUEST node with full tryptic cleavage constraints and dynamic methionine oxidation. Mass tolerances were set to 2 ppm for precursor ions and 0.02 Da for fragment ions. A 1% false discovery rate (FDR) was applied to peptide-spectrum matches (PSM) as the reporting threshold. Precursor Ions quantifier node was used to for protein abundance calculation and quantification.

Statistical Analysis

Performance Parameters

All data processing and statistical analyses were performed using Python (v3.10.11). Since all participants completed the same fatiguing protocol, changes in performance tests and ECG metrics were used to verify the induction of physical fatigue. Pre-fatigue heart rate (HR) and heart rate variability (HRV) were calculated as 30-minute averages before testing; post-fatigue values were averaged during and after the fatigue protocol. Normality was assessed using the Shapiro-Wilk test, and paired Student's t-tests were used to compare pre- and post-fatigue measures ($p < 0.05$), with effect sizes calculated using Cohen's d for paired samples.

Targeted Biomarkers

Descriptive statistics were calculated for each biomarker, and pre (time 1) versus post (time 4) differences were assessed using paired Student's t-tests, with effect sizes calculated using Cohen's d for paired samples. Mean values of duplicate measures for cortisol (μg/dL), testosterone (pg/mL), IgA (μg/mL), alpha-amylase (U/mL), uric acid (mg/dL), and IL-6 (pg/mL) were z-score standardized for use in model training. Multicollinearity was assessed using variance inflation factor (VIF). A support vector machine (SVM) model with leave-one-out cross-validation (LOOCV) was used to classify rested (pre-fatigue) vs. fatigued (post-fatigue) states.

Identified Proteins

Untargeted mass spectrometry of our samples yielded 2,284 proteins across time points, as deposited in the Open Science Framework (DOI: 10.17605/OSF.IO/2WM4P, version 1.0). Of the 2,284 proteins detected, those present in more than 50% of samples ($n = 1{,}355$) were retained. Missing values were imputed at 50% of the detection limit, and all data were log-normalized. Mixed-effects models were used to evaluate the effect of time of day and fatigue status on protein abundance, with participant as a random effect. Proteins significantly associated with fatigue only ($n = 1{,}149$) were selected for further analysis. Recursive Feature Elimination

Table 1. Top salivary proteins selected by recursive feature elimination for fatigue classification.

Protein	Gene Symbol	Model Importance	Function
Sodium/potassium-transporting ATPase subunit beta-1	ATP1B1	0.75	Non-catalytic component of the active enzyme, which catalyzes the hydrolysis of ATP coupled with exchange of Na^+ and K^+ ions across the plasma membrane. The beta subunit regulates, through assembly of alpha/beta heterodimers, the number of sodium pumps transported to the plasma membrane.
Fumarate hydratase, mitochondrial	FH	0.39	Catalyzes the hydration of fumarate to L-malate in the tricarboxylic acid (TCA) cycle to facilitate a transition step in the production of energy in the form of NADH.
Stomatin-like protein 2	STOML2	0.34	May regulate the biogenesis and activity of the mitochondria. Through regulation of mitochondrial function, may play a role in cell migration, proliferation, T-cell activation, calcium homeostasis, and cellular response to stress. Required for mitochondrial hyperfusion, a pro-survival response resulting in ATP production.
N-acetylmuramoyl-L-alanine amidase	PGLYRP2	0.32	Hydrolyses the link between N-acetylmuramoyl residues and L-amino acid residues in certain cell-wall glycopeptides.

DRAFT

(RFE) within cross-validation was used to identify a stable subset of proteins consistently selected across ≥4 of 5 folds. Similar to targeted biomarkers, an SVM model with LOOCV was used to classify rested (pre-fatigue) vs. fatigued (post-fatigue) states. For both models, performance was evaluated using a confusion matrix to calculate sensitivity and specificity, along with F1 score and Matthews Correlation Coefficient (MCC). Area under the curve (AUC) of the receiver operating characteristic (ROC) was also calculated for each biomarker and the combined models.

In addition to identifying a minimal predictive panel, broader pathway enrichment was performed to characterize the biological processes represented by the full set of proteins associated with fatigue to capture systems-level responses that may not be represented within a small feature-selected panel. Pathway analysis was conducted using the Reactome Analyze Gene List tool (18), followed by the Analyze Gene Expression tool using the PADOG method (19), which down-weights genes commonly found across multiple pathways. Resulting pathways were then cross-referenced with protein accession numbers corresponding to the four SVM-selected proteins to determine whether the top predictors of fatigue were embedded within broader fatigue-related biological networks.

Results

Change in Performance Parameters

Physical fatigue was defined as a categorical state of reduced physical performance, confirmed by significant declines in aerobic, anaerobic capacity and muscular endurance following the protocol. Mean and standard deviation of performance measures as well as targeted marker concentrations before and after the fatigue protocol are presented in Table 2. Post-fatigue, average heart rate increased while HRV decreased, accompanied by reductions in step test recovery performance, anaerobic capacity, maximum push-ups, and plank time. No significant changes were observed for vertical leap height or peak isometric force.

Predictive Accuracy of Targeted Molecules vs Discovered Proteins

Recursive feature elimination identified a stable subset of proteins ($n = 4$) consistently selected across ≥4 of 5 folds. Protein names, gene IDs, functions, and model importance are shown in Table 2. Figure 2 shows individual and combined ROC curves for both SVM models. The targeted small molecule model achieved 86% accuracy, with IgA and UA showing the highest discriminatory power. Testosterone, IL-6, alpha-amylase, and cortisol were less effective. In contrast, the protein-based model achieved 95% accuracy. ATP1B1 was the top-performing protein, followed by STOML2 and PGLYRP2, while FH showed limited individual predictive value.

Confusion matrices showed a higher true positive rate for the protein model (80%) compared to the molecule model (60%), and a slightly higher true negative rate (100% vs. 97%). The protein model also had superior F1 score (89%) and MCC (87%) compared to the molecule model (71% and 65%, respectively).

Pathway Analysis

Twenty biological pathways were associated with the four proteins identified by the SVM model (Figure 3). Thirteen pathways were upregulated, including "Processing of SMDT1" and "Cellular response to mitochondrial stress," both linked to STOML2 ($p = 0.015$). These pathways are involved in mitochondrial function, calcium transport, and cellular stress adaptation. Seven pathways were downregulated, notably "Cell surface interactions at the vascular wall" and "Basigin interactions," both associated with ATP1B1 ($p = 0.015$). These pathways play roles in immune cell trafficking, vascular integrity, and hemostasis.

Discussion

Salivary biomarkers offer a non-invasive way to monitor physiological stress, and prior studies have linked fatigue-induced biomarkers to performance outcomes in tactical and

DRAFT

Table 2. Changes in physical performance parameters pre- to post-fatigue protocol.

Performance Parameter	Pre-Fatigue Mean (SD)	Post-Fatigue Mean (SD)	Effect Size	p-value
Cardiorespiratory Responses				
Heart Rate (bpm)	67.2 (6.47)	136.4 (21.4)	3.04	<0.001
Heart Rate Variability (ms)	86.5 (33.1)	21.1 (10.8)	-1.84	<0.001
Recovery Heart Rate (bpm)	128.7 (23.7)	160.3 (18.46)	2.01	<0.001
Neuromuscular Performance				
Max Vertical Leap (cm)	37.0 (9.6)	37.5 (10.0)	0.16	0.62
IMTP Peak Vertical Force (N)	2334.7 (1056.7)	2223.2 (868.2)	-0.38	0.26
Max Hand Release Pushups (#)	38.2 (6.6)	32.1 (6.6)	-1.53	<0.001
Max Plank (s)	150.0 (48.9)	92.9 (24.6)	-1.88	<0.001
Metabolic Performance				
Anaerobic Capacity (W)	448.0 (133.9)	389.7 (125.3)	-1.36	<0.001
Salivary Biomarkers				
Cortisol (μg/dL)	0.44 (0.25)	0.79 (0.66)	0.57	0.10
Testosterone (pg/mL)	192.85 (103.41)	247.98 (128.33)	0.68	0.06
Immunoglobulin A (μg/mL)	212.30 (69.40)	381.25 (166.75)	1.16	<0.01
Alpha-amylase (U/mL)	130.37 (132.26)	285.88 (225.04)	0.81	<0.05
Uric Acid (mg/dL)	5.66 (1.82)	11.37 (4.91)	1.34	<0.01
Interleukin-6 (pg/mL)	2.45 (1.87)	4.68 (2.39)	2.06	<0.001

Note. Values are mean (SD); effect sizes are Cohen's d for paired comparisons.

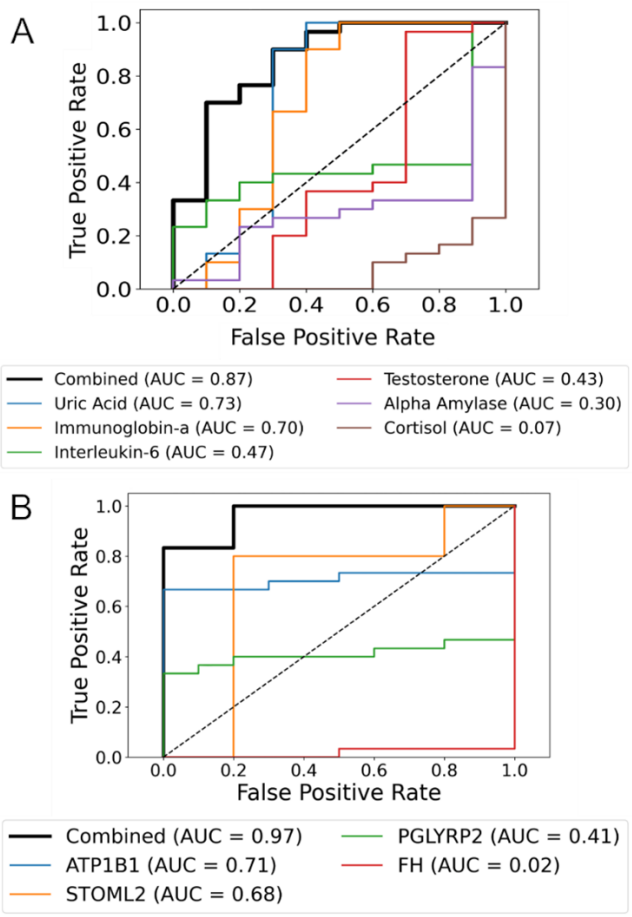

Fig. 2. Receiver operating characteristic (ROC) curves for predicting physical fatigue using targeted salivary biomarkers vs salivary proteins identified via untargeted mass spectrometry. Values in parentheses represents discriminatory performance in the classification model. A) ROC curves for targeted biomarkers, including cortisol, testosterone, immunoglobin A (IgA), alpha-amylase (AA), uric acid (UA), and interleukin-6 (IL-6), as well as a combined model incorporating all six molecules. B) ROC curves for salivary proteins identified through untargeted mass spectrometry including sodium/potassium-transporting ATPase subunit beta-1 (ATP1B1), stomatin-like protein 2 (STOML2), N-acetyl-L alanine amidase (PGLYRP2), and fumurate hydratase (FH).

athletic settings (20–22). This study builds on this work by comparing the predictive performance of two models for classifying physical fatigue: one based on traditional stress-related biomarkers and the other on proteins identified through untargeted proteomics. Our data suggests strong overall classification accuracy of fatigue, but the proteomics-based model outperformed the targeted biomarker model, which was driven by higher sensitivity.

Cortisol and dehydroepiandrosterone (a precursor to testosterone) have been studied as potential biomarkers for stress resilience during military training (23, 24) but showed poor predictive utility in our study when analyzed independently. Similarly, AA and IL-6, which are linked to sympathetic activity and inflammation, respectively (9, 10), were also weak predictors in our study. This may be due to the variability in secretion during fatigue, which is influenced by differences in hypothalamic-pituitary-adrenal (HPA) axis responsiveness (25), genetic predispositions (26, 27), and anticipatory stress responses (28). Additional factors such as diurnal rhythm (29), hydration status (30), and baseline psychological or physiological stress levels (15, 31) further contribute to inconsistent hormonal patterns across individuals. In contrast, IgA and UA exhibited stronger individual performance. IgA reflects mucosal immune function and is rapidly secreted in response to sympathetic activation (32), while UA is a robust marker of oxidative stress and metabolic turnover during exertion (33). These roles in immune defense and systemic antioxidant activity may inherently reduce inter-individual variability, making IgA and UA reliable indicators of acute physical fatigue. Together, these patterns highlight the advantages of immune and metabolic markers over systemic hormones for detecting acute fatigue.

Proteomics captures a broader physiological picture than isolated hormonal or inflammatory markers. The proteomics model achieved greater performance compared to the panel of targeted biomarkers (95% to 85% overall accuracy). We also identified four novel proteins related to fatigue: ATP1B1, FH, and SOML2, PGYLRP2. ATP1B1, a subunit of the Na^+/K^+-ATPase, reflects neuromuscular excitability and ion homeostasis, processes that degrade during repeated muscle activation and contribute to peripheral fatigue, but has never been linked to directly fatigue in saliva (34). Similarly, FH has not been previously reported as a salivary biomarker in healthy individuals or in fatigue-related studies, likely due to its low abundance and intracellular localiza-

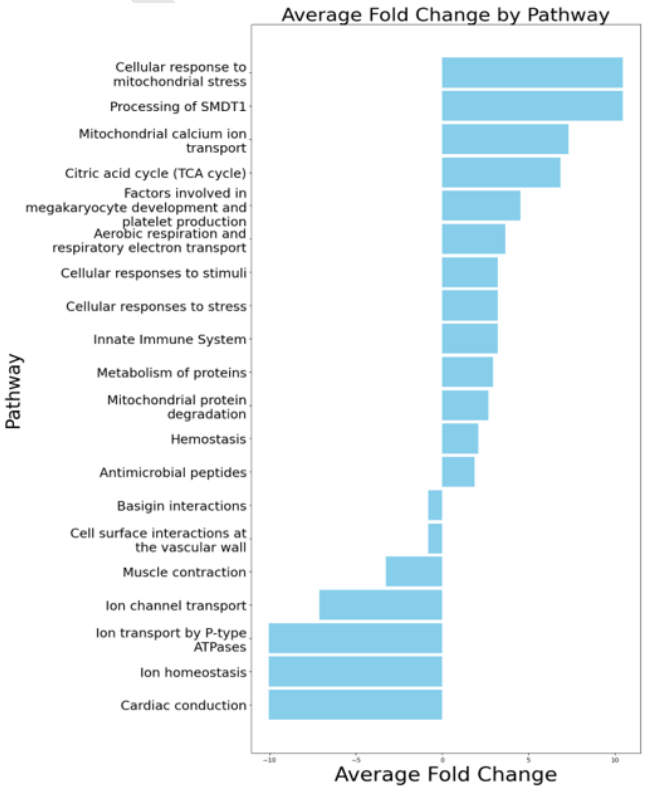

Fig. 3. Average fold change of significantly enriched biological pathways following the fatigue protocol. Bars represent the mean fold change across proteins assigned to each pathway, with positive values indicating upregulation and negative values indicating downregulation in the fatigued state relative to baseline. Enriched pathways reflect processes related to mitochondrial stress, energy metabolism, immune regulation, and cellular stress responses, highlighting coordinated pathway-level alterations associated with physical fatigue.

tion (35, 36)(37,38). STOML2, an inner mitochondrial membrane protein that organizes respiratory supercomplexes, reflects mitochondrial efficiency and the capacity to sustain ATP production under high metabolic demand; its presence in saliva may indicate exertion-induced mitochondrial stress or remodeling not captured by systemic biomarkers (37, 38). PGLYRP2, an innate immune protein involved in bacterial peptidoglycan recognition, reflects shifts in mucosal and systemic immune activity that can accompany acute physiological stress; its association with fatigue in this context suggests a role for rapid immune modulation during strenuous exertion (39).

The four novel proteins identified in this study as predictors of physical fatigue mapped to pathways consistent with prior work in military and civilian populations (12–14), emphasizing protein folding, metabolic stress, and immune regulation as central pathways in fatigue development. Specifically, pathway enrichment highlighted processes related to immune signaling, mitochondrial function, and cellular stress responses, which have previously been shown to differentiate fatigued from non-fatigued states. For example, Xu et al. (12) reported that immune signaling and cytokine-related pathways were strong indicators of fatigue, while Michael et al. (13) found that salivary biomarkers linked to energy metabolism and protein degradation predicted performance declines during strenuous training. Additional studies show that protein chaperones and metabolic genes increase in response to cellular stress, supporting recovery and adaptation (12–14). Furthermore, clusters of proteins involved in protein folding and metabolic stress demonstrated strong associations with fatigue onset (AUC > 0.8) during military training (14), and a 30-protein panel reflecting immune regulation, metabolic activity, and stress responses achieved 95.7% diagnostic accuracy for EEG-defined fatigue in emergency physicians (12). Alignment between the present pathway analysis and prior findings indicate that physical fatigue arises from coordinated changes in mitochondrial function, immune signaling, metabolic dynamics, and cellular stress responses, providing a comprehensive framework for understanding fatigue in occupational contexts.

The protein model's superior performance likely stems from its focus on low-abundance biomarkers enriched using nanoparticle capture. The *a priori* focus on low-abundance biomarkers helps to enrich biochemical targets that are likely modulated by specific cellular processes, rather than generalized markers of overall organ or system function. However, these biomarkers may still reflect localized dynamic changes within tissues or cells that contribute to the organ system's response to fatigue stress. In contrast, hormonal markers such as cortisol and testosterone are significantly influenced by extraneous factors, including psychological stress, diurnal rhythms, and individual variability in HPA axis responsiveness. Similarly, inflammatory markers like IL-6 can be elevated due to unrelated stressors such as infections or injuries. These external influences reduce their specificity and reliability as fatigue indicators. Salivary proteins offer enhanced temporal stability, capturing both acute and sustained responses to physical exertion, unlike the delayed or fluctuating kinetics of hormonal and inflammatory markers. Proteomic biomarkers also provide a broader biological context by integrating immune regulation, metabolic shifts, and cellular stress adaptations, enabling a more comprehensive understanding of fatigue. Furthermore, salivary proteins exhibit lower inter-individual variability, as they reflect localized and specific responses to exertion, ensuring greater consistency across individuals under similar conditions.

Strengths, Limitations, and Implications for Future Research

This study demonstrated the utility of nanoparticle-based biomarker enrichment combined with untargeted high-resolution LC-MS to explore the low-abundance salivary proteome. This innovative approach enabled comprehensive, unbiased identification of proteins associated with physical fatigue. Direct comparison between the proteomic and targeted small molecule models provided critical insights into their relative utility, highlighting the superior sensitivity and specificity of the protein-based approach. The fatigue protocol reliably induced measurable physiological fatigue, as confirmed by reductions in performance and changes in HR and HRV.

Despite these strengths, the study had limitations. While machine learning approaches such as SVM can identify promising biomarkers, the limited sample size and exploratory nature of this study introduce a high risk of overfitting and inflated effect estimates. Leave-one-out cross-validation (LOOCV) was employed to reduce bias in model performance estimates; however, these results are hypothesis-generating and must be validated in larger, independent cohorts before any clinical or operational application. The short protocol focused on acute fatigue and did not capture the chronic fatigue commonly experienced during extended training or deployment. Chronic fatigue arises from different mechanisms such as persistent inflammation (40), mitochondrial dysfunction (41)(43), and hormonal dysregulation (42) which were not evaluated. Future studies should incorporate multi-day or cumulative workload designs to better reflect operational demands (43). Larger and more diverse cohorts are essential for validation. Longitudinal assessments would help track biomarker changes over time, particularly in chronic fatigue and sustained stress contexts (4). Expanding to multi-omics approaches, including metabolomics, genomics, and transcriptomics, could provide a more integrated view of fatigue biology. Combining these with wearable monitoring (45), and considering sex-specific responses and individual variability, would improve model precision. Ultimately, translating these findings into targeted interventions could support effective fatigue management in high-demand environments.

Conclusions

This study demonstrated that salivary proteins identified through untargeted proteomics outperformed known biomarkers in classifying physical fatigue, and were related

DRAFT

to pathways involving mitochondrial regulation, immune responses, and metabolic adaptation. These findings support the use of proteomic approaches for monitoring physical readiness in demanding environments. Future research should validate these results in larger, more diverse cohorts, incorporate longitudinal designs to capture chronic fatigue, and integrate multi-omics and wearable technologies. Such advances could enable real-time fatigue monitoring and inform targeted interventions to improve performance and resilience.

Practical Applications

Findings from this exploratory study suggest that salivary proteomic profiling may offer a practical and potentially more discriminative method for monitoring physical fatigue than traditional stress-related biomarkers in occupational and tactical settings. This approach could inform training load management, fatigue risk mitigation, and operational readiness decisions.

Bibliography

1. Bradley C Nindl, Daniel C Billing, Jace R Drain, Meaghan E Beckner, Julie Greeves, Herbert Groeller, Hilde K Teien, Samuele Marcora, Anthony Moffitt, and Tara Reilly. Perspectives on resilience for military readiness and preparedness: report of an international military physiology roundtable. 21(11):1116–1124. ISSN 1440-2440.
2. G James Herrera. The fundamentals of military readiness.
3. Ross O Phillips. A review of definitions of fatigue–and a step towards a whole definition. 29: 48–56. ISSN 1369-8478.
4. Kristen J Koltun, Matthew B Bird, Jennifer N Forse, and Bradley C Nindl. Physiological biomarker monitoring during arduous military training: Maintaining readiness and performance. 26:S64–S70. ISSN 1440-2440.
5. Heikki Kyröläinen, Jari Karinkanta, Matti Santtila, Harri Koski, Matti Mäntysaari, and Teemu Pullinen. Hormonal responses during a prolonged military field exercise with variable exercise intensity. 102(5):539–546. ISSN 1439-6319.
6. Andrew J Steckl and Prajokta Ray. Stress biomarkers in biological fluids and their point-of-use detection. 3(10):2025–2044. ISSN 2379-3694.
7. Jamie L Tait, Jace R Drain, Sean L Corrigan, Jeremy M Drake, and Luana C Main. Impact of military training stress on hormone response and recovery. 17(3):e0265121. ISSN 1932-6203.
8. Panagiota Klentrou, Thomas Cieslak, Melanie MacNeil, Angela Vintinner, and Michael Plyley. Effect of moderate exercise on salivary immunoglobulin a and infection risk in humans. 87(2):153–158. ISSN 1439-6319.
9. Linda Becker, Lucas Semmlinger, and Nicolas Rohleder. Resistance training as an acute stressor in healthy young men: associations with heart rate variability, alpha-amylase, and cortisol levels. 24(3):318–330. ISSN 1025-3890.
10. James P McClung, Svein Martini, Nancy E Murphy, Scott J Montain, Lee M Margolis, Ingjerd Thrane, Marissa G Spitz, Janet-Martha Blatny, Andrew J Young, and Yngvar Gundersen. Effects of a 7-day military training exercise on inflammatory biomarkers, serum hepcidin, and iron status. 12(1):141. ISSN 1475-2891.
11. Josef Finsterer. Biomarkers of peripheral muscle fatigue during exercise. 13(1):218. ISSN 1471-2474.
12. Y-L Xu, C-X ZHAO, A-P XI, M Ding, Y Li, H Jianjun, X-H SU, F-L LIU, J-Z WANG, and Z-J LIU. Discovery and identification of fatigue-related biomarkers in human saliva. 22(23). ISSN 1128-3602.
13. Darren J Michael, Sheena Daugherty, Adrienne Santos, Brent C Ruby, and John E Kalns. Fatigue biomarker index: An objective salivary measure of fatigue level. 45:68–73. ISSN 0001-4575.
14. Justin McKetney, Conor C Jenkins, Catie Minogue, Phillip M Mach, Erika K Hussey, Trevor G Glaros, Joshua Coon, and Elizabeth S Dhummakupt. Proteomic and metabolomic profiling of acute and chronic stress events associated with military exercises. 18(4):279–295.
15. Shinsuke Tamai, Hiroaki Hiraoka, Kazuhiro Shimizu, Keisuke Miyake, Daisuke Hoshi, Kai Aoki, Koki Yanazawa, Takehito Sugasawa, Kazuhiro Takekoshi, and Koichi Watanabe. Variabilities of salivary human herpesvirus 6/7 and cortisol levels during a three-day training camp in judo athletes. 11(1):43–49. ISSN 2186-8131.
16. Claudia Fredolini, Francesco Meani, Alessandra Luchini, Weidong Zhou, Paul Russo, Mark Ross, Alexis Patanarut, Davide Tamburro, Guido Gambara, and David Ornstein. Investigation of the ovarian and prostate cancer peptidome for candidate early detection markers using a novel nanoparticle biomarker capture technology. 12(4):504–518. ISSN 1550-7416.
17. Elisa Pin, Emanuel F Petricoin, Nelson Cortes, Thomas G Bowman, Eni Andersson, Mathias Uhlén, Peter Nilsson, and Shane V Caswell. Immunoglobulin a autoreactivity toward brain enriched and apoptosis-regulating proteins in saliva of athletes after acute concussion and subconcussive impacts. 38(17):2373–2383. ISSN 0897-7151.
18. Karen Rothfels, Marija Milacic, Lisa Matthews, Robin Haw, Cristoffer Sevilla, Marc Gillespie, Ralf Stephan, Chuqiao Gong, Eliot Ragueneau, Bruce May, Veronica Shamovsky, Adam Wright, Joel Weiser, Deidre Beavers, Patrick Conley, Krishna Tiwari, Bijay Jassal, Johannes Griss, Andrea Senff-Ribeiro, Timothy Brunson, Robert Petryszak, Henning Hermjakob, Peter D'Eustachio, Guanming Wu, and Lincoln Stein. Using the reactome database. 3(4):e722. ISSN 2691-1299. doi: 10.1002/cpz1.722. Place: United States.
19. Adi Laurentiu Tarca, Sorin Draghici, Gaurav Bhatti, and Roberto Romero. Down-weighting overlapping genes improves gene set analysis. 13:136. ISSN 1471-2105. doi: 10.1186/1471-2105-13-136. Place: England.
20. Brian Heilbronn, Kenji Doma, Wade Sinclair, Jonathan Connor, Lachlan Irvine-Brown, and Anthony Leicht. Acute fatigue responses to occupational training in military personnel: A systematic review and meta-analysis. 188(5):969–977. ISSN 1930-613X 0026-4075. doi: 10.1093/milmed/usac144. Place: England.
21. K. E. Friedl, R. J. Moore, R. W. Hoyt, L. J. Marchitelli, L. E. Martinez-Lopez, and E. W. Askew. Endocrine markers of semistarvation in healthy lean men in a multistressor environment. 88(5):1820–1830. ISSN 8750-7587 0161-7567. doi: 10.1152/jappl.2000.88.5.1820. Place: United States.
22. Meaghan E. Beckner, Luana Main, Jamie L. Tait, Brian J. Martin, William R. Conkright, and Bradley C. Nindl. Circulating biomarkers associated with performance and resilience during military operational stress. 22(1):72–86. ISSN 1536-7290. doi: 10.1080/17461391.2021.1962983. Place: Germany.
23. Brian J. Martin, Martin Wright, Varun Patel, Adam Susmarski, Mita Lovalekar, Jennifer N. Forse, Meaghan E. Beckner, Andrew K. Ledford, and Bradley C. Nindl. Physiological, physical, and psychological determinants of success during the naval special warfare screener selection course. 39(2):e162–e170. ISSN 1533-4287 1064-8011. doi: 10.1519/JSC.0000000000004979. Place: United States.
24. Andrew K. Ledford, Deirdre Dixon, Celeste Raver Luning, Brian J. Martin, Patti C. Miles, Meaghan Beckner, Drew Bennett, John Conley, and Bradley C. Nindl. Psychological and physiological predictors of resilience in navy SEAL training. 46(3):290–301. ISSN 1940-4026 0896-4289. doi: 10.1080/08964289.2020.1712648. Place: United States.
25. L. Weibel, S. Maccari, and O. Van Reeth. Circadian clock functioning is linked to acute stress reactivity in rats. 17(5):438–446. ISSN 0748-7304. doi: 10.1177/074873002237138. Place: United States.
26. Stefan Wüst, Elisabeth F. C. Van Rossum, Ilona S. Federenko, Jan W. Koper, Robert Kumsta, and Dirk H. Hellhammer. Common polymorphisms in the glucocorticoid receptor gene are associated with adrenocortical responses to psychosocial stress. 89(2):565–573. ISSN 0021-972X. doi: 10.1210/jc.2003-031148. Place: United States.
27. J. L. Santos, E. Saus, S. V. Smalley, L. R. Cataldo, G. Alberti, J. Parada, M. Gratacòs, and X. Estivill. Copy number polymorphism of the salivary amylase gene: implications in human nutrition research. 5(3):117–131. ISSN 1661-6758 1661-6499. doi: 10.1159/000339951. Place: Switzerland.
28. Deirdre A. Katz and Melissa K. Peckins. Cortisol and salivary alpha-amylase trajectories following a group social-evaluative stressor with adolescents. 86:8–16. ISSN 1873-3360 0306-4530. doi: 10.1016/j.psyneuen.2017.08.021. Place: England.
29. U. M. Nater and N. Rohleder. Salivary alpha-amylase as a non-invasive biomarker for the sympathetic nervous system: current state of research. 34(4):486–496. ISSN 0306-4530. doi: 10.1016/j.psyneuen.2009.01.014. Place: England.
30. Mitchell E. Zaplatosch and William M. Adams. The effect of acute hypohydration on indicators of glycemic regulation, appetite, metabolism and stress: A systematic review and meta-analysis. 12(9). ISSN 2072-6643. doi: 10.3390/nu12092526. Place: Switzerland.
31. Jana Strahler, Nadine Skoluda, Mattes B. Kappert, and Urs M. Nater. Simultaneous measurement of salivary cortisol and alpha-amylase: Application and recommendations. 83: 657–677. ISSN 1873-7528 0149-7634. doi: 10.1016/j.neubiorev.2017.08.015. Place: United States.
32. Akefeh Ahmadiafshar, Mahmood Reza Mohsenifard, and Saeideh Mazloomzadeh. Evaluation of serum & salivary IgA in patients with type 1 diabetes. 10(4):e0122757. ISSN 1932-6203. doi: 10.1371/journal.pone.0122757. Place: United States.
33. Yuichiro Nishida, Minako Iyadomi, Yasuki Higaki, Hiroaki Tanaka, Megumi Hara, and Keitaro Tanaka. Influence of physical activity intensity and aerobic fitness on the anthropometric index and serum uric acid concentration in people with obesity. 50(19):2121–2128. ISSN 1349-7235 0918-2918. doi: 10.2169/internalmedicine.50.5506. Place: Japan.
34. Torben Clausen. Na+-k+ pump regulation and skeletal muscle contractility. 83(4):1269–1324. ISSN 0031-9333. doi: 10.1152/physrev.00011.2003. Place: United States.
35. Francisco Manuel Lemos Amado, Rui Miguel Pinheiro Vitorino, Pedro Miguel Dimas Neves Domingues, Maria João Calheiros Lobo, and José Alberto Ramos Duarte. Analysis of the human saliva proteome. 2(4):521–539. ISSN 1744-8387 1478-9450. doi: 10.1586/14789450.2.4.521. Place: England.
36. Weihong Yan, Rolf Apweiler, Brian M. Balgley, Pinmanee Boontheung, Jonathan L. Bundy, Benjamin J. Cargile, Steve Cole, Xueping Fang, Mireya Gonzalez-Begne, Timothy J. Griffin, Fred Hagen, Shen Hu, Lawrence E. Wolinsky, Cheng S. Lee, Daniel Malamud, James E. Melvin, Rajasree Menon, Michael Mueller, Renli Qiao, Nelson L. Rhodus, Joel R. Sevinsky, David States, James L. Stephenson, Shawn Than, John R. Yates, Weixia Yu, Hongwei Xie, Yongming Xie, Gilbert S. Omenn, Joseph A. Loo, and David T. Wong. Systematic comparison of the human saliva and plasma proteomes. 3(1):116–134. ISSN 1862-8354 1862-8346. doi: 10.1002/prca.200800140. Place: Germany.
37. Darah A. Christie, Caitlin D. Lemke, Isaac M. Elias, Luan A. Chau, Mark G. Kirchhof, Bo Li, Eric H. Ball, Stanley D. Dunn, Grant M. Hatch, and Joaquín Madrenas. Stomatin-like protein 2 binds cardiolipin and regulates mitochondrial biogenesis and function. 31(18):3845–3856, . ISSN 1098-5549 0270-7306. doi: 10.1128/MCB.05393-11. Place: United States.
38. Darah A. Christie, Panagiotis Mitsopoulos, Julianna Blagih, Stanley D. Dunn, Julie St-Pierre, Russell G. Jones, Grant M. Hatch, and Joaquín Madrenas. Stomatin-like protein 2 deficiency in t cells is associated with altered mitochondrial respiration and defective CD4+ t cell responses. 189(9):4349–4360, . ISSN 1550-6606 0022-1767. doi: 10.4049/jimmunol.1103829. Place: England.
39. Roman Dziarski and Dipika Gupta. The peptidoglycan recognition proteins (PGRPs). 7 (8):232. ISSN 1474-760X 1465-6906 1474-7596. doi: 10.1186/gb-2006-7-8-232. Place: England.
40. Katrine B Norheim, Grete Jonsson, and Roald Omdal. Biological mechanisms of chronic

DRAFT

fatigue. *Rheumatology*, 50(6):1009–1018, 2011.

41. Kristin Filler, Debra Lyon, James Bennett, Nancy McCain, Ronald Elswick, Nada Lukkahatai, and Leorey N. Saligan. Association of mitochondrial dysfunction and fatigue: A review of the literature. 1:12–23. ISSN 2214-6474. doi: 10.1016/j.bbacli.2014.04.001. Place: Netherlands.
42. Andrew S. Papadopoulos and Anthony J. Cleare. Hypothalamic-pituitary-adrenal axis dysfunction in chronic fatigue syndrome. 8(1):22–32. ISSN 1759-5037 1759-5029. doi: 10.1038/nrendo.2011.153. Place: England.
43. Paul C. Henning, Bong-Sup Park, and Jeong-Su Kim. Physiological decrements during sustained military operational stress. 176(9):991–997. ISSN 0026-4075. doi: 10.7205/milmed-d-11-00053. Place: England.

DRAFT