Unique and Overlapping Proteins of Subjective Feelings of Mental and Physical Energy and Fatigue: An Exploratory Study

Original Research

Ali Boolani[1,2], Bryndan Lindsey[3], Emanuel Petricoin[4], Weidong Zhou[4], Rayan Alhammad[5,6], Joshua T. Butcher[7], Joel Martin[8]

[1]*Human Performance and Nutrition Research Institute, Oklahoma State University, Stillwater, OK, USA*
[2]*Department of Physiology, Center for Health Sciences, Oklahoma State University, Tulsa, OK, USA*
[3]*State Key Laboratory of Mechanical System and Vibration, School of Mechanical Engineering, Shanghai Jiao Tong University, Shanghai, China*
[4]*Center for Applied Proteomics and Molecular Medicine, George Mason University, Fairfax, VA, USA*
[5]*Clinical Laboratory Department, College of Applied Medical Science, King Saud bin Abdulaziz University for Health Sciences, Al Ahsa, Saudi Arabia*
[6]*King Abdullah International Medical Research Center, Al Ahsa, Saudi Arabia*
[7]*Department of Physiological Sciences, College of Veterinary Medicine, Oklahoma State University, Stillwater, OK, USA*
[8]*Sports Medicine Assessment Research and Testing Lab, George Mason University, Fairfax, VA, USA*

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Abstract

Introduction: Recent evidence suggests that subjective feelings of mental energy (ME), mental fatigue (MF), physical energy (PE), and physical fatigue (PF) are unique and overlapping biological correlates. This exploratory study aimed to identify unique and overlapping proteomic markers and their associated pathways for ME, MF, PE, and PF.

Methods: Participants (n=10) completed a 3-day protocol with saliva samples collected at multiple time points, including pre- and post-fatigue (Day 2). Trait and state levels of ME, MF, PE, and PF were assessed each day, and absolute and relative changes in mood and proteomic markers were calculated. Top 1% of proteomic markers were identified using a genetic algorithm with Random Forest Regressor, followed by Community Louvain network analysis and STRING-based protein–protein interaction analysis.

Results: Our analysis identified unique and overlapping proteomic markers for each of the 4 moods. The PTP analysis revealed that energy was associated with ATP generation ($p<0.001$), activation ($p=0.003$) and regulation of adaptive immunity ($p<0.001$), and markers of cardiac myopathy ($p=0.028$) while fatigue was associated with catabolism ($p=0.008$), oxidative stress, innate immunity ($p<0.001$), and necroptosis.

Conclusions: Consistent with prior research, the results highlight the distinct biological profiles of energy and fatigue, supporting their assessment as separate mental and physical state and trait moods.

Key Words: proteomics; biomarkers; mood states.

Corresponding author: Joel Martin, jmarti38@gmu.ed

Introduction

Symptoms of psychological fatigue are a commonly occurring phenomena in approximately 20.4% of adults worldwide[1] resulting in a high societal and economic burden, including higher risk for car accidents,[2] increased absence in school children,[3] and decreased work productivity.[4] It is estimated that fatigue costs employers an estimated $136.4 billion annually in health-related loss productivity time.[4] Despite the prevalence and high social and economic burden, fatigue is poorly understood. One of the primary difficulties in understanding and treating psychological fatigue is the challenge faced by researchers in defining it.[5,6] While Philips et al.[5] has proposed defining fatigue as a sub-optimal psychophysiological condition, evidence suggests that there are distinct advantages to measuring the psychological and physical constructs of fatigue separately.[7–9]

Adding to this complexity is the evidence that suggests that energy and fatigue are two distinct but overlapping moods, each with unique biological,[7,10–12] biomechanical,[13,14] and behavioral[11,15,16] correlates. For instance, evidence supports that feelings of energy are associated with dopamine,[17,18] glucose,[10,19] annexin A1,[20] resting metabolic rate,[11] peripheral skeletal muscle O_2,[11] VO_{2Max},[21] carbohydrate metabolizing gut microbiota,[12] cardiorespiratory coordination,[7] and changes in total blood carbon dioxide,[7] blood anion gap,[7] blood hemoglobin[7] and hematocrit concentration.[7] Concomitantly, evidence supports that feelings of fatigue are associated with serotonin,[17,22] histamine,[23] inflammatory blood and salivary TNF-α,[17,20] IL-6,[24] leptin,[25] pro-inflammatory gut microbiota,[12] salivary cortisol,[7] and blood chloride.[7] Biomechanical changes also differ between these states with feelings of lower energy linked to increased movement errors,[13,14] while feelings of greater fatigue tend to result in slower movement.[13,14] Further, behaviors such as polyphenol consumption,[26] cognitive workload,[11,26] typical caffeine intake,[26] and the interaction between sleep and diet[27] can be differentially associated with either feelings of energy or fatigue.

While these findings highlight the distinct nature of energy and fatigue, there are also overlapping features. For example, genetic evidence suggests that energy and fatigue, while separate constructs, may share common genetic underpinnings.[28] Additionally, post-hoc analyses of exercise over-training studies demonstrate that resting metabolic rate can be associated with both energy and fatigue.[29] A recent study also found that blood sodium levels decreased significantly in a sub-set of individuals who self-reported lower feelings of both mental energy or higher feelings mental fatigue post aerobic exercise.[30] Non-exercise behavioral studies suggest complexity in the interaction between behavior and these mood states, with poor sleep quality negatively impacting both,[16] however, with nuanced sex-related differences.[15] There are also differing results relating to the interaction between these moods, physical activity,[11,16,26] and sedentary behavior.[8,11,16]

There is also considerable variability in human responses to common fatigue-modifying interventions, such as caffeine,[31] acute,[32] and chronic exercise.[33] This heterogeneity likely reflects the complex interplay of biological, psychological, and environmental factors. For example, psychological factors, such as individual traits (e.g., predisposition to certain mood states) and social functioning, significantly influence the effectiveness of interventions aimed at altering energy and fatigue. Such interventions include caffeinated beverages,[34] workplace ergonomics,[35] and aquatic exercise for physical therapy.[36] Studies consistently report substantial variability in psychological responses to standardized stimuli. For instance, one investigation found that 33% of participants experienced increased feelings of energy following the same exercise protocol, even under adverse conditions such as heat stress and dehydration, while others reported decreased energy.[7] Research designed to induce fatigue, using a variety of standardized protocols, reveal substantial variation in the outcomes.[34–37] Collectively, these findings[34–37] suggest that individuals can experience concurrent feelings of both energy and fatigue, adding further complexity to understanding the underlying biomarkers of energy and fatigue mood states.

While prior studies have suggested distinct and overlapping physiological interactions involving metabolism, inflammation, and neuroendocrine signaling, it remains unclear whether distinct molecular signatures exist for different forms of energy and fatigue or if they share common biological underpinnings. Therefore, this study aims to investigate the proteomic profiles associated with subjective reports of physical energy (PE), mental energy (ME), physical fatigue (PF), and mental fatigue (MF). By leveraging high-dimensional proteomics data and network-based analyses, we seek to: (1) identify biomarkers that uniquely define state and trait mental and physical energy and fatigue, (2) determine shared molecular pathways across these states and traits, and (3) explore the functional relevance of key proteins through pathway enrichment and network modelling. Importantly, we investigate these proteomic profiles within the context of a physically fatiguing protocol that elicited varied cognitive, physiological, biomechanical, and mood responses. Findings from this work could advance precision approaches for monitoring, predicting, and managing fatigue by identifying molecular targets for individualized interventions.

Methods

Study design

A within-subjects repeated measures experimental design was used, structured into three phases: Baseline (Day 1), Fatigue Induction (Day 2), and Recovery (Day 3). This structure was chosen to capture both acute and delayed physiological responses to a controlled fatiguing intervention, allowing for within-subject comparison of physiological and psychological responses over time.

Participants

Participants eligible for the study were healthy adults aged 18 to 55 years. Individuals with any existing injuries or surgeries within the past three months, or those reporting pain that would limit their ability to run, perform a push-up, pull-up, or bodyweight squat, were excluded. Additionally, participants 1) with a history of cardiovascular, pulmonary, renal, or metabolic diseases were excluded and 2) were not engaging in at least 30 minutes of daily physical activity (structured exercise or activities of daily living) were excluded. Finally, participants must have completed, or self-reported the ability to complete, a 2+ mile ruck carrying 20kg within the past three months.

Participants were recruited through word of mouth and flyers. This study received approval from the George Mason University Institutional Review Board (IRB #2004820). Ten recreationally active participants (6 male, 4 female) ranging from 21 to 50 years (32.4 ± 9.1, mean ± SD) were recruited to participate. Values for height, weight, and percent body fat were as follows: 172.3 ± 10.1 cm, 75.0 ± 14.4 kg, 19.8 ± 5.7%. All participants provided written informed consent before commencing any study procedures.

Protocol

Saliva samples (~5mL per collection) were collected using the mLife True oral fluid collection device (mLife, TX, USA), at 7 timepoints across 3-days: Baseline (day 1), Fatigue Induction (day 2), and Recovery (day 3) (Figure 1). On days 1 and 3, samples were collected at ~ 0800 and 1300 hours; participants were instructed to avoid strenuous exercise 24 hours prior to day 1 and throughout the protocol outside the prescribed fatiguing protocol on day 2. On day 2, participants performed a 1-hour physical fatiguing protocol, while wearing an ECG monitor (Zephyr Bioharness 3.0, Zephyr Technology Corporation, Annapolis, MD, USA), donned approximately 1-hour before, during, and 1-hour after the fatiguing protocol.

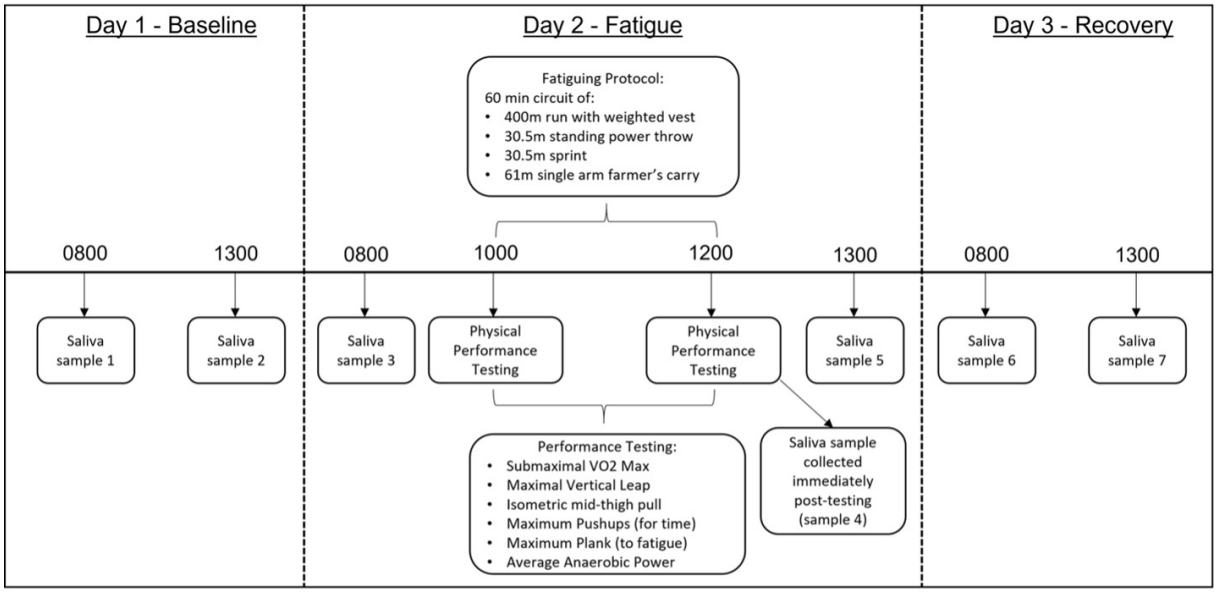


Figure 1. Timeline of saliva collection and fatiguing protocol

The fatigue protocol consisted of 1 hour of continuous circuits involving a 400m run, 30.5m standing power throw (4.5kg), 30.5m sprint, and a 61m single arm farmer's carry (M = 22.7kg, F = 15.9kg) while wearing a weighted vest (9.1kg). This circuit was designed simulate occupational tasks common to operational and military training environments. Immediately before and after the protocol, participants completed a physical performance battery

including Queen's step test,[38] countermovement jump (CMJ) and isometric mid-thigh pull (IMTP) on force plate (ForceDecks, Vald Performance, Brisbane, Queensland, Australia), maximum hand-release pushups (HRPU), maximum plank hold, and average anaerobic power (watts) over 60s max sprint on an airbike ergometer (max power; Rogue Echo Bike, Rogue, Columbus, Ohio, USA). The performance battery was designed to assess aerobic capacity, muscular strength and endurance, power, and anaerobic capacity, and was administered in the same order for each participant with 5-minute rest periods between tests. Prior analyses showed that participants were physically fatigued after the protocol, as evidenced by declines in aerobic/anaerobic capacity and muscular endurance. Post-fatigue, heart rate increased, HRV decreased, and step test recovery, anaerobic capacity, push-up maximum, and plank duration declined, whereas vertical jump height and peak isometric force were unchanged.[39] On day 2, saliva samples were collected at ~0800, 1200 (immediately post-assessment), and 1300 (1 hour post-assessment). All samples were stored on dry ice until the end of each collection day, then preserved at -80°C at the Center for Proteomics and Molecular Medicine (CAPMM) until analysis.

State Mental and Physical Energy and Fatigue

The state aspect of the O'Connor State-Trait scale was used to assess state mental and physical energy and fatigue [40]. The state aspect of the scale uses a 12-item Visual Analog Scale (VAS), with each state outcome containing three items. Each item was anchored by "No feelings at all" (0) to "The highest imaginable feeling" (100). The Cronbach's α for these moods has been reported to range from 0.88 to 0.90.[8,15] The Cronbach's α for the current study ranged from 0.89 to 0.95 (State PE = 0.89, State PF = 0.94, State ME = 0.88, State MF = 0.95).

Trait Mental and Physical Energy and Fatigue

The trait aspect of the O'Connor State-Trait scale was used to assess trait mental and physical energy and fatigue.[40] The trait aspect of the scale uses a 12-item, 5-point Likert Scale, with each trait outcome containing three items. Representative statements included "I feel I have energy" and "I have feelings of being worn out". Responses ranged from "never" (0) to "always" (4). Cronbach's α for previous studies has ranged from 0.73 to 0.88.[26,34,35] The Cronbach's α for the current study ranged between 0.72 and 0.85 (Trait PE = 0.85, Trait PF = 0.73, Trait ME = 0.79, Trait MF = 0.72).

Targeted Analysis

Approximately 0.5mL of each saliva sample was sent to Salimetrics (Carlsbad, CA) for quantification of targeted markers, including cortisol, testosterone, immunoglobulin (IgA), alpha-amylase (AA), interleukin-6 (IL-6), and uric acid (UA) using commercial assays. 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. Details on sample test volume, limits of sensitivity and inter-assay coefficients of variation for all assays are displayed in Table 1.

Table 1. Details of commercial immunoassays used to quantify targeted markers.

Assay	Sample Volume per Test	Lower Limit of Sensitivity	Standard Curve Range	Intra-assay Coefficient of Variation (%)	Inter-assay Coefficient of Variation (%)
Cortisol (Cat. No. 1-3002)	25 µL	0.007 µg/dL	0.012-3.0 µg/dL	4.60	6.00
Testosterone (Cat. No. 1-2402)	25 µL	1 pg/mL	6.1-600 pg/mL	4.60	9.85
Immunoglobulin-A (Cat. No. 1-1602)	10 µL of 5X diluted saliva	2.5 µg/mL	2.5-600 µg/mL	5.60	8.79
Alpha-amylase (Cat. No. 1-1902)	8 µL of 200X diluted saliva	0.4 U/mL	N/A	5.47	4.7
Uric Acid (Cat. No. 1-3802)	10 µL of saliva	0.26 mg/mL	0-20 mg/dL	1.88	4.46
Interleukin-6	25 µL of saliva	N/A	N/A	<15	<15

Proteomics Analysis

The remaining 4.5mL 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.[41] 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

All data was pre-processed using Python 3.13.2. A total of 2,284 proteins were extracted from saliva, in addition to the 6 additional targeted biomarkers. Proteomics and targeted biomarkers were combined, and data were excluded if more than 10% of data were missing within the first six timepoints. After this exclusion step, 1,182 biomarkers remained. All data was scaled using StandardScaler.[42] Missing values were then imputed using Multiple Imputation by Chained Equations (MICE), implemented via the sklearn library. Following imputation, biomarker data were merged with all other data. Absolute (Δ), and relative ($\frac{previous\ time\ point - current\ time\ point}{previous\ time\ point}$) (Δ%) were calculated for all data per participant.

Data Selection and Feature Engineering

To account for the inherent stability of trait mental and physical energy and fatigue, analysis was restricted to three primary timepoints (Day 1~ 0800 and 1300; Day 2 ~0800). To account for the relative temporal stability of trait measures, trait scores were held constant across the three selected time points. In contrast, to capture state-based fluctuations, we derived three feature types for each biomarker: (1) the raw value at each time point (current state), (2) the absolute change from the prior time point, and (3) the relative percent change from the prior time point. These features were examined in relation to corresponding changes in physical energy, mental energy, physical fatigue, and mental fatigue. This multi-dimensional approach ensures that the analytic pipeline can detect both the baseline presence and the dynamic responsiveness of the proteomic profile.

High-Dimensional Analytical Strategy

Given the high-dimensional nature of this study, characterized by a small sample size (n=10) relative to a wide proteomic profile (>2,200 proteins), our analytical pipeline was specifically engineered to mitigate five critical risks: 1) overfitting, 2) data leakage, 3) false positives, 4) feature instability/small sample bias, and 5) correlated predictors that produce spurious results (biological noise). To address these challenges, we implemented a multi-stage consensus strategy that reduced dimensionality through regularized feature selection, penalized unstable predictors via stability thresholding, and ensured generalizability through cross-individual validation.

To control for overfitting and data leakage, we utilized a Leave One Group Out (LOGO) validation strategy. The repeated measures, within participant design of this study inherently creates autocorrelation among the data. Thus, using cross-validation techniques such as K-fold cross-validation and/or Leave One Out (LOO) can result in

information from the same participant appearing in both the training and testing dataset. This causes information leakage and inflates predictive performance. Thus, using LOGO cross validation solves this issue by holding out all data for an entire participant, training the models on the other participants and testing it on the held-out participant. This approach ensures independence of the training and testing data and generalization across individuals.[43]

To address the challenges of false positives in a high dimensional dataset, we used three complementary approaches to reduce the data to the top 1% of biomarkers associated with each mood. We used the Boruta algorithm and then reached a consensus between genetic algorithms (GA) and random forest recursive feature elimination (RF-RFE). The Boruta algorithm identifies all relevant features by comparing the real features to randomized shadow features. This identifies non-linear, robust correlated features and reduces dimensionality without strict sparsity assumptions.[44] Further by combing both GA and RF-RFE to get a mathematical consensus, we ensured that the selected biomarkers were not just artifacts of one specific mathematical approach and instead they were selected by both approaches.[45] To ensure that these algorithms did not overfit, we restricted the maximum depth of 3 and used square root regularization. By limiting the depth, we forced the model to find global patterns rather than hyper-specific interactions that only exist in one or two subjects, while the square root subsampling prevented a single dominant, potentially spurious protein from overshadowing other potentially relevant biomarkers across the forest.[46,47] Further, to control for feature instability and small sample bias we set a stability score (S) ≥ 0.70 across 10 iterations where biomarkers that achieved a stability score ≥ 0.70 for both the GA and RF-RFE algorithms were included in the biomarker profile.

To ensure that the reduced biomarker samples were true biological signals and not random background noise, we first applied a topographic filter using a network analysis using Louvain and Greedy Modularity algorithms to identify communities within out network. A network analysis allows for identification of functional modules, detection of co-regulated biomarker clusters, and assessment of system-level biology such that we can identify biomarkers that are mathematically central to each mood. By splitting the biomarkers into communities, we are reducing the testing burden and identifying coordinated biological responses to each mood.[48,49] The Louvain algorithm allowed us to uncover the hierarchical structure of the complex networks by optimizing modularity,[48] while the Greedy Algorithm was used to find community partitions by maximizing the strength of the division within the network.[49]

In high dimensional datasets, a single protein might show statistical correlation by chance however, it is mathematically improbable for a noise protein to also hold high centrality (betweenness) score within a functional network. Thus, by prioritizing these hubs, we filtered statistical artifacts in favor of proteins that are structurally integrated into the body's response to each mood.[50] The proteins for the network were detected using Vector Autoregression (VAR) models. While standard correlations only show a snapshot, due to the repeated measures, within participant design of this study, a VAR allowed us to see if a biomarker precedes a change in mood, a critical step to establishing causality rather than just association.[51,52] The VAR used LOGO for cross validation. Upset plots were created to identify unique and overlapping biomarkers. All analyses were completed using Python 3.13.2, using networkx, deap, sklearn, statsmodel, boruta, community, and matplotlib libraries.

Protein-Protein Interaction Network

While individual proteins can be noisy, the STRING protein-protein interaction (PPI) and Functional Enrichment analysis can aggregate the signal from multiple proteins to identify biological pathways. By identifying pathways, we reduced the likelihood that the protein was a random protein, to a pathway-level significant, reducing risks for false discovery.[53] This analysis allowed us to reduce the risk of selecting a random protein mathematically to a near 0 probability. A PPI network was constructed using the STRING database for Figures 1-4. Functional enrichment analysis of the protein-protein interaction networks was conducted using STRING (https://string-db.org/). Proteins from all 4 moods, including the trait, state, absolute and relative changes of each moods were entered into STRING by their corresponding gene names (see Table 2). This allowed us to identify whether our analysis captured a real biological module or random proteins.[54] For each relevant group, all genes were unbiasedly entered together and the interaction network viewed. Clusters were identified via k-means clustering using the minimal number of centroids and color coded using the query proteins and first shell of interactions. The interactions between network nodes (proteins) were determined by STRING and presented unaltered. Known interactions are observed with a light blue (from curated databases) or light purple (experimentally determined) edge. Predicted interactions are shown as green (gene neighborhood), red (gene fusion), or blue (gene co-occurrence) edges. Other edges presented include text mining (light green), co-expression (black), or light blue (protein homology). Functional enrichments in each cluster were based on a significant ($p < 0.05$) false discovery rate, corrected for multiple testing within each category using the Benjamini–Hochberg False Detection Rate (FDR) procedure. In many cases, a large number of significant enrichments

were observed; therefore, emphasis was placed on the most significant functional enrichments within a Biological Process (Gene Ontology), KEGG Pathways, and Reactome Pathways, specifically those with high strength and signal. When a protein lacked an identifiable gene name in STRING, an associated gene was identified to serve as a surrogate marker. For testosterone and uric acid, HSD17B3 (Testosterone 17-beta-dehydrogenase 3) and XDH (Xanthine dehydrogenase/oxidase) were selected, respectively. For glutathione S-transferase theta-1 isoform a (GSTT1), a GST in the same family (theta) with significant homology was selected, namely GSTT2B. Adjusted p-values for all significant biological and KEGG pathways are reported.

Results

Energy vs. Fatigue

Energy only: When combining the proteomic network data for trait, state, absolute change, and relative changes in Energy we found 3 well-defined clusters ($p<0.05$) with several overlaps (Figure 2). The largest cluster contained important elements driving the biological processes of a humoral immune response ($p<0.001$), a negative regulation of blood coagulation pathways ($p<0.001$), and zymogen activation ($p<0.001$). This is further supported by significant enrichment of the KEGG pathway regulating complement and coagulation cascades ($p=0.007$). Further, there is potential for a role for Vitamin D, as several components of the Vitamin D receptor pathway are present (CAMP, S100A9, CST1). The second cluster is also quite large, with 27 genes that are innately linked to cellular metabolism (ribosome phosphate biosynthesis ($p<0.001$), nucleoside triphosphate biosynthesis ($p=0.002$), purine ribonucleotide biosynthesis ($p=0.002$)) and ATP production (ATP metabolic process $p=0.003$) with a KEGG pathway of oxidative phosphorylation ($p<0.001$). While significantly smaller, the cluster 3 genes can be described most appropriately as influencing a disease-phenotype, specifically intrinsic cardiomyopathy ($p=0.028$). A closer look at the functions of the proteins includes a role in regulating folate metabolism and genomic integrity. Taken together, subjective feelings of energy can be linked to blood homeostasis, inflammation, and immune activation/wound healing (Figure 2).

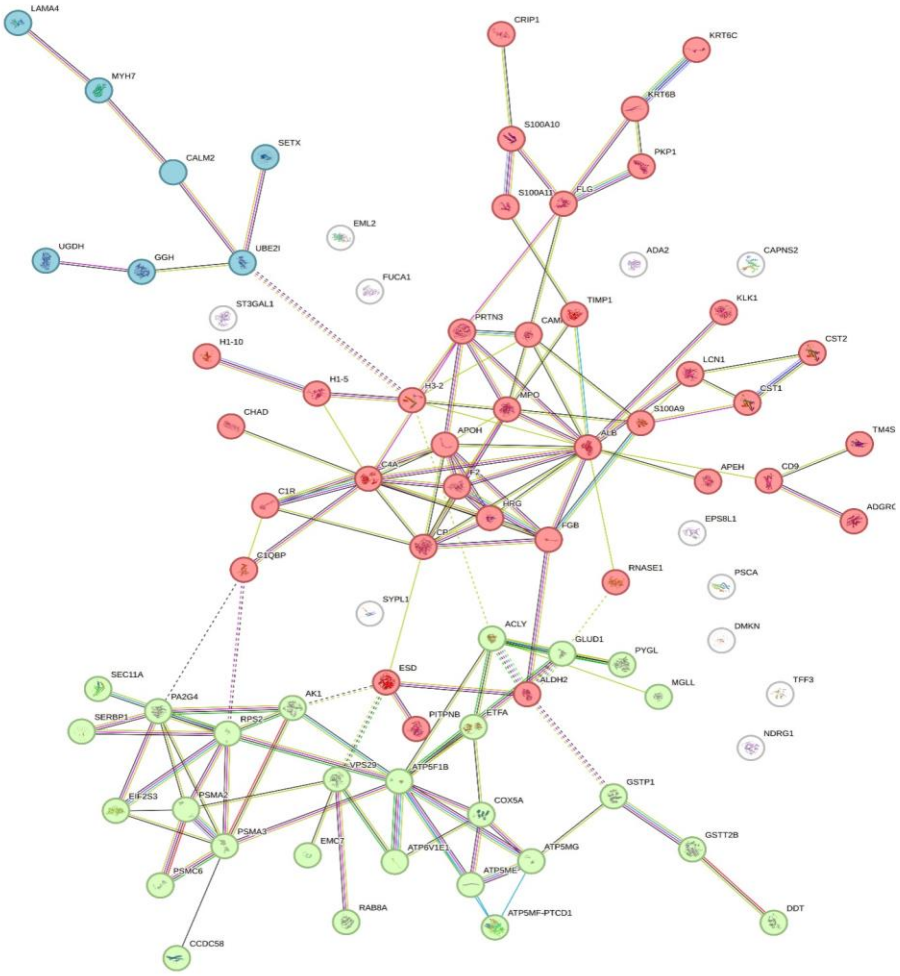


Figure 2. Pathway interactions across Energy. All groups (8) that contained energy were included, for a total of 88 genes, with 5 duplicates. K-means clustering utilized 3 clusters.

Cluster 1 (red, n = 37 genes) demonstrates significant functional enrichments in the biological processes involved in the humoral immune response and negative regulation of blood coagulation (Table 2). The only significant KEGG Pathway signal falls within the complement and coagulation cascades. Cluster 2 (green, n = 27 genes) demonstrates significant functional enrichments in the biological processes around the biosynthesis of ribose phosphate, nucleoside triphosphate, purine ribonucleotide, and ATP. The KEGG pathway that has the highest signal is oxidative phosphorylation. Albeit while containing only 7 genes, Cluster 3 (blue) demonstrates significant functional enrichments in disease-gene associations that are described as influencing intrinsic cardiomyopathy.

Table 2. Clustered proteins linked to subjective states of energy and fatigue.

Subject State Mood	Cluster 1	Cluster 2	Cluster 3	Cluster 4	Cluster 5
Energy only	ADGRG1, ALB, ALDH2, APEH, APOH, C1QBP, C1R, C4A, CAMP, CD9, CHAD, CP, CRIP1, CST1, CST2, ESD, F2, FGB, FLG, H1-10, H1-5, H3-2, HRG, KLK1, KRT6B, KRT6C, LCN1, MPO, PITPNB, PKP1, PRTN3, RNASE1, S100A10, S100A11, S100A9, TIMP1, TM4SF1s	ACLY, AK1, ATP5F1B, ATP5ME, ATP5MF-PTCD1, ATP5MG, ATP6V1E1, CCDC58, COX5A, DDT, EIF2S3, EMC7, ETFA, GLUD1, GSTP1, GSTT2B, MGLL, PA2G4, PSMA2, PSMA3, PSMC6, PYGL, RAB8A, RPS2, SEC11A, SERBP1, VPS29	CALM2, GGH, LAMA4, MYH7, SETX, UBE2I, UGDH	-	-
Physical Energy Only	AK1, ATP5ME, ATP5MF-PTCD1, ATP5MG, ATP6V1E1, CCDC58, COX5A, EIF2S3, ESD, ETFA, GSTP1, PITPNB, PSMA2, PSMA3, RAB8A, VPS29	C1R, C4A, CHAD, F2, H1-5, HRG	CST1, CST2, LCN1	GLUD1, PYGL	FLG, CAMP
Mental Energy Only	ACLY, ADGRG1, AK1, ALB, ALDH2, APEH, APOH, ATP5F1B, C1QBP, CD9,	PKP1, KRT6B, KER6C	DDT, GSTT2B		

	CP, FGB, GGH, H3-2, KLK1, MPO, PA2G4, PRTN3, RNASE1, RPS2, S100A9, SEC11A, SERBP1, SETX, TIMP1, UBE2I			
Fatigue Only	A2ML1, ACSL1, ACTN4, ALDOA, ANP32E, ANPEP, ARHGAP1, ARPC1B, ATP5PF, ATP6V1G1, CANX, CIT, DLST, DNAJB9, EEF1G, GAPDH, GOT1, GPX3, H2BC12, HSP90B1, IL1RN, IMMT, LMNB1, NCF4, PGK1, PSCA, PSMD11, PSMD7, RAB1B, RPL36, RPL4, RPS13, S100A8, SDCBP, SERBP1, SET, SFN, SRSF7, TMPRSS11B, TPT1, UBA1, UQCRC1, XDH, XRCC6	C1QC, C4A, CFI, CHAD, CLU, F5, IGLL5, LOC102723407, SBSN, SERPINC1, SERPINF1	S100A16, S100A14	
Physical Fatigue Only	ACTN4, ALDOA, ANP32E, ANPEP, C1QC, C4A, CFI, CHAD, CLU, F5, GOT1, GPX3, IGLL5, LOC102723407, SBSN, SERPINF1, TMPRSS11B,	CANX, DNAJB9, RAB1B, SDCBP	PSMD11, RPL36, RPL4, RPS13	ARHGAP1, CIT

	UQCRC1, XDH, XRCC6		
Mental Fatigue Only	ACSL1, ARHGAP1, DLST, DNAJB9, GAPDH, H2BC12, HSP90B1, IMMT, LMNB1, NCF4, PGK1, S100A8, SET, SFN, UBA1	EEF1G, PSMD7, RPS13, SERBP1, SRSF7, TPT1	CLU, SBSN, SERPINC1

Fatigue Only

When combining trait, state, absolute change, and relative changes in subjective indices of mental and physical fatigue, fatigue had the most duplicated genes across the 2 clusters (adjusted $p<0.05$) with 18 duplicates and a triplicate. The largest cluster enriched in biological processes is appropriately characterized by catabolism ($p=0.008$), with a high prevalence of proteins that play a role in ubiquitination and the Unfolded Protein Response (UPR). Within the KEGG pathways are the interacting pathways of carbon metabolism ($p=0.002$), biosynthesis of amino acids ($p=0.004$), and glycolysis/gluconeogenesis, in addition to the reactome pathway of gluconeogenesis ($p=0.001$). There are also key regulators of oxidative stress and uric acid production seen in cluster 1. The presence of multiple ribosomal proteins (RPLs) is noted in this cluster as well. Cluster 2 has functional enrichments of biological processes that highlight the classical pathway of complement activation ($p<0.001$) and activation of the innate immune response ($p=0.001$). IGLL5, also known as immunoglobulin lambda like polypeptide 5, is believed to play a role with B-cell development (Figure 3).

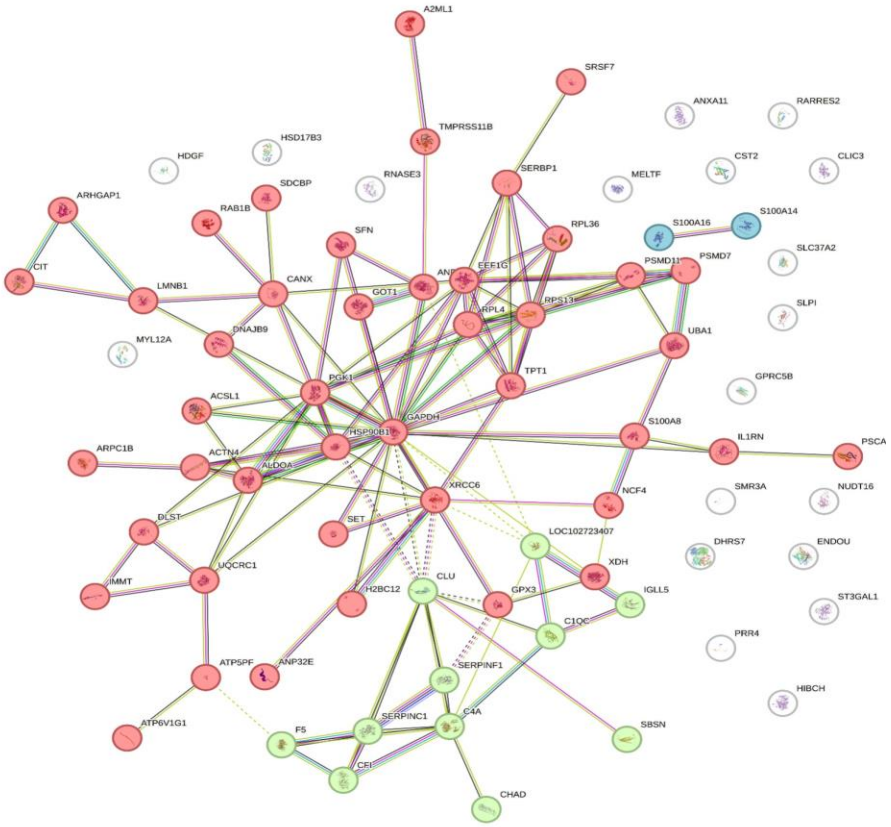
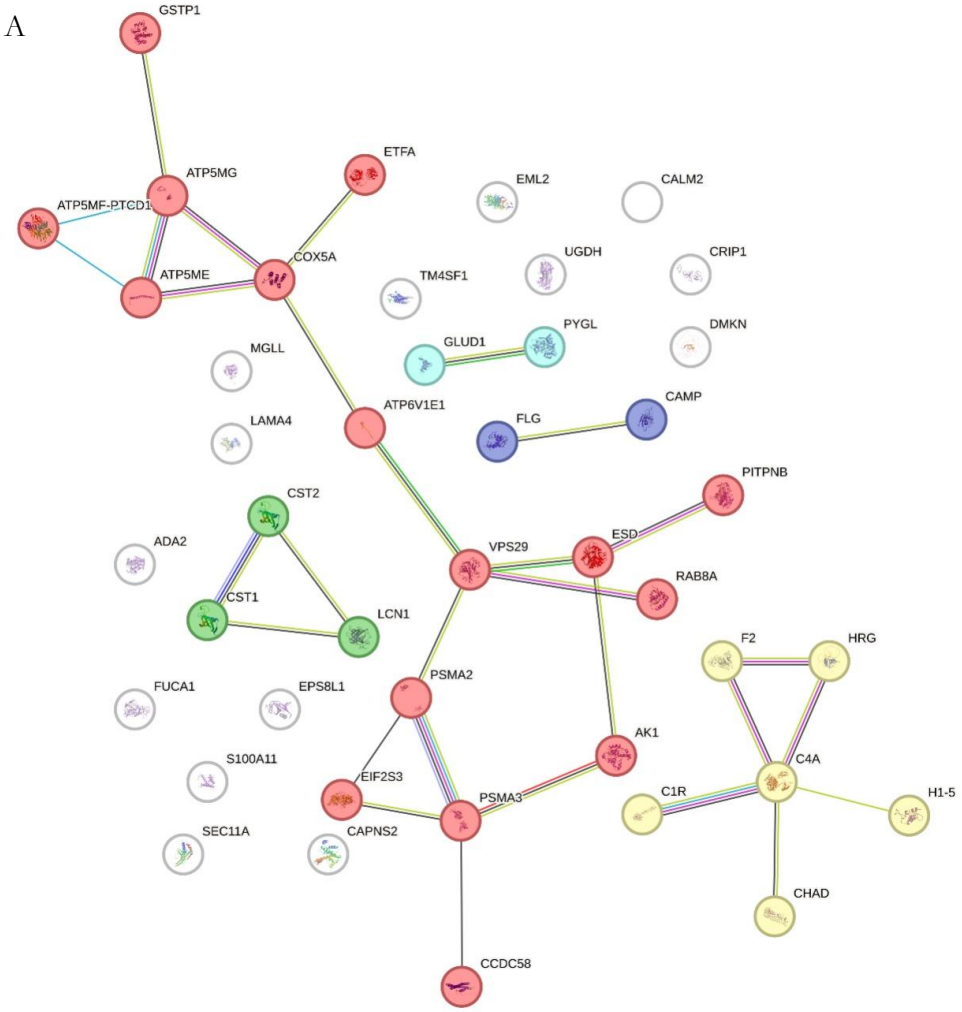


Figure 3. Pathway interactions across Fatigue. All groups (8) that contained Fatigue were included, for a total of 88 genes, with 18 duplicates and 1 triplicate. K-means clustering utilized 3 clusters.

Cluster 1 (red, *n* = 44 genes) demonstrated only one significant functional enrichment in the biological processes and that was catabolism (Table 2). Significant signals from KEGG pathways included those involved in carbon metabolism, biosynthesis of amino acids, and glycolysis/gluconeogenesis. Cluster 2 (green, *n* = 11 genes) demonstrated several significant functional enrichments in the biological processes but the highest signal by far was the complement activation, classical pathway. The next highest signal was the innate immune response. The KEGG Pathway that was also significant was the complement and coagulation cascades. Cluster 3 (blue, *n* = 2 genes) only contained two genes characterized by the S-100/ICaBP type calcium binding domain: S100A16 and S100A14.

Physical Energy only: As key part of the global network describing energy in Figure 2, physical energy (Figure 4A) yielded 5 clusters, with the largest (red) being dominated by the nucleoside triphosphate biosynthetic (p=0.03) and ATP metabolic process (p=0.04), respectively. Cluster 2 was substantially smaller with only 6 genes and describes key components of the humoral immune response (p=0.008). Notably, cluster 2 contains CHAD, a regulator of chondrocytes. Cluster 3 highlights sensory perception of taste (p<0.001) and the KEGG pathway of salivary secretion (p=0.02). Cluster 4, despite having only two members, contains two metabolic proteins involved in the TCA cycle and carbohydrate metabolism, in GLUD1 (glutamate dehydrogenase 1) and PYGL (glycogen phosphorylase) respectively. Cluster 5 did not have any enrichments.



B

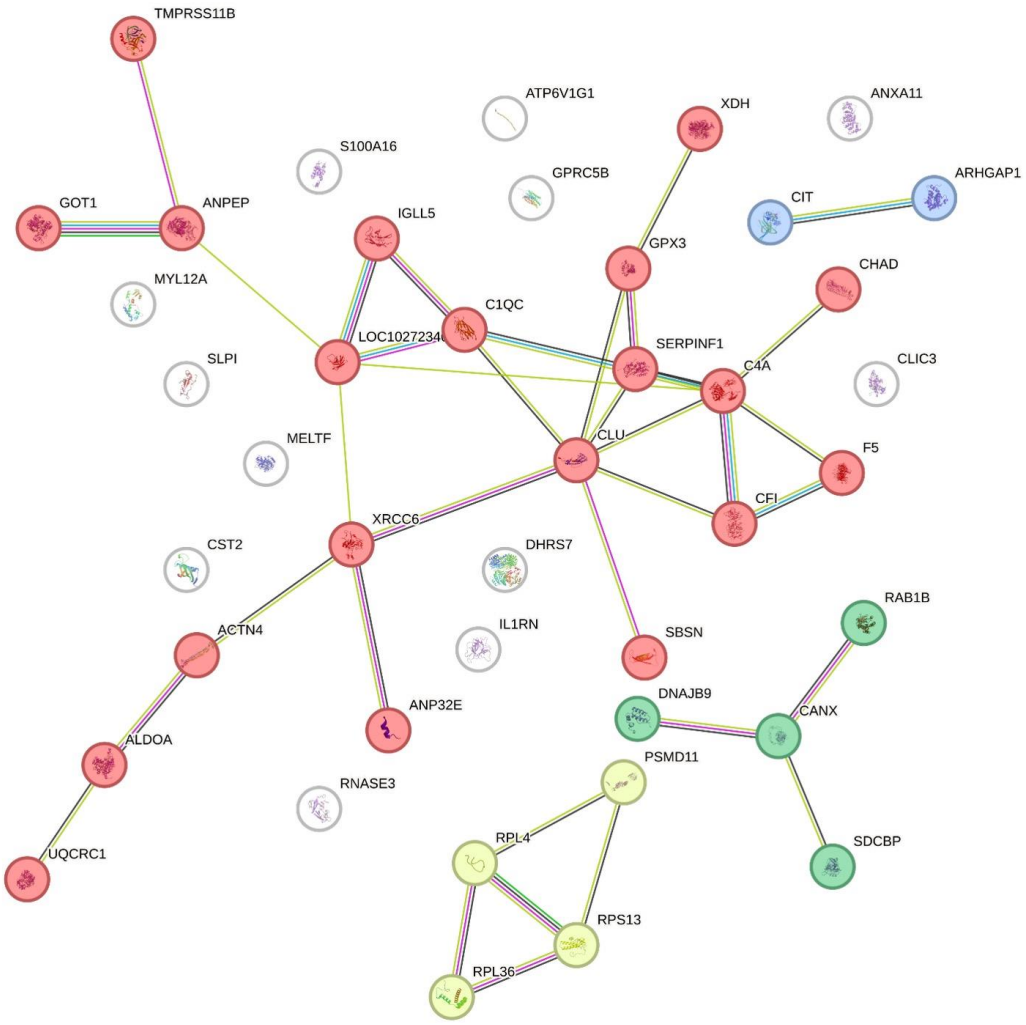


Figure 4A & B. Pathway interactions within Physical Energy and Physical Fatigue. All groups (4) that contained physical energy (A) or physical fatigue (B) were included, for a total of 44 genes. K-means clustering utilized 5 clusters within physical energy and 4 clusters within physical fatigue.

Cluster 1 (red, $n = 16$ genes) demonstrated two significant functional enrichments in two biological processes, including the nucleoside triphosphate biosynthesis and ATP metabolic processes. KEGG pathways highlight significance within oxidative phosphorylation. Cluster 2 (yellow, $n = 6$ genes) demonstrated one significant functional enrichment in the biological process of the humoral immune response. The top KEGG pathway identified is the complement and coagulation cascades but the remaining three are driven by infection and disease through C4A and C1R, including pertussis, staphylococcus aureus infection, and systemic lupus erythematosus. Cluster 3 (green, $n = 3$ genes) demonstrated two significant functional enrichments in the biological processes, and these relate to the sensory perception of taste and the detection of chemical stimulus involved in the sensory perception of bitter taste. Significance is also found with the KEGG pathway of salivary secretion. Cluster 4 (blue, $n = 2$ genes) demonstrated no significant functional enrichments in the biological processes but did have one KEGG pathway identified as significant, necroptosis. Cluster 5 (purple) also contained two genes (FLG, CAMP) but no significant enrichments.

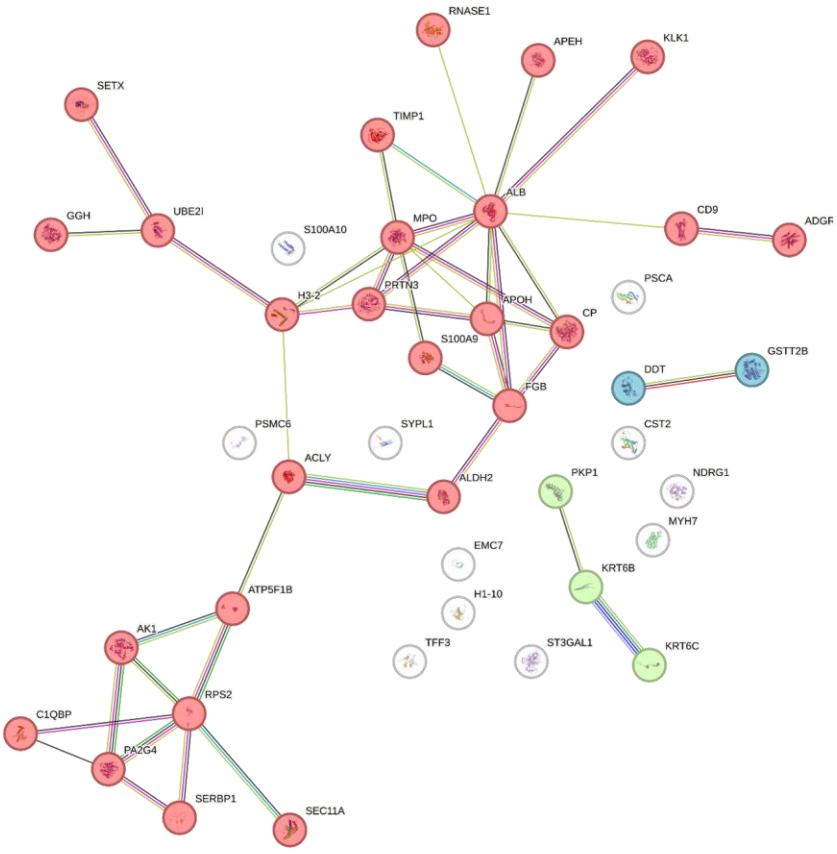
Physical Fatigue only: There were 4 clusters identified but the primary (e.g., largest cluster) is significantly enriched with the classical pathway of complement activation ($p<0.001$), activation of the immune response ($p<0.001$), and the innate response ($p=0.006$). In addition, there are also several roles for these genes including structural (ACTN4, ALDOA),

protein degradation (ANPEP), chondrocyte adhesion (CHAD), redox balance (XDH, GPX3,) and DNA repair (XRCC6). Additional impacts include hydrogen sulfide (GOT1) and angiogenesis (SERPINF1), all of which seem intuitively tied to physical fatigue (Figure 4B).

Cluster 1 (red, *n* = 20 genes) demonstrated significant functional enrichment of biological processes, which the largest signal by far being complement activation, classical pathway (Table 2). Additional significant enrichment was observed with activation of the immune response and the innate immune response. Cluster 2 (green, *n* = 4 genes) demonstrated no functional enrichments of biological processes but did have cellular components of the melanosome, ER membrane, and extracellular exosome. Indeed, all these proteins do function as assembly/transport proteins. Cluster 3 (yellow, *n* = 4 genes) demonstrated one significant functional enrichment of the biological process of cytoplasmic translation, which was further demonstrated by the significance of the ribosome KEGG pathway. Baring the PSMD11, which is part of the proteasome, the remaining 3 genes play a direct role in the ribosome, and work together to balance protein synthesis/breakdown. Cluster 4 (blue) only contained two genes (ARHGAP1, CIT). While there are no functional enrichments in biological processes or KEGG pathways, these two genes have a significant signal in 3 reactome pathways, including the RHOA/B/C GTPase cycle.

Mental Energy only: Despite the large primary cluster, there were no significant functional enrichments. However, the top two cellular components were secretory and azurophil granule lumen, in combination with reactome pathways that include platelet (p=0.001) and neutrophil degranulation (p=0.001), clotting cascade (p=0.01), and regulation of IGF transport and uptake (p=0.01). Both secretory and azurophil granules are well-characterized for their role in inflammation with the typical packages including lysosomal enzymes, MPO, proteases and anti-microbial defensins. We also see key metabolic components with ACLY, AK1, ATP5F1B and three zinc binding proteins. Cluster 2 contains only 3 genes, with significance in the biological process of intermediate filament organization (p<0.001). All have structural roles in the cytoskeleton, either as intermediate filaments or the closely associated specialized structures called junctional plaques.

A



B

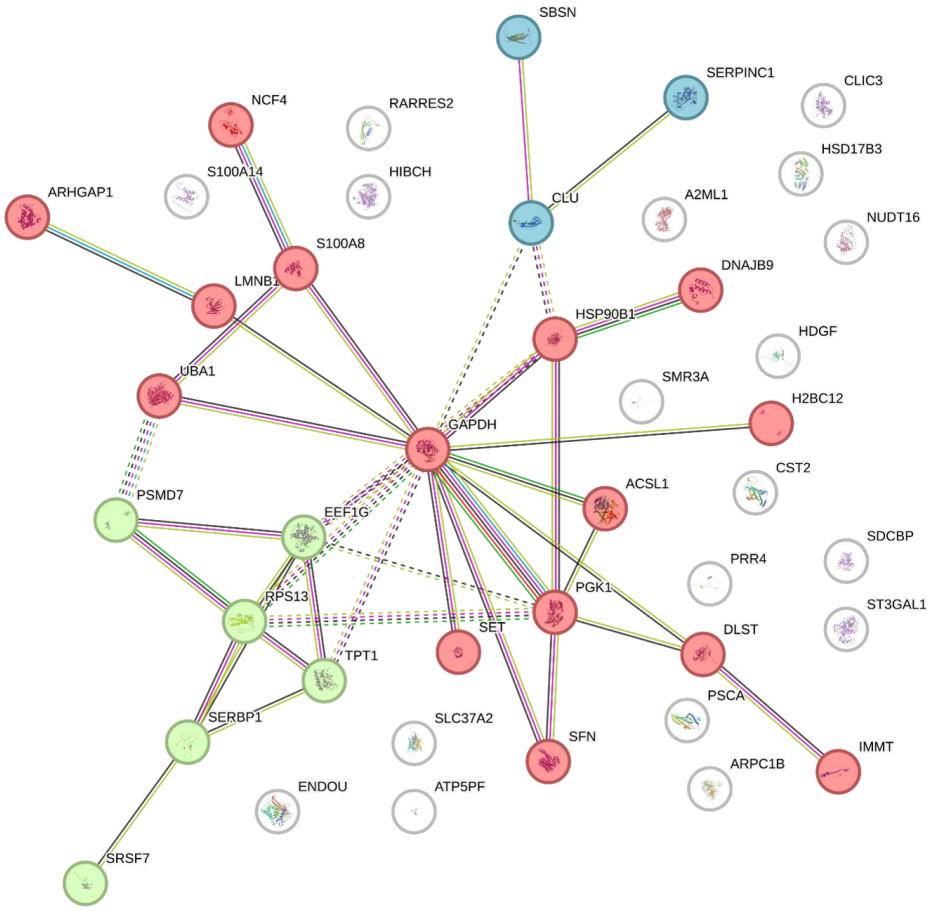


Figure 5A & B. Pathway interactions within Mental Energy and Mental Fatigue. All groups (4) that contained mental energy (A) or mental fatigue (B) were included, for a total of 44 genes. K-means clustering utilized 3 clusters within mental energy and 3 clusters within mental fatigue.

Cluster 1 (red, *n* = 26 genes) did not demonstrate any significant functional enrichments or KEGG pathways (Figure 5A). Interestingly, the top two cellular components with significant signals were in secretory and azurophil granule lumens, which is also consistent with reactome pathways targeting platelet and neutrophil degranulation. Cluster 2 (green, n = 3 genes) demonstrated one significant enrichment in the biological process of intermediate filament organization and a reactome pathway involving formation of the cornified envelope. Cluster 3 (blue, n = 2 genes) demonstrated no significant functional enrichments and only 2 genes (DDT, GSTT2B).

Mental Fatigue only: Interestingly, despite no functional enrichments of biological processes, the KEGG pathway of carbon metabolism was significant and the reactome pathways indicated Rho GTPases signaling and effectors. Metabolism is key, with ACSL1, DLST, GAPDH, and PGK1 illustrating this along with mitochondrial stability with IMMT. It is also indicative of a role in regulating nuclear stability, with H2BC12 being a key component of the nucleosome, LMNB1 being a framework for the nuclear envelope, SET being a part of nucleosome assembly, and UBA1 for DNA repair.

Cluster 1 (red, *n* = 15 genes) demonstrated no significant functional enrichments but did have one significantly upregulated KEGG pathway, specifically that of carbon metabolism ($p<0.001$). Cluster 2 (green, *n* = 6 genes) also did not demonstrate any functional or KEGG pathway enrichments but as a cellular component all were described within the extracellular exosome. Cluster 3 (blue, *n* = 3 genes) has a significantly upregulated KEGG pathway of complement and coagulation cascades ($p=0.01$) (Figure 5B).

Discussion

To our knowledge, this is the first study to identify the putative biological pathways and patterns associated with specific genes and pathways for the distinct mood states of mental and physical energy and fatigue. While these two states are often co-occurring, and previous findings have suggested unique yet some shared biological markers,[7,10–12,20] our findings provide molecular insights into these associations, further emphasizing the complexity of the physiological systems that are associated with these moods. Our findings suggest that when integrating both trait and state-based changes, as well as absolute and relative changes in subjective indices of energy and fatigue there are distinct molecular underpinnings of these moods. Although this is the first study using exploratory proteomics analyses, the distinct pathways identified for each mood state are in line with previously published works.[11,17,20] Taken together, these findings support previous works, while adding to the literature by identifying candidate pathways that are statistically associated with each mood state.

When examining subjective feelings of energy, our analysis identified three primary clusters, each reflecting distinct biological processes. The first cluster, with 37 genes, shows significant functional enrichment in humoral immune response and blood coagulation pathways, particularly within the complement and coagulation cascades. These findings align with previous studies that have identified hypoactivity in humoral immune response[55] and blood coagulation,[56] when fatigue was measured as the opposite of energy. In a prior exploratory study, Dupree and colleagues[20] reported that Annexin A1, a protein involved in humoral immunity and blood coagulation,[57] was associated with subjective feelings of energy only. Additionally, proteins such as CAMP, S100A9 and CST1 suggest a potential role for Vitamin D signaling, highlighting the importance of immune regulation and antioxidant activities[58] in sensation of energy. Emerging evidence also points to Annexin A1's role in regulating Vitamin D receptors (VDr),[59] further supporting this connection.

Previous literature has reported a link between subjective feelings of energy and resting metabolic rate,[11] skeletal muscle O_2 consumption,[11] $VO_{2Max,}$[21] and cardiorespiratory coordination.[7] Our findings corroborate these observations, as these parameters are intricately tied to the body's ability to produce ATP through oxidative phosphorylation, a process identified in Cluster 2. Further, changes in blood CO_2, anion gap, hemoglobin and hematocrit concentrations[7] are indirectly linked to ATP production through oxidative phosphorylation. These parameters help reflect the efficiency of aerobic ATP generation and the body's ability to maintain proper metabolic and acid-base balance. Collectively, the previously reported parameters[7,11,21] and those identified in our study reflect the efficiency of energy metabolism, and the role of ATP biosynthesis and purine nucleotide synthesis as potential contributors to subjective feelings of energy. Finally, we report a small cluster, with 7 genes that is primarily linked to intrinsic cardiomyopathy. Interestingly, these findings align with previous work that measured vitality/energy and fatigue as two distinct constructs, which find that vitality is associated with cardiovascular outcomes[60] and not with fatigue.

Fatigue was characterized by the largest number of duplicated genes, demonstrating complex molecular underpinnings with multiple overlapping pathways. This study found that Cluster 1 is predominately associated with catabolism and includes genes involved in ubiquitination, unfolded protein response, as well as key regulators of oxidative stress and uric acid production. These processes align with previous findings linking fatigue to serotonin,[17,22] histamine,[23] cortisol,[7] and inflammatory markers like TNF-α[17,20] and IL-6,[24] as these neurohormones and inflammatory markers play significant roles in inflammatory responses and cellular stress. Additionally, the presence of multiple ribosomal proteins suggests a connection to protein turnover and synthesis, an outcome that is influenced by cortisol.[61]

Our analysis identified a second cluster that is associated with the classical complement activation pathway and immune response activation, which aligns with previously published findings.[12,17,20] The presence of complement and coagulation cascades, identified in both this study and previous studies,[25,62] indicate that fatigue may not be a direct consequence of energy depletion but could also involve immune system activation, possibly due to ongoing inflammation or infection responses. These observations support the anthropological supposition made by Loy and colleagues[17] that subjective feelings of fatigue serve to influence avoidance-oriented behavior, such as promoting rest, thereby enhancing recovery from injury or illnesses.

Lastly, we also report a small third cluster containing only two genes (S100A16 and S100A14), suggesting potential novel insights into calcium-binding proteins. While these genes are primarily associated with cancer development and progression, they are also involved in calcium ion binding and may have indirect links to ion channel regulation.[63] This may explain the findings of Boolani and colleagues who reported an association between changes in blood chloride and sodium with changes in fatigue.[7]

When comparing physical and mental energy, we find distinct yet overlapping clusters, each associated with specific biological processes, metabolic pathways, and cellular components. While both sets of clusters contribute to the understanding of global network of energy, they highlight unique physiological mechanisms and emphasize different pathways related to metabolism, immune response, and cellular function. Physical energy clusters show a dominant focus on ATP production (cluster 1), metabolic regulation, including carbohydrate metabolism (cluster 4), and immune regulation (cluster 2). The inclusion of oxidative phosphorylation and ATP biosynthesis links physical energy directly to cellular bioenergetics. Conversely, mental energy lacks significant functional enrichment related to metabolism and focuses more on inflammatory processes and structural roles in cells (Clusters 1 and 2). Although metabolic components are present, such as ACLY and ATP5F1B, the primary focus is on inflammatory granules and immune pathways that support mental alertness. Genes S100A9 and FGB influence the TLR2/4 signaling pathway, a key mediator of the antimicrobial inflammatory response were also present. This contradicts prior findings which suggests that fatigue is associated with innate immunity, such pro-inflammatory gut microbiota[12], inflammatory cytokines and immune system activation.[17,20] These findings support literature reporting an association between physical energy and cardiorespiratory coordination,[7] intensity of exercise,[7] skeletal muscle oxygenation,[11] resting metabolic rate,[11] carbohydrate metabolizing gut microbiota,[12] blood hemoglobin and hematocrit concentration;[7] and associations between mental energy and Annexin A1.[20]

Although our findings contradict those of Marcora and colleagues,[21,64] an explanation for these differences may be that Marcora and colleagues only collected data on mental energy and mental fatigue and did not collect data on changes physical energy and physical fatigue. Previous findings from our lab suggest that individuals who are asked to perform seated mental tasks also report a decline in physical energy.[35,65,66] Another discrepancy between our findings and those previously published findings is the association between blood anion gap and CO_2 concentrations and mental energy.[7] While changes in blood anion gap and CO_2 concentrations are best explained by clusters 1 (ATP metabolism and oxidative phosphorylation) and 4 (TCA cycle and carbohydrate metabolism) of physical energy, there may be some indirect insight from mental energy clusters, particular through immune response and inflammatory pathways, which may influence CO_2 levels via effects on lung function and acid-base balance. However, their direct impact on blood CO_2 and anion gap is less pronounced compared to the metabolic pathways found in physical energy.

Both physical and mental fatigue clusters reveal distinct biological processes, yet there are overlapping elements particularly in immune activation and protein metabolism. While both forms of fatigue share an element of immune response activation, physical fatigue emphasizes complement activation and inflammation, while mental fatigue shows involvement with the complement and coagulation cascades. Our findings support previously published literature that identified leptin impacting physical fatigue[17,67] as leptin could potentially play a large role in cluster 4 of physical fatigue, by influencing metabolic processes. In cluster 4, which involves the TCA cycle and carbohydrate metabolism, leptin could affect ATP production and energy metabolism by disrupting nutrient utilization and energy balance.[68] Further serotonin a neurotransmitter known to influence physical fatigue[17,28] has an indirect connection to Cluster 3 in physical fatigue, which is focused on protein synthesis. Serotonin is often linked to muscle weakness and fatigue,[69] which might be mediated by protein degradation and disruption of muscle recovery processes associated with Cluster 3 in physical fatigue.

Further, previous findings suggest that histamine impacts mental fatigue only.[17,23] Histamine is involved in neuroinflammation[70] and neurotransmission,[71] and could contribute to neuroinflammation in cluster 2 of mental fatigue, affecting neuron-to-neuron communication through extracellular exosome mechanisms. TNF-α and IL-6 are both associated with mental fatigue and may be associated with both cluster 1 (carbon metabolism, mitochondrial stability, and DNA repair) and 3 (complement and coagulation pathways) in mental fatigue. Elevated TNF-α and IL-6 may contribute to neuroinflammation in the brain,[72] exacerbating mental fatigue by impairing cognitive function and synaptic plasticity.[73] Sodium and chloride imbalances also known to negatively impact mental fatigue[7] could amplify these cytokine responses.[74] Further, cortisol, also known to be associated with mental fatigue[7] may play a role in cluster 1 of mental fatigue by impairing brain function and energy production in the brain leading to mental fatigue.[75] Another interesting finding is that both forms of fatigue involve DNA repair mechanisms (XRCC6 in physical fatigue, UBA1 in mental fatigue), pointing to the idea that cellular damage and the inability to maintain genomic integrity may be key features of both physical and mental fatigue.

This study has several limitations including the small sample size; however, the findings of this study align well with previously published findings suggesting that even with a small sample size the findings may not be spurious. Another

potential limitation of this study is the heterogeneous response in both subjective and objective responses to the fatiguing intervention. Although the fatiguing intervention was standardized by time, the workload during the intervention was relative, which may have resulted in varied responses. However, the varied responses may have helped identify the proteins associated with these mood outcomes, since directionally the changes in proteins and mood outcomes would have to be similar. Another potential limitation is the reliance on enrichment tools, and a lack of replication.

Conclusions

The objective of this study was to identify the underlying biological processes and their associations with specific genes and pathways of the distinct mood states of mental and physical energy and fatigue. Using an interventional trial, we found that in line with previous findings energy is primarily associated with the complement and coagulation cascades and metabolic pathways, while fatigue is associated with catabolism, complement activation, and innate immune response. Interestingly, this study also identified two unique functional enrichments, with energy being associated with disease-genes for intrinsic cardiomyopathy, while fatigue was associated 2 genes associated with calcium-dependent signaling and regulation of various cellular processes. These findings provide new insights into the molecular underpinnings of subjective feelings of mental and physical energy and fatigue and may offer hypotheses for future research aimed at identifying therapeutic targets for mitigating these moods.

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Conflict of Interest. The authors declare no conflicts of interest.

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