Preliminary insights into salivary proteomic versus targeted biomarker profiles associated with acute physical fatigue

Bryndan Lindsey[1], Kenneth Bowden[1], Yosef Shaul[2], Emanuel Petricoin[3], Shane V. Caswell[2], Rayan Alhammad[3,4], Anissa N. Elayadi[1], Joel R Martin[2*]

[1] Research and Exploratory Development Department, Johns Hopkins Applied Physics Laboratory, Laurel, MD

[2] Sports Medicine Assessment Research & Testing (SMART) Laboratory; George Mason University, Virginia, USA

[3] Center for Applied Proteomics and Molecular Medicine, School of Systems Biology, George Mason University, Manassas, Virginia

[4] Clinical Laboratory Department, College of Applied Medical Sciences, King Saud bin Abdulaziz University for Health Sciences, Al Ahsa, Saudi Arabia

*Corresponding author

Email: jmarti@gmu.edu

Abstract:

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 in classifying 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.

1. Introduction:

Operational readiness, the ability of military forces to fight and meet the demands of the assigned missions, is shaped by various factors such as dehydration, lack of sleep, caloric deficits, and physical fatigue amongst others (1). Among these, physical fatigue plays a critical role, particularly in demanding operational environments (2). Defined as a measurable decline in muscular performance or increased perceived exertion, (3), physical fatigue can impair function, elevate injury risk, and undermine mission success (4). Following sustained activity, readiness is often diminished, yet rapidly quantifying this decline remains a challenge.

Traditional approaches to assess physical readiness include body composition, imaging, movement screens, and wearable physiological monitors (4). Body composition, bone imaging and movement screens provide measures of the general health and the potential injury risk of the warfighter. However, assessments require cumbersome equipment and a time and expertise burden. Additionally, there is still mixed evidence as to the validity of some of these measures to predict injury (5). Importantly, while these measures offer relatively stable indicators of readiness over short periods (e.g., days, weeks), physical readiness itself can fluctuate rapidly in response to operational demands, even in warfighters with elite physical attributes. As such, physiological monitors have been used to measure internal loading through signals like heart rate (HR), heart rate variability (HRV), and oxygen saturation (SpO2), while accelerometers and inertial measurement units have been used to measure external load (4). While these technologies are useful for providing continuous data during specific windows, their functionality is limited by battery life and device internal storage.

Physical exertion-induced changes in circulating levels of chemical compounds in the body have also shown potential as markers of excessive training stress and delayed recovery (6). Prolonged physical exertion triggers changes in the autonomic nervous system (ANS), modulating the activation of hormones like cortisol and testosterone amongst other compounds (7). Biochemical compounds like cortisol and testosterone (8), immunoglobin-A (IgA) (9), alpha-amylase (AA) (10), interleukin-6 (IL-6) (11), and uric acid (UA) (12) have been shown to significantly alter as a result of military related stressors, and therefore, have been proposed as potential biomarkers of physical readiness. Saliva offers a non-invasive, easily collected medium for assessing biochemical markers of physiological state.

Unlike blood or urine, saliva sampling can be conducted in field environments without the need for specialized equipment or personnel. Additionally, salivary biomarkers have been shown to reflect systemic physiological changes, including hormonal and immune responses, making them a viable alternative for assessing readiness and fatigue in military operational settings. Recently, untargeted proteomic approaches have been used to identify panels of salivary proteins related to immunoregulation, inflammation, and metabolism that accurately predict fatigue status in both civilian (13,14) and military settings (15). These proteins have been hypothesized to be more specific markers of physical fatigue as opposed to hormones like cortisol and testosterone which respond to potentially confounding factors like diurnal rhythm and psychological stressors (16). However, the sensitivity and specificity of identified proteomic markers compared to previously studied small molecules (i.e., cortisol, testosterone, IgA, IL-6, UA, AA) to predict physical fatigue have yet to be evaluated within the same cohort.

Therefore, this study aimed to compare the predictive performance of salivary proteins identified via untargeted liquid chromatography-mass spectrometry (LC-MS) with that of targeted stress-

related small molecules in classifying physical fatigue. We hypothesized that the protein panel would demonstrate superior sensitivity and specificity in detecting fatigue status following a controlled fatiguing protocol in healthy individuals.

2. Materials and 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 ≥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) in accordance with the ethical standards set by the Helsinki Declaration.

Because this study was designed as an exploratory biomarker discovery effort, no a priori power analysis was conducted for the proteomic outcomes. The primary goal was to identify candidate biomarkers of acute physical fatigue for future validation. Performance-based metrics were used to confirm induction of a fatigued state and provide reference effect sizes to inform sample size calculations in subsequent studies.

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.

Insert Fig 1 Here

Fig 1. Schematic timeline of the 3-day protocol including saliva sample collection (1–7), performance assessments, and fatiguing exercise. The fatigue protocol on Day 2 consisted of a 60-minute circuit involving weighted running, sprints, carries, and power throws. Saliva was collected pre- and post-protocol as well as one-hour post-exercise in addition to baseline and recovery days.

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 (17), 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 x 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.

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 (18,19). 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 ($p < 0.05$).

Targeted Small Molecules

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. 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. Model 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.

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 = 149) were selected for further analysis (Figure 2).

Insert Fig 2 Here

Fig 2. Volcano plot of Salivary Protein Significance in Response to Fatigue and Time. The x-axis represents -log10(TimeP-value), and the y-axis represents -log10(FatigueP-value). Each point represents an individual protein with colors indicating statistical significance categories. Dashed lines indicate the significance thresholds used for classification. Proteins in the upper left quadrant were differentially abundant in response to fatigue only, and were further analyzed for predictive utility.

Recursive Feature Elimination (RFE) within cross-validation identified a stable subset of proteins (n = 4) consistently selected across ≥4 of 5 folds. An SVM model was trained using these proteins and evaluated using LOOCV. Performance metrics included sensitivity, specificity, F1 score, Matthews Correlation Coefficient (MCC), and area under the ROC curve (AUC). Protein names, gene IDs, functions, and model importance are shown in Table 1. Pathway analysis was performed on the identified proteins using the Reactome Analyze Gene List tool,(20) followed by the Analyze Gene Expression tool with the PADOG method,(21) which down-weights genes commonly found across multiple pathways. The resulting pathway lists were manually cross-referenced with the protein accession numbers identified by the SVM model to link the top predictors of fatigue status to specific biological pathways.

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

Protein	Gene ID	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-malae 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 the mitochondrial function, may play a role in several biological processes including cell migration, cell proliferation, T-cell activation, calcium homeostasis and cellular response to stress. It is required for mitochondrial hyperfusion, a pro-survival cellular response to stress which results in ATP production.
N-acetylmuramoyl	PGLYRP2	0.32	Hydrolyses the link between N-acetylmuramoyl residues and L-amino acid residues in certain cell-wall glycopeptides.

3. Results

Change in Performance Parameters

Mean and standard deviation of performance measures before and after the fatigue protocol are presented in Table 2. Average heart rate increased, while HRV decreased significantly post-fatigue. Step test recovery HR, anaerobic capacity, maximum pushups, and plank time also declined significantly. No significant changes were observed for vertical leap height or peak force during the mid-thigh pull.

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

Performance Parameter	Pre-Fatigue Mean (SD)	Post-Fatigue Mean (SD)	Effect Size	*t-value*	*p-value*
Heart Rate (bpm)	67.2 (6.47)	136.4 (21.4)	2.56	-7.47	<0.01
Heart Rate Variability (ms)	86.5 (33.1)	21.1 (10.8)	1.44	4.20	<0.01
Recovery Heart Rate (bpm)	128.7 (23.7)	160.3 (18.46)	1.49	-3.33	<0.01
Max Vertical Leap (cm)	37.0 (9.6)	37.5 (10.0)	-0.15	-0.52	0.62
IMTP Peak Vertical Force (N)	2334.7 (1056.7)	2223.2 (868.2)	0.35	1.21	0.26
Max HRPU (#)	38.2 (6.6)	32.1 (6.6)	1.40	4.84	<0.01
Max Plank (s)	150.0 (48.9)	92.9 (24.6)	1.71	5.93	<0.01
Anaerobic Capacity (W)	448.0 (133.9)	389.7 (125.3)	1.24	4.29	<0.01

Predictive Accuracy of Targeted Molecules vs Discovered Proteins

Figure 3 shows individual and combined ROC curves for both 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.

Insert Fig3 Here

Figure 3. Receiver Operating Characteristic (ROC) Curves for Predicting Physical Fatigue Using Targeted Small Molecules and Proteomic Biomarkers. Values in parentheses represents discriminatory performance in the classification model. A) ROC curves for targeted small molecules, 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).

Confusion matrices (Table 3) 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).

Table 3. Confusion Matrices for Classification of Fatigue Status Using Targeted Small Molecules and Discovered Proteins.

	Predicted Fatigue Status	

			Targeted Molecules		Discovered Proteins		
			Rested	Fatigued	Rested	Fatigued	Total
Observed	Count	Rested	29	1	30	0	30
		Fatigued	4	6	2	8	10
	%	Rested	97.7	3.3	100.0	0.0	100.0
		Fatigued	40.0	60.0	20.0	80.0	100.0

Pathway Analysis

Twenty biological pathways were associated with the four proteins identified by the SVM model (Figure 4). Thirteen pathways were upregulated, including “Processing of SMDT1” and “Cellular response to mitochondrial stress,” both linked to STOML2 (p-value = 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-value = 0.015). These pathways play roles in immune cell trafficking, vascular integrity, and hemostasis.

Insert Fig4 Here

4. Discussion:

This study compared the predictive performance of two models for classifying physical fatigue: one based on traditional stress-related small molecules and the other on salivary proteins

identified through untargeted, nanoparticle-enriched proteomics. Fatigue was defined as a categorical state of reduced physical performance, which was achieved as evidenced by significant declines in aerobic and anaerobic capacity, muscular endurance, and changes in HR and HRV pre- to post-protocol. Additionally, these responses aligned with prior findings on acute fatigue in military training contexts,(22) Circulating biomarkers offer a means to monitor physiological stress, with prior studies linking hormonal and enzymatic shifts to performance outcomes in tactical and athletic settings.(23,24) In this study, both models showed strong overall classification accuracy, but the protein-based model outperformed the small molecule model by 9%, driven by higher sensitivity (true positive rate).

Although Cortisol and dehydroepiandrosterone (a precursor to testosterone) have shown promise as surrogate markers for stress resilience during military training ,(25,26) both showed poor predictive utility in this cohort (AUCs: 0.07 and 0.43). These results may be attributed to the large interindividual variability in both baseline cortisol and testosterone and their responses observed pre- to post-fatigue. Previous research has consistently highlighted significant variability in the secretion of these hormones during physical stress, influenced by factors such as hypothalamic-pituitary-adrenal (HPA) axis responsiveness,(27) genetic predispositions,(28) and stress anticipation.(29) Additionally, external factors like diurnal rhythm further contribute to the inconsistency of hormonal responses, potentially reducing their reliability as fatigue biomarkers.(16)

Similarly, AA and IL-6, linked to sympathetic activity and inflammation, respectively,(10,11) were also weak predictors in our sample (AUCs: 0.30 and 0.47). Like cortisol and testosterone, AA and Il-6 are subject to high inter-individual variability influenced by factors such as genetics,(30) diurnal rhythms,(31) hydration,(32) and baseline stress levels.(33) Furthermore,

although we attempted to control for factors like hydration and food intake in our study design, it is possible that variations in nutritional timing may have affected AA response. In contrast, IgA and UA exhibited stronger individual performance. IgA reflects mucosal immune function and is rapidly secreted in response to sympathetic activation.(34) while UA is a robust marker of oxidative stress and metabolic turnover during exertion.(35) These roles in localized immune defense and systemic antioxidant activity may inherently reduce inter-individual variability, making IgA and UA reliable indicators of acute physical fatigue.

The complete panel of identified proteins achieved greater performance compared to the panel of targeted small molecules (95% to 85% overall accuracy). Proteomics captures a broader physiological picture than isolated hormonal or inflammatory markers. This includes integrated responses involving immune signaling, mitochondrial function, and metabolic adaptation. The four proteins identified in this study were linked to fatigue-relevant pathways, consistent with prior military and civilian studies identifying similar proteomic signatures.(13,15) For instance, specific clusters of biomarkers related to protein folding and metabolic stress demonstrated strong correlations with fatigue onset (AUC > 0.8) during military training.(15) Additionally, a panel of 30 proteins linked immune regulation, metabolic processes, and stress response achieved a diagnostic accuracy of 95.7% for slow-wave EEG-diagnosed fatigue in emergency room physicians.(13)

The pathways identified in this study as predictive of physical fatigue closely align with those highlighted in related research,(13–15) underscoring the interconnected roles of these biological processes. Notably, the pathways "Processing of SMDT1" and "Cellular response to mitochondrial stress" emphasize mitochondrial function, mirroring findings by McKetney et al.,(15) which identified protein folding and metabolic stress pathways as central to fatigue onset.

Both studies highlight mitochondrial adaptation in managing cellular energy demands and fostering stress resilience during prolonged exertion. Similarly, the immune and inflammatory pathways identified in the present study, including the downregulated "Cell surface interactions at the vascular wall" and "Basigin interactions," are consistent with Xu et al.,(13) which identified immune-related pathways and cytokine regulation as critical indicators of fatigue. These findings suggest a shared mechanism of immune activation and regulation in response to physical and environmental stressors.

Proteins associated with metabolic regulation emerged as key discriminators of fatigue states, with the 'Transporting of Small Molecules' pathway emphasizing the physiological need to prioritize energy balance during fatigue. This is consistent with the work of Michael et al.,(14) where salivary biomarkers linked to energy metabolism and protein degradation were predictive of fatigue. Furthermore, the inclusion of the "Cellular Response to Stimuli" pathway in the current findings supports broader research emphasizing stress-responsive pathways. For instance, studies have shown that protein chaperones and metabolic genes are upregulated to counteract cellular stress, facilitating recovery and adaptation.(13–15) Together, these findings suggest that physical fatigue results from a complex interplay of mitochondrial regulation, immune signaling, metabolic adaptation, and stress responses, offering a comprehensive framework for understanding the molecular mechanisms underpinning fatigue across diverse 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 for 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. Consequently, this protein archive may contain candidate biomarkers that are directly linked to fatigue-related pathways, such as mitochondrial function, protein folding, and metabolic regulation, providing greater specificity to the physiological mechanisms underlying physical fatigue.

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. Additionally, 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 markers 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,(36) mitochondrial dysfunction,(37) and hormonal dysregulation(38) which were not evaluated. Additionally, while the protocol was relevant to military tasks, it may not fully reflect real-world operational complexity.

Future studies should incorporate multi-day or cumulative workload designs to better reflect operational demands (39). 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 (39), 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 targeted small molecule biomarkers in classifying physical fatigue, with associated 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.

5. Acknowledgements:

N/A

6. References:

1. Nindl BC, Billing DC, Drain JR, Beckner ME, Greeves J, Groeller H, et al. Perspectives on resilience for military readiness and preparedness: Report of an international military physiology roundtable. Journal of science and medicine in sport / Sports Medicine Australia. 2018 Nov;21(11):1116–24.

2. Herrera GJ. The fundamentals of military readiness. 2020;

3. Phillips RO. A review of definitions of fatigue–And a step towards a whole definition. Transportation research part F: traffic psychology and behaviour. 2015;29:48–56.

4. Koltun KJ, Bird MB, Forse JN, Nindl BC. Physiological biomarker monitoring during arduous military training: Maintaining readiness and performance. Journal of Science and Medicine in Sport. 2023 Jun;26(S1):S64–70.

5. Rhon DI, Molloy JM, Monnier A, Hando BR, Newman PM. Much work remains to reach consensus on musculoskeletal injury risk in military service members: a systematic review with meta-analysis. European Journal of Sport Science. 2022;22(1):16–34.

6. Kyröläinen H, Karinkanta J, Santtila M, Koski H, Mäntysaari M, Pullinen T. Hormonal responses during a prolonged military field exercise with variable exercise intensity. European journal of applied physiology. 2008;102:539–46.

7. Steckl AJ, Ray P. Stress biomarkers in biological fluids and their point-of-use detection. ACS sensors. 2018;3(10):2025–44.

8. Tait JL, Drain JR, Corrigan SL, Drake JM, Main LC. Impact of military training stress on hormone response and recovery. Plos one. 2022;17(3):e0265121.

9. Klentrou P, Cieslak T, MacNeil M, Vintinner A, Plyley M. Effect of moderate exercise on salivary immunoglobulin A and infection risk in humans. European journal of applied physiology. 2002;87:153–8.

10. Becker L, Semmlinger L, Rohleder N. Resistance training as an acute stressor in healthy young men: associations with heart rate variability, alpha-amylase, and cortisol levels. Stress. 2021;24(3):318–30.

11. McClung JP, Martini S, Murphy NE, Montain SJ, Margolis LM, Thrane I, et al. Effects of a 7-day military training exercise on inflammatory biomarkers, serum hepcidin, and iron status. Nutrition journal. 2013;12:1–4.

12. Finsterer J. Biomarkers of peripheral muscle fatigue during exercise. BMC musculoskeletal disorders. 2012;13:1–13.

13. Xu Y, Gong Y, Xiao D, Zhao C, Gao X, Peng X, et al. Discovery and identification of fatigue-related biomarkers in human saliva. Eur Rev Med Pharmacol Sci. 2018;22(23):8519–36.

14. Michael DJ, Daugherty S, Santos A, Ruby BC, Kalns JE. Fatigue biomarker index: An objective salivary measure of fatigue level. Accident Analysis & Prevention. 2012;45:68–73.

15. McKetney J, Jenkins CC, Minogue C, Mach PM, Hussey EK, Glaros TG, et al. Proteomic and metabolomic profiling of acute and chronic stress events associated with military exercises. Mol Omics. 2022;18(4):279–95.

16. Tamai S, Hiraoka H, Shimizu K, Miyake K, Hoshi D, Aoki K, et al. Variabilities of salivary human herpesvirus 6/7 and cortisol levels during a three-day training camp in judo athletes. The Journal of Physical Fitness and Sports Medicine. 2022;11(1):43–9.

17. Narkhede P, Jaimala S, Amita M. Comparison of maximal oxygen consumption values estimated from six minute walk test and Queens College step test. Indian Journal of Physiotherapy and Occupational Therapy. 2014;8(1):154.

18. Fredolini C, Meani F, Luchini A, Zhou W, Russo P, Ross M, et al. Investigation of the ovarian and prostate cancer peptidome for candidate early detection markers using a novel nanoparticle biomarker capture technology. The AAPS journal. 2010;12:504–18.

19. Pin E, Petricoin E, Cortes N, Bowman T, Andersson E, Uhlen M, et al. IgA autoreactivity towards brain enriched and apoptosis regulating proteins in saliva of athletes after acute concussion and subconcussive impacts. Journal of neurotrauma. 2021;

20. Rothfels K, Milacic M, Matthews L, Haw R, Sevilla C, Gillespie M, et al. Using the Reactome database. Current protocols. 2023;3(4):e722.

21. Tarca AL, Draghici S, Bhatti G, Romero R. Down-weighting overlapping genes improves gene set analysis. BMC bioinformatics. 2012;13:1–14.

22. Heilbronn B, Doma K, Sinclair W, Connor J, Irvine-Brown L, Leicht A. Acute fatigue responses to occupational training in military personnel: A systematic review and meta-analysis. Military Medicine. 2023;188(5–6):969–77.

23. Friedl KE, Moore RJ, Hoyt RW, Marchitelli LJ, Martinez-Lopez LE, Askew EW. Endocrine markers of semistarvation in healthy lean men in a multistressor environment. Journal of Applied Physiology. 2000;

24. Beckner ME, Main L, Tait JL, Martin BJ, Conkright WR, Nindl BC. Circulating biomarkers associated with performance and resilience during military operational stress. European Journal of Sport Science. 2022 Jan 2;22(1):72–86.

25. Martin BJ, Wright M, Patel V, Susmarski A, Lovalekar M, Forse JN, et al. Physiological, Physical, and Psychological Determinants of Success During the Naval Special Warfare Screener Selection Course. The Journal of Strength & Conditioning Research. 2022;10–1519.

26. Ledford AK, Dixon D, Luning CR, Martin BJ, Miles PC, Beckner M, et al. Psychological and physiological predictors of resilience in Navy SEAL training. Behavioral Medicine. 2020;46(3–4):290–301.

27. Weibel L, Maccari S, Van Reeth O. Circadian clock functioning is linked to acute stress reactivity in rats. Journal of biological rhythms. 2002;17(5):438–46.

28. Wüst S, Van Rossum EF, Federenko IS, Koper JW, Kumsta R, Hellhammer DH. Common polymorphisms in the glucocorticoid receptor gene are associated with adrenocortical responses to psychosocial stress. The Journal of Clinical Endocrinology & Metabolism. 2004;89(2):565–73.

29. Katz DA, Peckins MK. Cortisol and salivary alpha-amylase trajectories following a group social-evaluative stressor with adolescents. Psychoneuroendocrinology. 2017;86:8–16.

30. Santos J, Saus E, Smalley S, Cataldo L, Alberti G, Parada J, et al. Copy number polymorphism of the salivary amylase gene: implications in human nutrition research. Journal of nutrigenetics and nutrigenomics. 2012;5(3):117–31.

31. Nater UM, Rohleder N. Salivary alpha-amylase as a non-invasive biomarker for the sympathetic nervous system: current state of research. Psychoneuroendocrinology. 2009;34(4):486–96.

32. Zaplatosch ME, Adams WM. The effect of acute hypohydration on indicators of glycemic regulation, appetite, metabolism and stress: a systematic review and meta-analysis. Nutrients. 2020;12(9):2526.

33. Strahler J, Skoluda N, Kappert MB, Nater UM. Simultaneous measurement of salivary cortisol and alpha-amylase: application and recommendations. Neuroscience & Biobehavioral Reviews. 2017;83:657–77.

34. Ahmadiafshar A, Mohsenifard MR, Mazloomzadeh S. Evaluation of serum & salivary IgA in patients with type 1 diabetes. Plos one. 2015;10(4):e0122757.

35. Nishida Y, Iyadomi M, Higaki Y, Tanaka H, Hara M, Tanaka K. Influence of physical activity intensity and aerobic fitness on the anthropometric index and serum uric acid concentration in people with obesity. Internal medicine. 2011;50(19):2121–8.

36. Norheim KB, Jonsson G, Omdal R. Biological mechanisms of chronic fatigue. Rheumatology. 2011;50(6):1009–18.

37. Filler K, Lyon D, Bennett J, McCain N, Elswick R, Lukkahatai N, et al. Association of mitochondrial dysfunction and fatigue: a review of the literature. BBA clinical. 2014;1:12–23.

38. Papadopoulos AS, Cleare AJ. Hypothalamic–pituitary–adrenal axis dysfunction in chronic fatigue syndrome. Nature Reviews Endocrinology. 2012;8(1):22–32.

39. Henning PC, Park BS, Kim JS. Physiological decrements during sustained military operational stress. Military medicine. 2011;176(9):991–7.

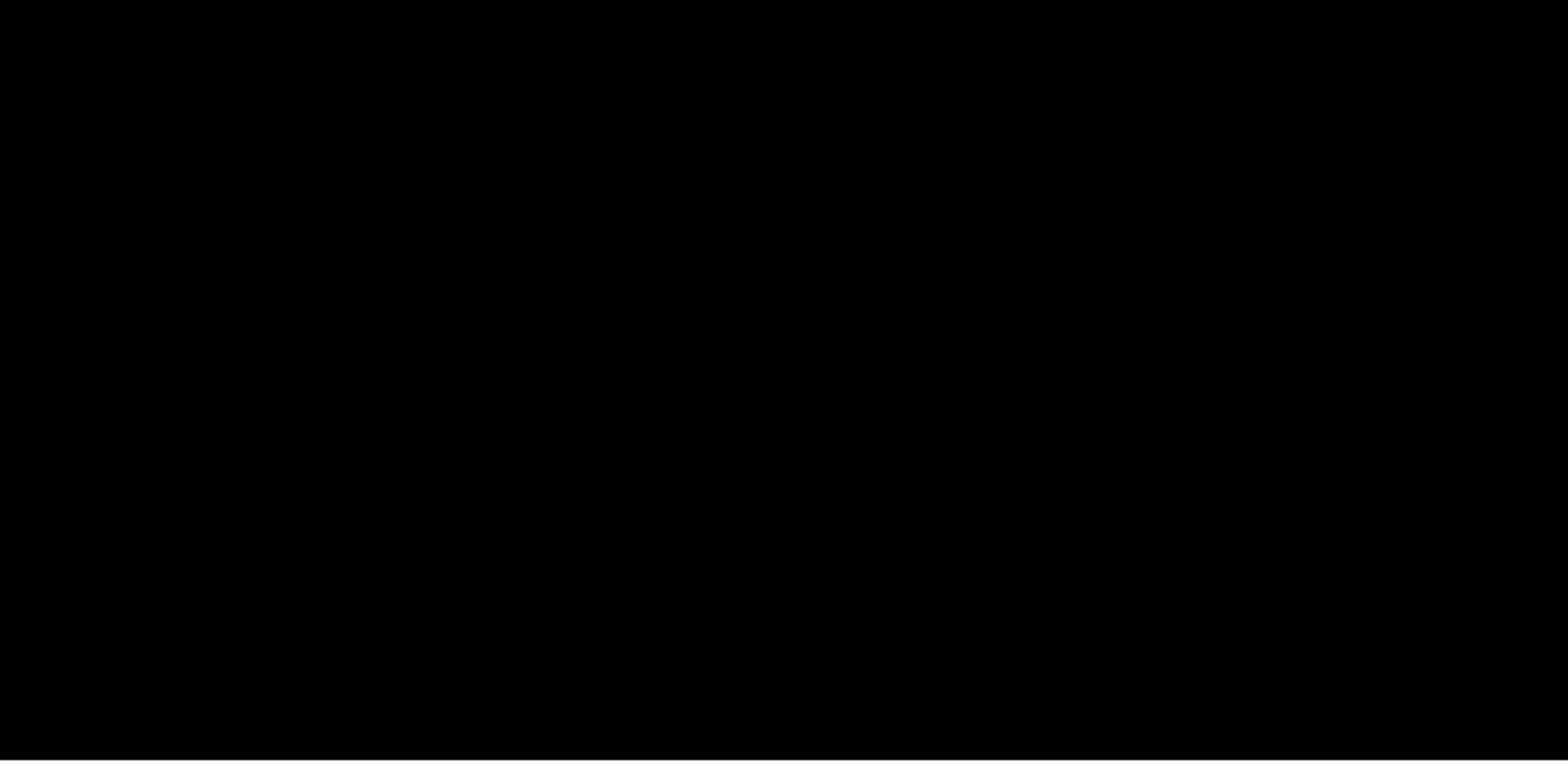


Figure 1

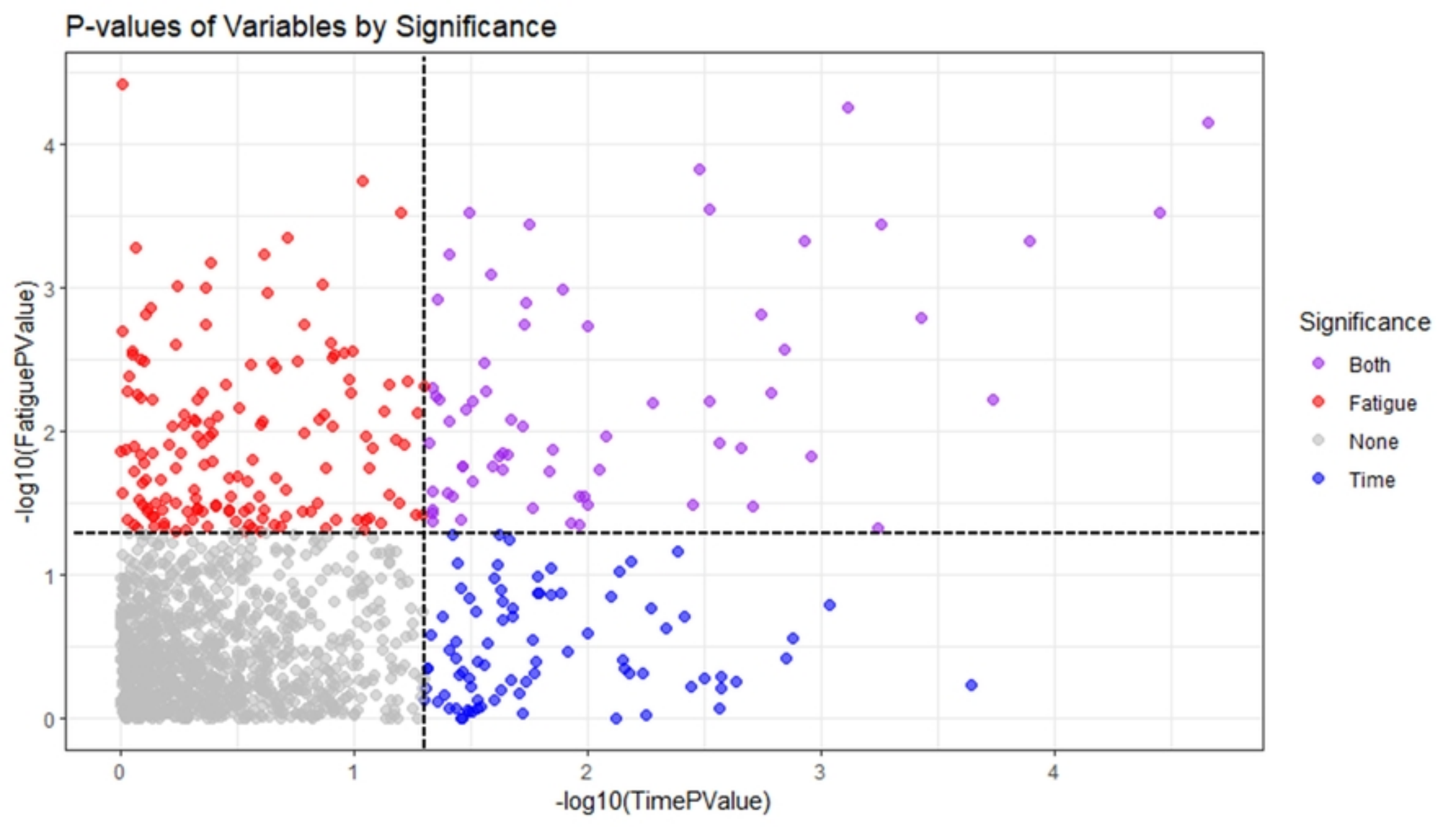
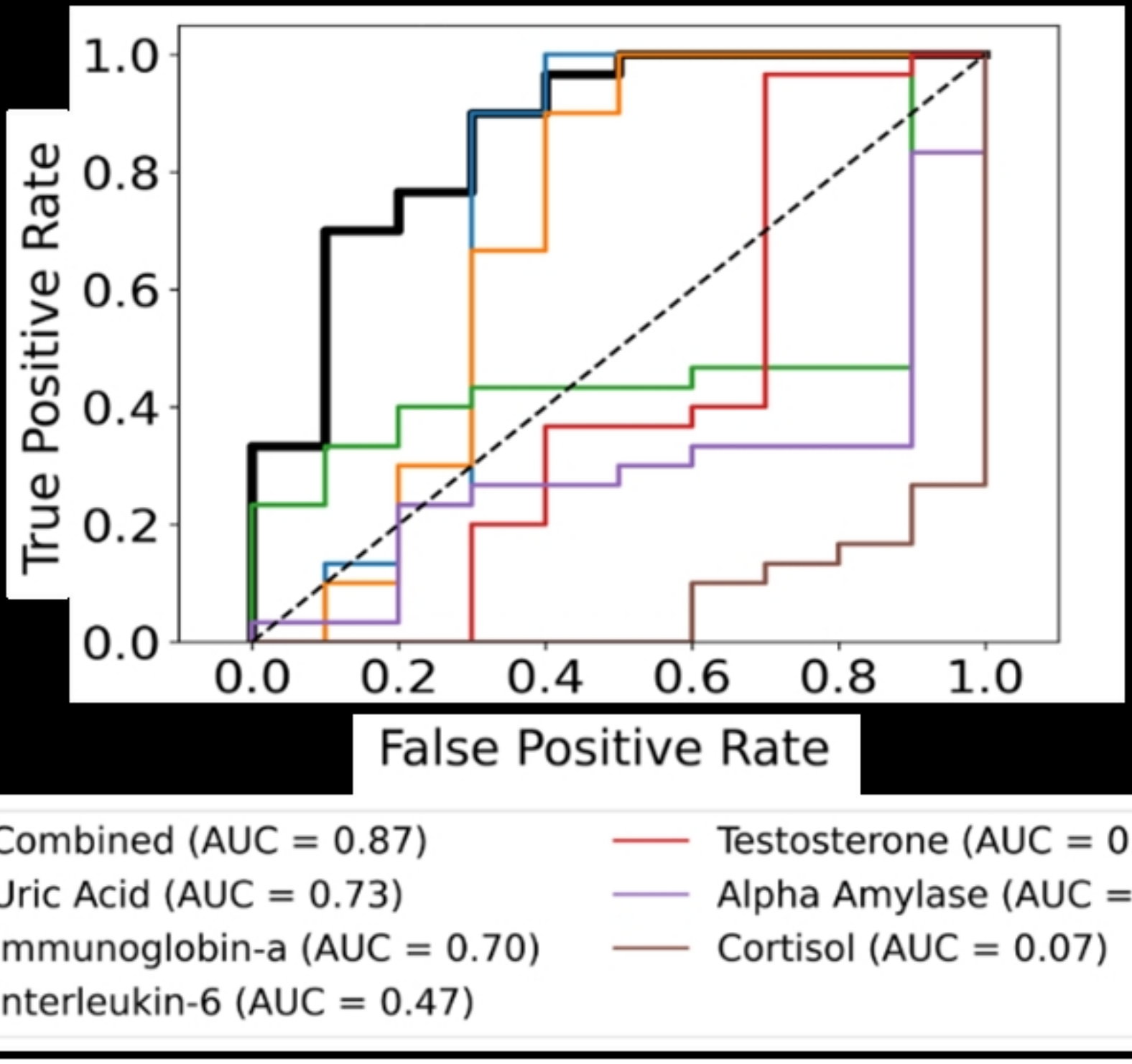

P-values of Variables by Significance
-log10(FatiguePValue)
-log10(TimePValue)
0
1
2
3
4
Significance
Both
Fatigue
None
Time

Figure 2


True Positive Rate
False Positive Rate
0.0
0.2
0.4
0.6
0.8
1.0
Combined (AUC = 0.87)
Uric Acid (AUC = 0.73)
Immunoglobin-a (AUC = 0.70)
Interleukin-6 (AUC = 0.47)
Testosterone (AUC = 0.43)
Alpha Amylase (AUC = 0.30)
Cortisol (AUC = 0.07)

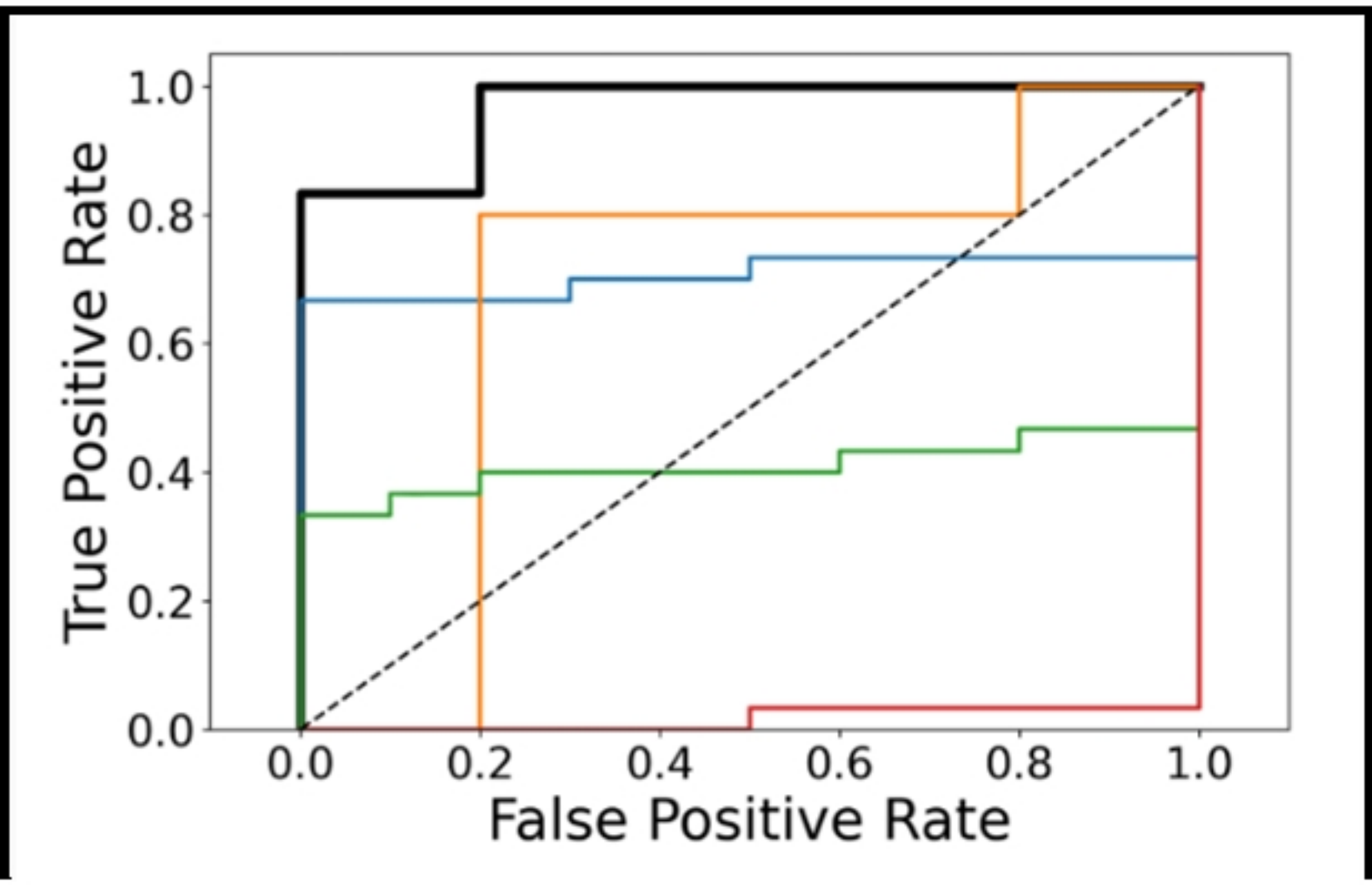

True Positive Rate
False Positive Rate
0.0
0.2
0.4
0.6
0.8
1.0
Combined (AUC = 0.97)
ATP1B1 (AUC = 0.71)
STOML2 (AUC = 0.68)
PGLYRP2 (AUC = 0.41)
FH (AUC = 0.02)

Figure 3

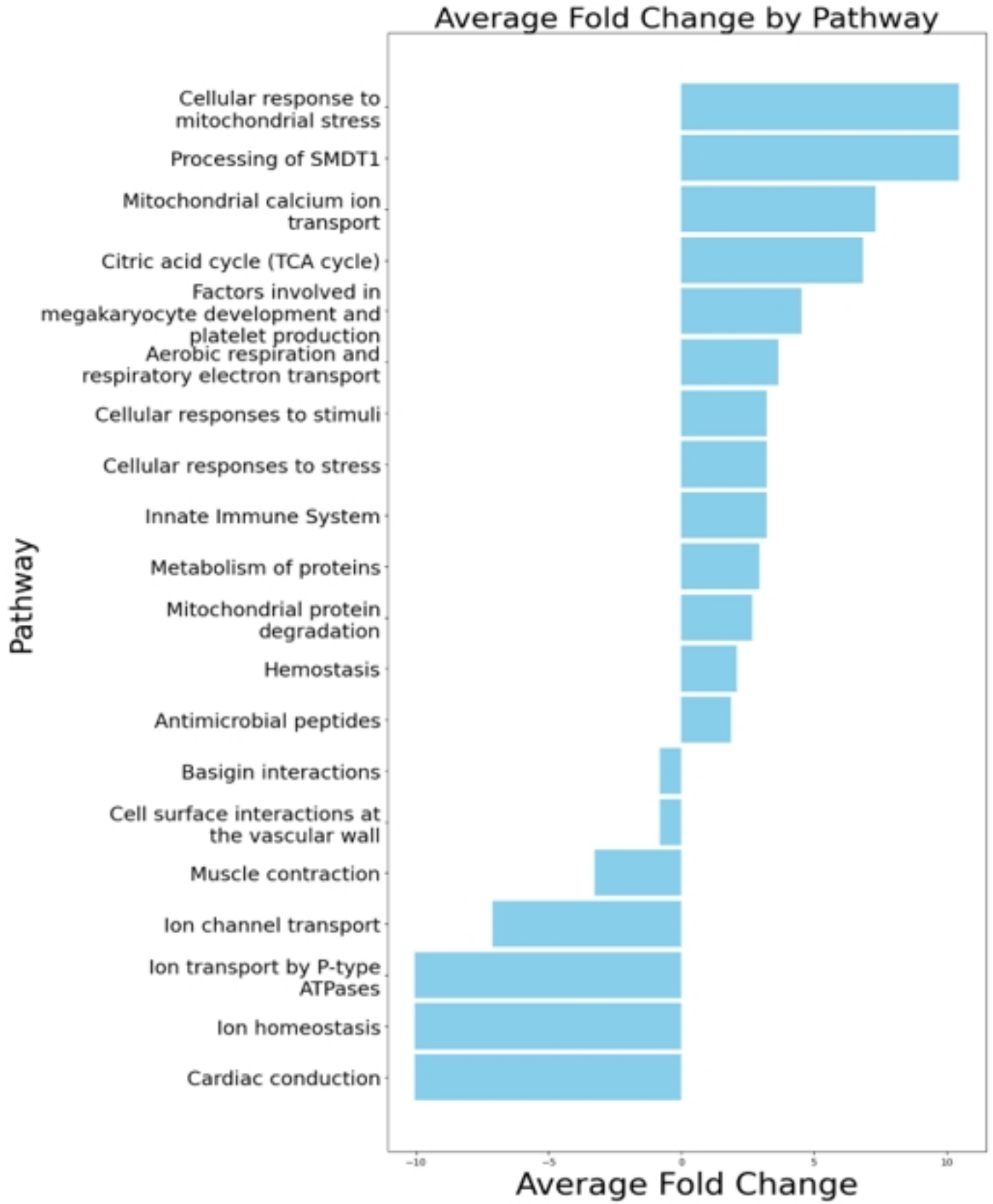

Average Fold Change by Pathway
Cellular response to mitochondrial stress
Processing of SMDT1
Mitochondrial calcium ion transport
Citric acid cycle (TCA cycle)
Factors involved in megakaryocyte development and platelet production
Aerobic respiration and respiratory electron transport
Cellular responses to stimuli
Cellular responses to stress
Innate Immune System
Metabolism of proteins
Mitochondrial protein degradation
Hemostasis
Antimicrobial peptides
Basigin interactions
Cell surface interactions at the vascular wall
Muscle contraction
Ion channel transport
Ion transport by P-type ATPases
Ion homeostasis
Cardiac conduction
Pathway
−10
−5
0
5
10
Average Fold Change

Figure 4