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

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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.

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

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

114 Therefore, this study aimed to compare the predictive performance of salivary proteins identified
115 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.

136

137 **Data Collection Protocol**

138

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

148

149 *Insert Fig 1 Here*

150

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

155

156 The fatigue protocol involved participants performing one hour of continuous circuits of a 400m
157 run, 30.5m standing power throw (4.5kg), 30.5m sprint, and a 6m single arm farmer's carry (M =
158 22.7kg, F = 15.9kg) while wearing a weighted vest (9.1kg). This circuit was developed to simulate

159 common occupational tasks and movements in operational settings. Directly before and after the
160 fatiguing protocol, participants performed a series of physical performance tests including Queen's
161 step test (17), maximum standing vertical leap (CMJ) and isometric mid-thigh pull (IMTP) on
162 force plate (ForceDecks, Vald Performance, Brisbane, Queensland, Australia), maximum hand-
163 release pushups (HRPU) and plank hold, and average anaerobic power (watts) over 60s max sprint
164 on cycle ergometer (max power; Rogue Echo Bike, Rogue, Columbus, Ohio, USA). On the fatigue
165 day, saliva samples were collected at approximately 0800, 1200 (directly after completion of the
166 post-fatigue protocol performance assessment) and 1300 (one hour after post- fatigue protocol
167 performance assessment). Samples were stored on dry-ice until cessation of each data collection
168 day after which they were preserved at -80°C at the Center for Proteomics and Molecular Medicine
169 (CAPMM) until analysis.
170

171 **Targeted Analysis**

172

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

181

182 **Proteomic Analysis**

183

184 The remaining 4.5 mL from each saliva sample was analyzed at the Center for Applied Proteomics
185 and Molecular Medicine (CAPMM) at George Mason University to identify and quantify salivary
186 proteins. Low abundance proteins were enriched by selective capture using core-shell hydrogel
187 nanoparticle based technology as previously described (18,19). After multiple washes to remove
188 unbound proteins, the protein-nanoparticle complexes were eluted by incubation with 4% sodium
189 dodecyl sulfate (SDS) in 50 mM ammonium bicarbonate at room temperature. The SDS was then
190 removed using detergent removal columns according to manufacturer instructions.

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

199 Data acquisition was performed in data-dependent mode, with one full MS scan (300 -1500 m/z,
200 60,000 resolving power) followed by MS/MS scans of the most abundant molecular ions,
201 dynamically selected and fragmented by higher-energy collisional dissociation (HCD) at a
202 collision energy of 27%. Tandem mass spectra were searched against the NCBI human database
203 using Proteome Discover v 2.4 (Thermo Fisher Scientific). Database searches employed the

204 SEQUEST node with full tryptic cleavage constraints and dynamic methionine oxidation. Mass
205 tolerances were set to 2 ppm for precursor ions and 0.02 Da for fragment ions. A 1% false
206 discovery rate (FDR) was applied to peptide-spectrum matches (PSM) as the reporting threshold.
207 Precursor Ions quantifier node was used to for protein abundance calculation and quantification.

208

209 **Statistical Analysis**

210

211 **Performance Parameters**

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

218 **Targeted Small Molecules**

219

220 Mean values of duplicate measures for cortisol (µg/dL), testosterone (pg/mL), IgA (µg/mL), alpha-
221 amylase (U/mL), uric acid (mg/dL), and IL-6 (pg/mL) were z-score standardized. Multicollinearity
222 was assessed using variance inflation factor (VIF). A support vector machine (SVM) model with
223 leave-one-out cross-validation (LOOCV) was used to classify rested (pre-fatigue) vs. fatigued
224 (post-fatigue) states. Model performance was evaluated using a confusion matrix to calculate

225 sensitivity and specificity, along with F1 score and Matthews Correlation Coefficient (MCC). Area
226 under the curve (AUC) of the receiver operating characteristic (ROC) was also calculated for each
227 biomarker and the combined models.
228

229 **Identified Proteins**

230

231 Untargeted mass spectrometry of our samples yielded 2,284 proteins across time points, as
232 deposited in the Open Science Framework (DOI: 10.17605/OSF.IO/2WM4P, version 1.0). Of the
233 2,284 proteins detected, those present in more than 50% of samples (n = 1,355) were retained.
234 Missing values were imputed at 50% of the detection limit, and all data were log-normalized.
235 Mixed-effects models were used to evaluate the effect of time of day and fatigue status on protein
236 abundance, with participant as a random effect. Proteins significantly associated with fatigue only
237 (n = 149) were selected for further analysis (Figure 2).

238

239 *Insert Fig 2 Here*

240

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

247

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

258

259 **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-

278 based model achieved 95% accuracy. ATP1B1 was the top-performing protein, followed by
279 STOML2 and PGLYRP2, while FH showed limited individual predictive value.
280
281 *Insert Fig3 Here*
282
283 **Figure 3.** Receiver Operating Characteristic (ROC) Curves for Predicting Physical Fatigue Using
284 Targeted Small Molecules and Proteomic Biomarkers. Values in parentheses represents
285 discriminatory performance in the classification model. A) ROC curves for targeted small
286 molecules, including cortisol, testosterone, immunoglobin A (IgA), alpha-amylase (AA), uric acid
287 (UA), and interleukin-6 (IL-6), as well as a combined model incorporating all six molecules. B)
288 ROC curves for salivary proteins identified through untargeted mass spectrometry including
289 sodium/potassium-transporting ATPase subunit beta-1 (ATP1B1), stomatin-like protein 2
290 (STOML2), N-acetyl-L alanine amidase (PGLYRP2), and fumurate hydratase (FH).
291
292 Confusion matrices (Table 3) showed a higher true positive rate for the protein model (80%)
293 compared to the molecule model (60%), and a slightly higher true negative rate (100% vs. 97%).
294 The protein model also had superior F1 score (89%) and MCC (87%) compared to the molecule
295 model (71% and 65%, respectively).
296
297 **Table 3.** Confusion Matrices for Classification of Fatigue Status Using Targeted Small Molecules
298 and Discovered Proteins.
299

	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

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

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

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

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

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

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

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

370 Proteins associated with metabolic regulation emerged as key discriminators of fatigue states,
371 with the 'Transporting of Small Molecules' pathway emphasizing the physiological need to
372 prioritize energy balance during fatigue. This is consistent with the work of Michael et al.,(14)
373 where salivary biomarkers linked to energy metabolism and protein degradation were predictive
374 of fatigue. Furthermore, the inclusion of the "Cellular Response to Stimuli" pathway in the
375 current findings supports broader research emphasizing stress-responsive pathways. For instance,
376 studies have shown that protein chaperones and metabolic genes are upregulated to counteract
377 cellular stress, facilitating recovery and adaptation.(13–15) Together, these findings suggest that
378 physical fatigue results from a complex interplay of mitochondrial regulation, immune signaling,
379 metabolic adaptation, and stress responses, offering a comprehensive framework for
380 understanding the molecular mechanisms underpinning fatigue across diverse contexts.

381 The protein model's superior performance likely stems from its focus on low-abundance
382 biomarkers enriched using nanoparticle capture. The a priori focus on low-abundance biomarkers
383 helps to enrich for biochemical targets that are likely modulated by specific cellular processes,
384 rather than generalized markers of overall organ or system function. However, these biomarkers
385 may still reflect localized dynamic changes within tissues or cells that contribute to the organ

386 system's response to fatigue stress. Consequently, this protein archive may contain candidate
387 biomarkers that are directly linked to fatigue-related pathways, such as mitochondrial function,
388 protein folding, and metabolic regulation, providing greater specificity to the physiological
389 mechanisms underlying physical fatigue.

390 In contrast, hormonal markers such as cortisol and testosterone are significantly influenced by
391 extraneous factors, including psychological stress, diurnal rhythms, and individual variability in
392 HPA axis responsiveness. Similarly, inflammatory markers like IL-6 can be elevated due to
393 unrelated stressors such as infections or injuries. These external influences reduce their
394 specificity and reliability as fatigue indicators. Additionally, salivary proteins offer enhanced
395 temporal stability, capturing both acute and sustained responses to physical exertion, unlike the
396 delayed or fluctuating kinetics of hormonal and inflammatory markers. Proteomic markers also
397 provide a broader biological context by integrating immune regulation, metabolic shifts, and
398 cellular stress adaptations, enabling a more comprehensive understanding of fatigue.
399 Furthermore, salivary proteins exhibit lower inter-individual variability, as they reflect localized
400 and specific responses to exertion, ensuring greater consistency across individuals under similar
401 conditions.

402 **Strengths, limitations, and implications for future research:**

403 This study demonstrated the utility of nanoparticle-based biomarker enrichment combined with
404 untargeted high-resolution LC-MS to explore the low-abundance salivary proteome. This
405 innovative approach enabled comprehensive, unbiased identification of proteins associated with
406 physical fatigue. Direct comparison between the proteomic and targeted small molecule models
407 provided critical insights into their relative utility, highlighting the superior sensitivity and

408 specificity of the protein-based approach. The fatigue protocol reliably induced measurable
409 physiological fatigue, as confirmed by reductions in performance and changes in HR and HRV.

410 Despite these strengths, the study had limitations. While machine learning approaches such as
411 SVM can identify promising biomarkers, the limited sample size and exploratory nature of this
412 study introduce a high risk of overfitting and inflated effect estimates. Leave-one-out cross-
413 validation (LOOCV) was employed to reduce bias in model performance estimates; however, these
414 results are hypothesis-generating and must be validated in larger, independent cohorts before any
415 clinical or operational application. The short protocol focused on acute fatigue and did not capture
416 the chronic fatigue commonly experienced during extended training or deployment. Chronic
417 fatigue arises from different mechanisms such as persistent inflammation,(36) mitochondrial
418 dysfunction,(37) and hormonal dysregulation(38) which were not evaluated. Additionally, while
419 the protocol was relevant to military tasks, it may not fully reflect real-world operational
420 complexity.

421 Future studies should incorporate multi-day or cumulative workload designs to better reflect
422 operational demands (39). Larger and more diverse cohorts are essential for validation.
423 Longitudinal assessments would help track biomarker changes over time, particularly in chronic
424 fatigue and sustained stress contexts (4). Expanding to multi-omics approaches, including
425 metabolomics, genomics, and transcriptomics, could provide a more integrated view of fatigue
426 biology. Combining these with wearable monitoring (39), and considering sex-specific responses
427 and individual variability, would improve model precision. Ultimately, translating these findings
428 into targeted interventions could support effective fatigue management in high-demand
429 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

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568

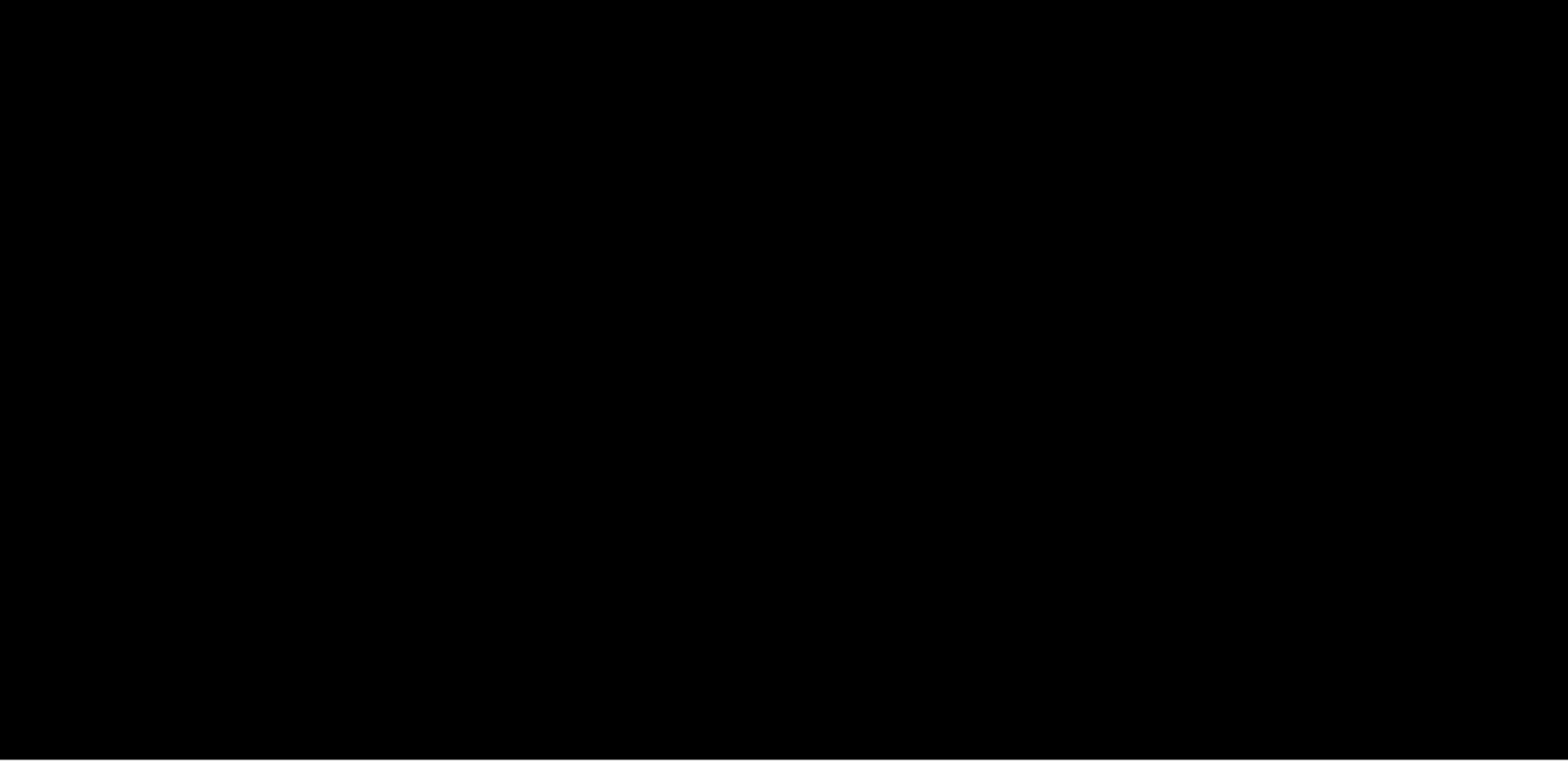
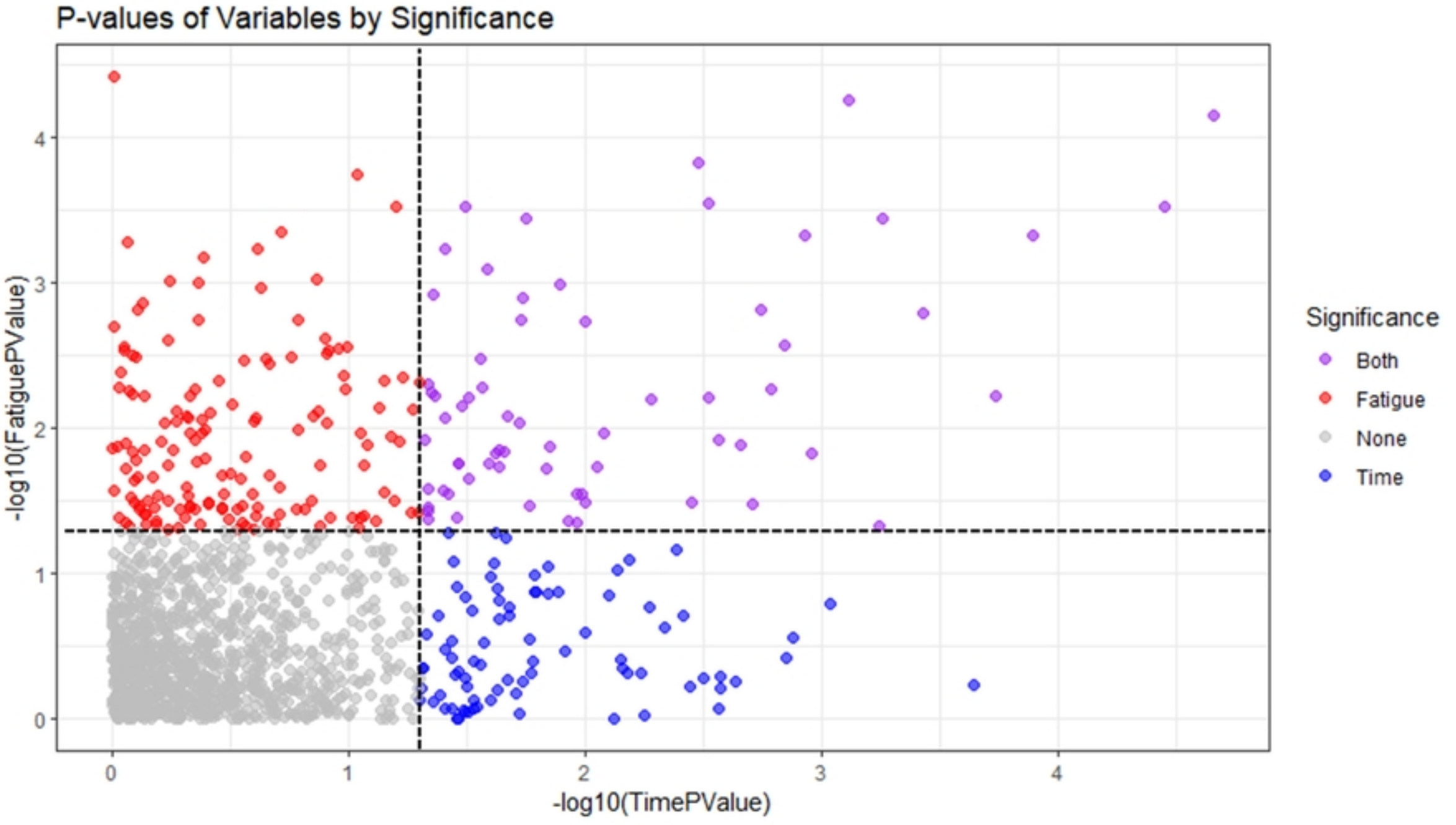


Figure 1


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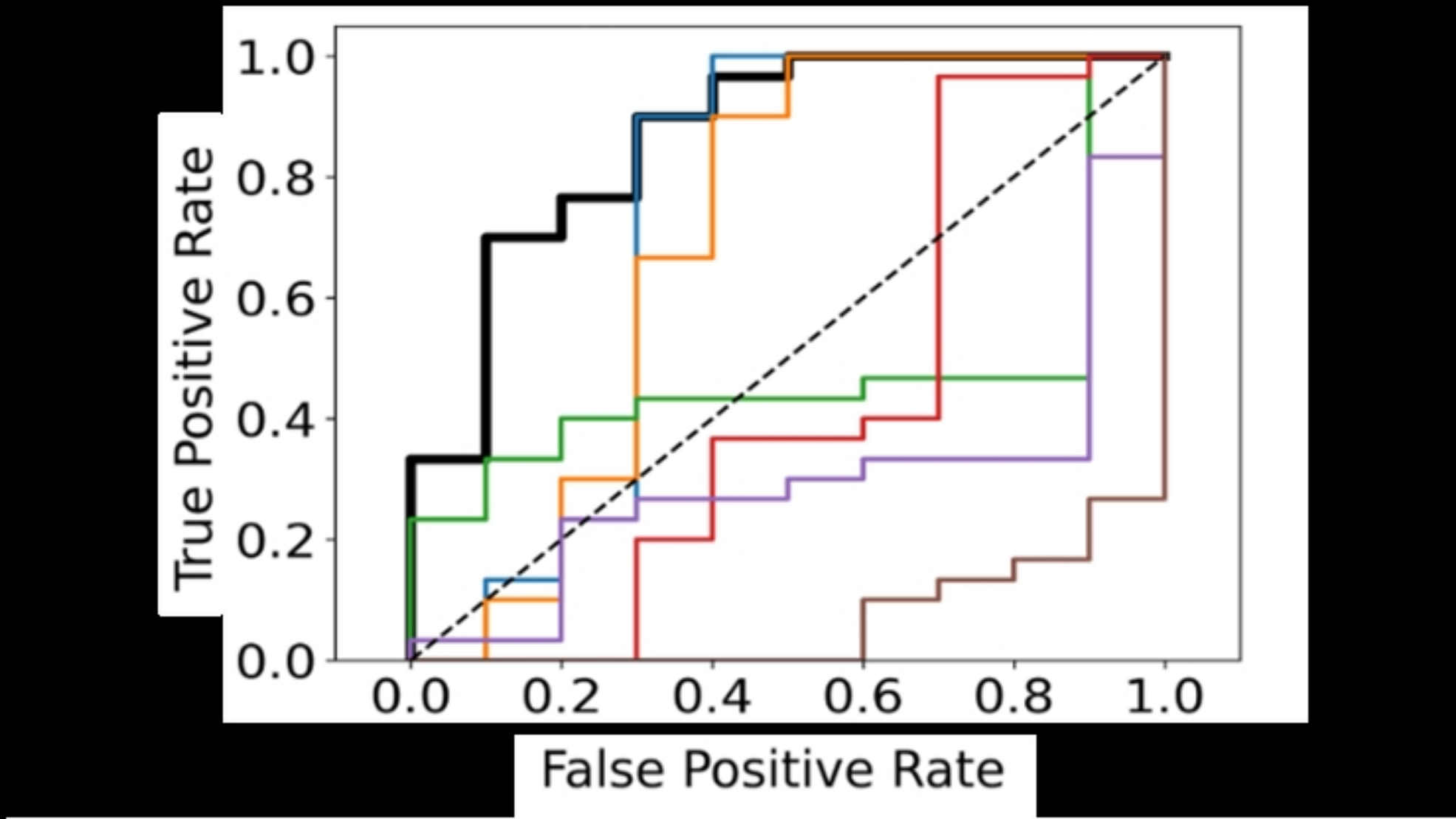
-log10(TimePValue)
0
1
2
3
4
Significance
Both
Fatigue
None
Time

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)

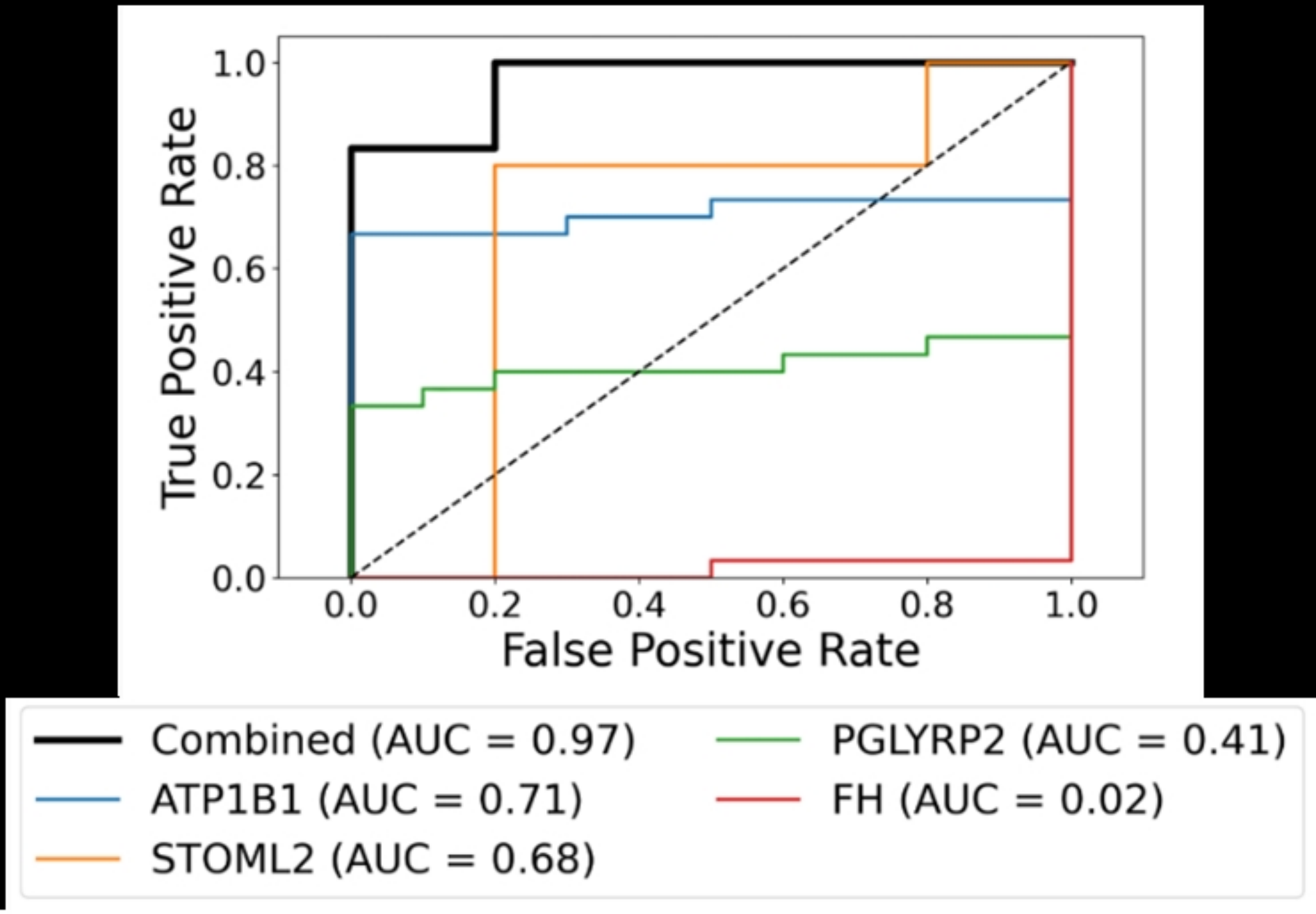

True Positive Rate
False Positive Rate
0.0
0.2
0.4
0.6
0.8
1.0
Combined (AUC = 0.97)
ATP1B1 (AUC = 0.71)
STOML2 (AUC = 0.68)
PGLYRP2 (AUC = 0.41)
FH (AUC = 0.02)

Figure 3

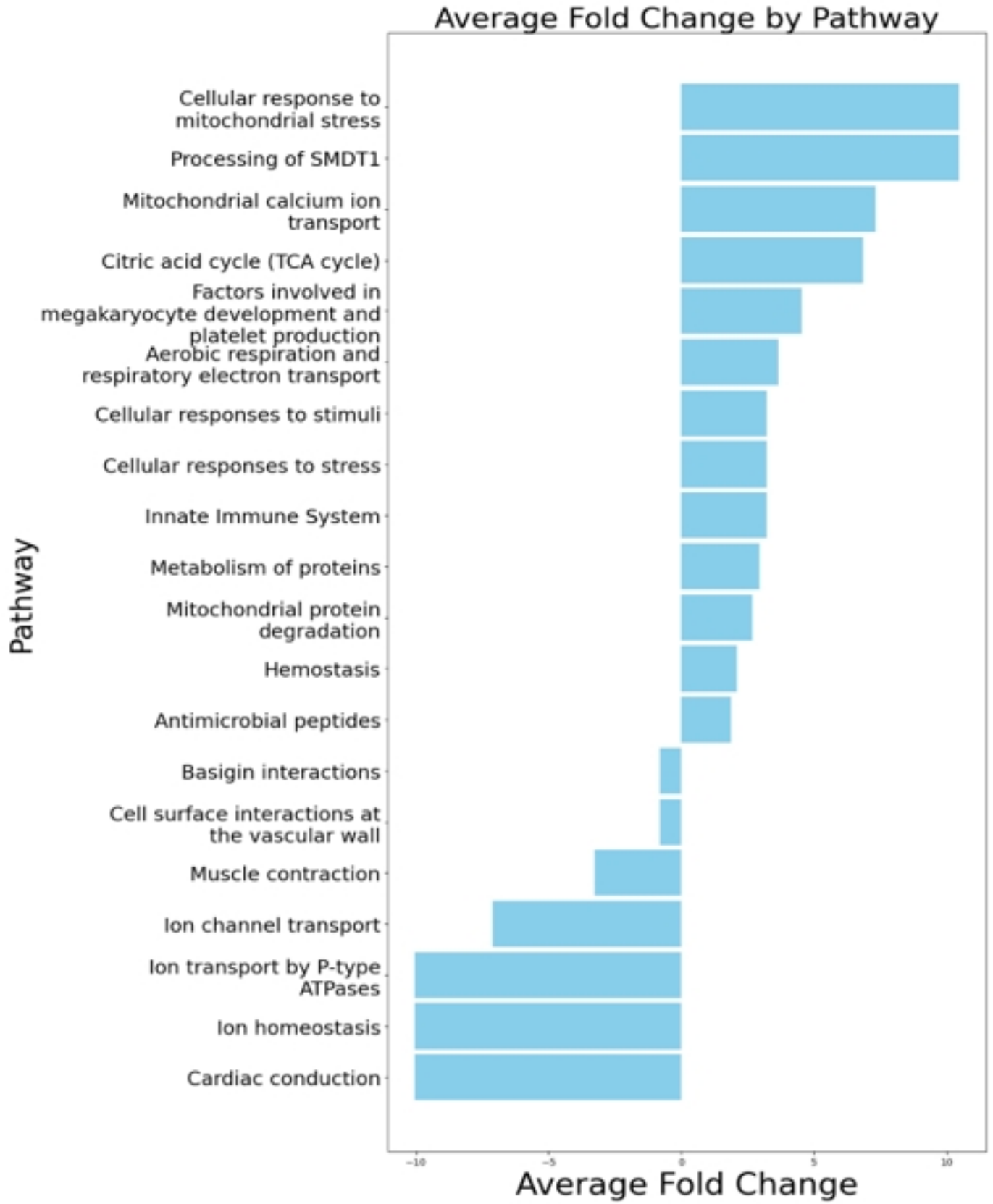

Average Fold Change by Pathway
Cellular response to mitochondrial stress
Processing of SMDT1
Mitochondrial calcium ion transport
Citric acid cycle (TCA cycle)
Factors involved in megakaryocyte development and platelet production
Aerobic respiration and respiratory electron transport
Cellular responses 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