

WITHDRAWN: LC-MS-Based Urine Metabolomics for the Identification of Biomarkers Related to The Occurrence and Severity of Flight Fatigue

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EDITORIAL NOTE:

The full text of this preprint has been withdrawn by the authors while they make corrections to the work. Therefore, the authors do not wish this work to be cited as a reference. Questions should be directed to the corresponding author.

Abstract

Background: Aircrew fatigue is a major contributor of operational errors in civil and military aviation, which translates a comprehensive performance associated with both neuromuscular and cognitive states. Here we have used untargeted and non-invasive urinary metabolomics to explore flight fatigue-related aberrations. In this sense, we aimed to identify biomarkers that could better monitor pilot fatigue and also assess its severity to prevent 'nonfunctional over-reached' state, thus promoting flight safety.

Methods: In this study, 22 active-duty male pilots, who conducted different flight hour duties, were recruited to mimic different levels of fatigue. For this, respective urine samples were collected, before and after flight, and analyzed by liquid chromatography/mass spectrometry (LC/MS).

Results: Except for the fatty acids and some amino acids, significant changes on metabolite levels were observed during the progression of flight fatigue. Most of these metabolites corresponded to acyl carnitines, carbohydrates, purines and indoles. The majority of amino acids were downregulated after the flight mission. A total of 61 metabolites were found to be significantly changed along with the extent of flight fatigue. To efficiently discriminate the occurrence of flight fatigue, three candidate biomarkers (beta-guanidinopropionic acid, 3-dehydro-L-gulonate and 2-propylpent-3-enoic acid) were further characterized. Lastly, Bayes discriminant function models were established to stratify pilots with severity of fatigue and, therefore, to aid in flight risk management.

Conclusion: To our knowledge, this study inaugurally provides a metabolic profiling in response to flight fatigue, thus offering a novel and effective way to monitor and manage this physiological condition.

Introduction

Contemporary military aviation is stressful and complex in nature. As such, some substantial demand on attention and cognitive ability are required in order to provide safe operations (1), which potentially make pilots prone to mental fatigue, accompanied by certain physical stress. Fatigue can largely impact the success of an air mission, mostly due to its effects on a number of performance variables including reaction time, accuracy, attention, and executive decision (2, 3). In fact, fatigue has been recognized as major contributing factors to operational errors (4). This condition is considered a common problem in military aviation (5), and growing evidence have indicated that fatigue can degrade neurobehavioral performance and fundamental piloting skills. The National Aeronautics and Space Administration's aviation safety report has disclosed that almost 20% of flight accidents are directly or indirectly related to fatigue (6). Flight fatigue is the most common physiological factor that leads to military accidents, costing hundreds of millions of dollars in lost equipment in addition to the incalculable value of well-trained pilots (7).

Although flight fatigue is one of the most prominent issues faced by the aviation health protection labor, there is still a lack of fast and straightforward evaluation methods as well as effective countermeasures. The standard practice to determine whether a pilot is fit to fly is mainly based on the commander's

personal assessment and experience, or on responses to subjective self-report measures (8, 9). As a result, the fatigue severity is evaluated according to a performance status scale (3). Still, subjective indicators of fatigue are problematic, especially in the military (10, 11), due to their limited sensitivity to small variations and the susceptibility to biases arising from personal and motivational factors, such as social acceptance (12, 13). In this context, health care professionals are frequently frustrated by the lack of an objective technology that may assist with a more precise and faster diagnosis, so the development of non-invasive methods to monitor and detect fatigue in pilots is an area of great interest (14, 15).

Flight fatigue is a comprehensive state caused by many factors (16), which result in changes not only in a psychological level, but also in regard to the biochemistry of the human body as an integrated system. Nevertheless, the underlying biochemical mechanisms correlated with this physiological condition are poorly understood. Metabolomics represents a comprehensive area of study, characterized by the high sensitivity and selectivity of metabolites that may correlate with biochemical perturbations in different organisms. This scientific area has been able to enhance the understanding of pathophysiological mechanisms (17–19) and, upon the establishment of novel diagnostic markers (20–22), it has been already used for exploring the underlying mechanisms of physical (23–25) and mental fatigue (22). However, few metabolomics studies have specifically aimed the issue of flight fatigue, which is mainly related to mental fatigue but accompanied by certain physical exhaustion. Understanding and monitoring these particular processes, in order to manage exposure to the continuum of fatigue, may play a potentially important role in flight safety. Hence, here we have evaluated the potential differences in the metabolic phenotypes of a representative number of pilots, based on the collection of urine samples before and after long-term flight missions. These samples were presently utilized to develop a novel, highly sensitive and non-invasive methodology to monitor pilot fatigue, linking the metabolic phenotype with flight fatigue state to the discovery of potential biomarkers. In addition, we further explored the molecular mechanism of flight fatigue, thus providing new insights for the development of molecularly targeted drugs that could accelerate fatigue elimination.

Materials And Methods

Study Populations

Twenty-two active-duty man pilots, with ages ranging from 26 to 45 (34.36 ± 4.55) years old, were recruited for this study. Eight pilots flew for 3 hours during the day, while seven others flew for 4 hours and the remaining ($n = 7$) had a single flight time of three hours during the day and four hours during the night. Urine samples from respective pilots were collected, before and after the long-term flight missions, and then stored at -80°C until analysis. This study was approved by the ethics committee of the Air Force Medical Center in accordance with the Declaration of Helsinki.

Sample Preparation and Metabolomics Analysis

After thawing the urine samples on ice, a 100 μl aliquot (per sample) was extracted with 300 μl methanol by vigorous vortexing. Subsequently, extracts were centrifuged at $13,000 \times g$ for 15 mins to pellet each

protein precipitate. A total of 100 μ L supernatant per sample was transferred to a 200 μ L vial insert for further analysis. A 1 μ L aliquot of each supernatant was injected into a Thermo Scientific™ Dionex™ UltiMate™ 3000 Rapid Separation LC (RSLC) system, to further perform an ultra-high performance liquid chromatography separation with a reversed phase C18 column (2.1 x 100 mm, 1.7 μ m, waters), operated at 45°C. The gradient mobile phase was composed of methanol (A) and water (B) with 0.1% formic acid and 10mmol/L ammonium acetate. Gradient elution was performed at the flow rate of 0.3 mL/minute. For this, an initial condition of 80% of mobile phase A were maintained for 1 min, followed by a linear gradient to 0% A for 12 mins. After a 0.5-minute washing step, the column was equilibrated to the initial condition for 1.5 mins.

The eluate was then introduced by electrospray ionization into the Q Exactive™ hybrid quadrupole Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) that operated in positive (ESI+) and negative (ESI-) electrospray ionization modes (one run for each mode) with a spray voltage of 3.7 kV and 3.5kV, respectively. The HESI (heated electrospray ionization) source utilized, for both modes, a capillary temperature of 320°C, heater temperature of 300°C, sheath gas pressure of 30 psi, and auxiliary gas pressure of 10 psi. During the full-scan acquisition (ranging from 50 to 1500 m/z), the instrument operated at 70,000 resolution (m/z = 200), with an automatic gain control (AGC) target of 1×10^6 charges and a maximum injection time (IT) of 50 ms.

To avoid artifacts due to the order of injected samples, these were randomly processed. Quality control (QC) samples were prepared by pooling equal volumes of each serum sample and injected every 6–8 samples throughout the analytical run to monitor the stability and reproducibility of the system.

Data processing

The peak picking, identification, alignment and normalization of the acquired data were all conducted by the Progenesis Q1 data analysis software (Nonlinear Dynamics, Newcastle, UK). The processed data were imported into SIMCA-P 13.0 software (Umetrics, Umeå, Sweden) for multivariate pattern recognition analysis. PCA was performed to detect outliers, and the distribution of different groups. OPLS-DA was carried out to obtain an overview of the complete data set after centering mean values and scaling unit variance (UV) (18).

Statistical Analysis

Respective data were expressed as the mean \pm standard deviation (SD) or standard error of the mean (SEM). Paired two-tailed Student's t-tests were conducted using GraphPad Prism 5.0 software. Metabolites with variable importance in projection (VIP) scores that were greater than 1.5 and $P < 0.05$ were considered statistically significant. Hierarchical cluster analysis (HCA) was conducted using the MeV software package (version 4.9.0). A correlation network was constructed using the Cytoscape software package. Receiver operating characteristic curve (ROC), binary logistic regression and Bayes discriminant analysis were also conducted with the SPSS software. The correlation analysis between variables were performed in R version 3.6.2.

Results

The metabolic profiling of the urine samples was performed in a random order and the representative total ion current (TIC) chromatograms of pilots before and after performing long-term flight are shown in Figure S1 (in the Supplementary Material).

Altered Metabolic Profile Under Flight Fatigue

The metabolic profiling of each urine sample was performed randomly. After multivariate statistical analyses were conducted (SIMCA-P version 13.0), the principle component analysis (PCA) scores plots of both ESI+ and ESI- showed a clear cluster of the QC samples ($R^2X = 0.611$, $Q^2 = 0.140$ for ESI+; $R^2X = 0.582$, $Q^2 = 0.113$ for ESI-), validating the high stability and reproducibility of the instrument (Figs. 1A and 1D). The models of supervised orthogonality partial least squares discriminant analysis (OPLS-DA) were then performed after excluding respective outliers (Figs. 1B and 1E). These models exhibited apparent metabolic variations in metabolome profiling when comparing before and after flight groups (BF vs. AF). As indicated, metabolites with $VIP > 1.0$ were colored in red (Figs. 1C and 1F).

Paired two-tailed Student's *t*-tests were performed, where 70 and 72 metabolites exhibited significant changes ($VIP > 1.0$, $P < 0.05$ and $CV < 30\%$) after long time flight in ESI+ and ESI- modes, respectively. The relative normalized quantities of the differential metabolites identified in AF (as compared to those in BF group) were further visualized in a heat map, according to their Pearson correlation coefficients (Figs. 2A and 2B). As shown, almost two thirds of these metabolites were decreased in response to long time flight (Figs. 2A and 2B). According to a Spearman correlation analysis, we further examined how the metabolite abnormalities could be related to the status of flight fatigue (Figs. 2C and 2D). A strong correlation between the severity of fatigue with several metabolite classes were identified. Fatty acids and nine amino acids were in general positively correlated with flight fatigue, while acyl carnitines, carbohydrates, purines, indoles and the majority of amino acids were negatively correlated. Overall, the levels of these important metabolites were significantly altered in urine samples after long-term flight, and then considered to be associated with flight fatigue.

The biological pathways involved in the metabolism of these differential metabolites as well as their biological roles were determined by enrichment analysis using MetaboAnalyst (Fig. 2E). All matched pathways were shown according to (i) the *p*-values from the pathway enrichment analysis (y-axis) and (ii) the pathway impact values from pathway topology analysis (x-axis) (26, 27) (most impacted pathways are colored in red, Fig. 2E). As a result, nine biochemical pathways including (i) phenylalanine, tyrosine and tryptophan biosynthesis, (ii) pyrimidine metabolism, (iii) tryptophan metabolism, (iv) lipoic acid metabolism, (v) phenylalanine metabolism, (vi) ascorbate and aldarate metabolism, (vii) purine metabolism, (viii) pentose and glucuronate interconversions, and (ix) valine, leucine and isoleucine biosynthesis, were considered closely related to flight fatigue.

Differential Metabolites Related to Flight Fatigue

Our cohort of active-duty male pilots were further divided into three groups, according to their flight time: (i) pilots who flew for three hours during the day (before flight group: BFL, after flight group: AFL), (ii) pilots who flew for four hours during the day (BFM and AFM groups), and (iii) pilots who had a single flight time of three hours during the day and four hours during the night (BFH and AFH groups). Since flight time is frequently correlated with the extent of fatigue, we hypothesized that changes in the levels of certain metabolites could emerge during the early stages of flight fatigue and then evolve according to the extent of fatigue. Consequently, OPLS-DA models for both ESI+ and ESI- have shown a clear separation of each pilot group before and after the flight, as well as among the three distinct groups (See Supplementary Figure S2), indicating that metabolic alterations occurred during flight fatigue progression. Thus, pair-wise comparisons were carried out based on OPLS-DA models. As a result, the levels of 29 metabolites in ESI+ mode (25 significantly reduced and 4 increased) and of 32 metabolites in ESI- mode (26 metabolites significantly reduced and 6 increased) were consistently altered (Fig. 3). These metabolites gradually changed according to the extent of fatigue, suggesting some strong and direct correlation between certain metabolites and flight fatigue (See Supplementary Figure S3).

Biomarkers of Flight Fatigue

To screen metabolites that could serve as potential biomarkers in flight fatigue states, we validated the differential metabolites in both AF and BF pilot groups. Based on the significantly changed metabolites in ESI+ (n = 70) and ESI- (n = 72) modes, a binary logistic regression was conducted to identify an optimal combination of metabolites as the potential biomarkers for AF. As a result, one metabolite for ESI+ (i.e. beta-guanidinopropionic acid) and two metabolites for ESI- (i.e. 3-dehydro-L-gulonate and 2-propylpent-3-enoic acid) were identified as potential biomarkers for flight fatigue. The area under the curve (AUC) of the ROC was subsequently calculated to evaluate the diagnostic performance of these biomarkers. ROC curves were also used for this same purpose (Figs. 4A and 4B). The area under the curve (AUC) of the ROC for ESI+ was 0.975, with an accuracy of 93.18%, a sensitivity of 95.45% and a specificity of 90.91%, while the AUC of the ROC for ESI- was 0.986, with an accuracy of 95.45%, a sensitivity of 90.91% and a specificity of 100%.

Multiple Discriminant Analysis of the Extent of Flight Fatigue

Although flight fatigue is a prominent issue that impacts mission success and flight safety, there is still a lack of direct evaluation methods to distinguish the fatigue severity and, therefore, to determine whether a pilot is prepared to fly. To address this issue, a Bayes discriminant function model was established by stepwise discriminant analysis of differential metabolites. According to this analysis, 10 and 7 statistically significant variables (related to ESI+ and ESI- modes, respectively) were included in the final discriminant function models. A retrospective discrimination was conducted among the individuals presently tested (Figs. 5A and 5B), which resulted into an excellent discriminant performance, with an accuracy of 95.5% and 97.7%, respectively (in Supplementary Tables S1-S4).

Discussion

Flight fatigue is the most frequently cited physiological factor affecting aircraft pilots, which contributes to the occurrence of accidents in the military aviation. As such, higher levels of attention and cognitive ability are required due to the stressful and complex nature of this condition. Besides, flight timing is expected to be an important determinant of pilot fatigue and fatigue level. Thus, the discovery of straightforward and sensitive indices that may detect fatigue and also monitor fatigue levels is critical to assess any associated safety risk and prevent air-related catastrophes (28, 29).

Urine is a non-invasive fluid to collect which contains many biomolecules of valuable diagnostic information(30). In this study, we retrieved urine samples from a cohort of 22 military pilots, before and after performing different periods of flight missions, for a comprehensive metabolomic investigation to particularly explore the metabolic characteristics of flight fatigue. To our knowledge, this is the first report involving the metabolic profiling of aircraft pilots exposed to different levels of fatigue severity.

In the present study, some significant metabolic alterations were observed during the progression of flight fatigue. Apart of fatty acids and a number of amino acids, most of the metabolites were downregulated after conclusion of flight mission. Carnitines play an important role in the transport of fatty acids across the inner mitochondrial membrane. According to the current literature, carnitines can also improve energy levels and physical function by reducing fatigue and improving cognitive functions (31). In our study, carnitines are significantly decreased in fatigue pilots, suggesting that flight-related stress may alter energy metabolism. We may speculate that, while performing stressful flight missions for a long period of time, pilots may consume more lipids to acquire sufficient energy. Furthermore, carbohydrate levels were expectedly decreased after flight. In contrast, the levels of isocitrate (intermediate involved in energy conversion via the Krebs cycle) were obviously elevated, highlighting the activation of aerobic pathways during long-time flight. Consistent with the findings in mental fatigue (32), exhaustive exercise (33) and chronic fatigue syndrome (34), most of the amino acids presently identified (75.68%) were negatively correlated with flight fatigue, implicating that amino acid consumption were accelerated in subjects performing extensive mental or physical tasks. Besides their utilization in protein synthesis, amino acids have also been involved in various metabolic activities in the brain. The serotonin precursor 5-Hydroxy-L-tryptophan (5-HTP) can easily cross the blood-brain barrier (BBB) and effectively increase the synthesis of serotonin in the central nervous system (CNS) (35). According to our data, the reducing urinary levels of 5-HTP suggest that mental activity may accelerate the uptake of this amino acid derivative into the brain. Interestingly, we have also observed a significant decrease of purines and purine derivatives in the urine samples of pilots after flight. The decreased excretion of purines may be consistent with the increased synthesis and/or turnover (flux) of ATP and GTP under high tension state. As the end product of purine metabolism and also an important antioxidant, uric acid has also increased significantly in our studies. In general, flight fatigue can cause prominent alterations in the urinary metabolome.

Lastly, potential biomarkers of flight fatigue were presently revealed by multivariate statistical analysis, combined with the binary logistic regression analysis (Fig. 4). Specifically, three metabolites were

selected as candidate biomarkers, with remarkable sensitivity and specificity to accurately identify fatigue state. Furthermore, Bayes discriminant function models were established for the diagnosis of fatigue severity. Such insights can be potentially applied for the early detection of fatigue conditions, thus providing some valuable information about whether aircrew would be authorized to fly. As such, this information serves as a critical tool for primary prevention and evaluation of flight safety. Monitoring some of these biomarkers levels may help optimizing flight conditions and/or pilot training to achieve a 'functional over-reached' state, in order to promote positive adaptation and increased performance following rest. Accordingly, we may prevent the development of a 'nonfunctional over-reached' state, which is a consequence of intense training leading to decrement in ability even with adequate rest (36).

Conclusion

Pilots fatigue is the most frequently cited physiological factor contributing to the occurrence of accidents in the military aviation, which requires high attention and cognitive ability due to its stressful and complex nature. Therefore, the discovery of objective and sensitive indices to detect fatigue and monitor fatigue levels is critical to assess the associated safety risk and prevent accidents and catastrophes. In this study, we collected the urine samples from recruit 22 military pilots before and after performing different time of flight missions for comprehensive metabolomics investigations to explore the metabolic characteristics of flight fatigue, and to identify objective and sensitive potential biomarkers for monitoring fatigue levels.

Monitoring the recovery of severe or chronic flight fatigue is of particular importance for commanders to effectively program training regimes/schedules to better emphasize positive adaptation. Given that these metabolomic markers have been primarily observed in a relatively small cohort, additional research is required using larger sample sizes to validate our current findings and then identify stronger relationships among the multiple variables involved.

Declarations

Author contributions

R.G. and F.J. were responsible for the execution of experiments, data analysis and preparation of the manuscript. F.Z.J. and M.Y.Z. helped with the statistical analysis of the data and contributed to the interpretation of the results. W.J.C. and Y.H.L. designed and supervised the study. All authors critically commented on and approved the final submitted version of the paper.

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Availability of data and materials

The data and materials used in the current study are all available from the corresponding author upon reasonable request.

Ethics approval

This study was approved by the ethics committee of the Air Force Medical Center.

Conflict of Interest

The authors have declared that they have no conflict of interests.

Consent for publication

Not applicable

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Supplementary Files

Supplementary Figures and Tables are not available with this version.

Figures

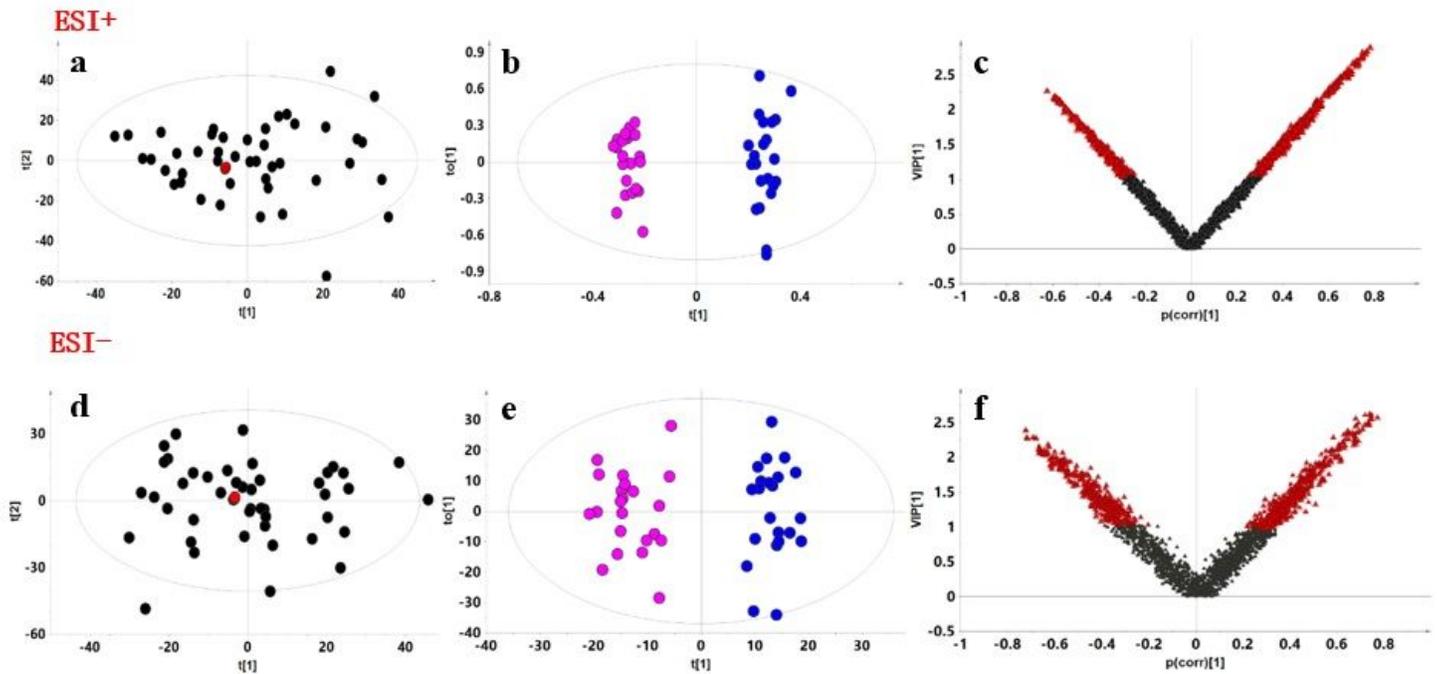


Figure 1

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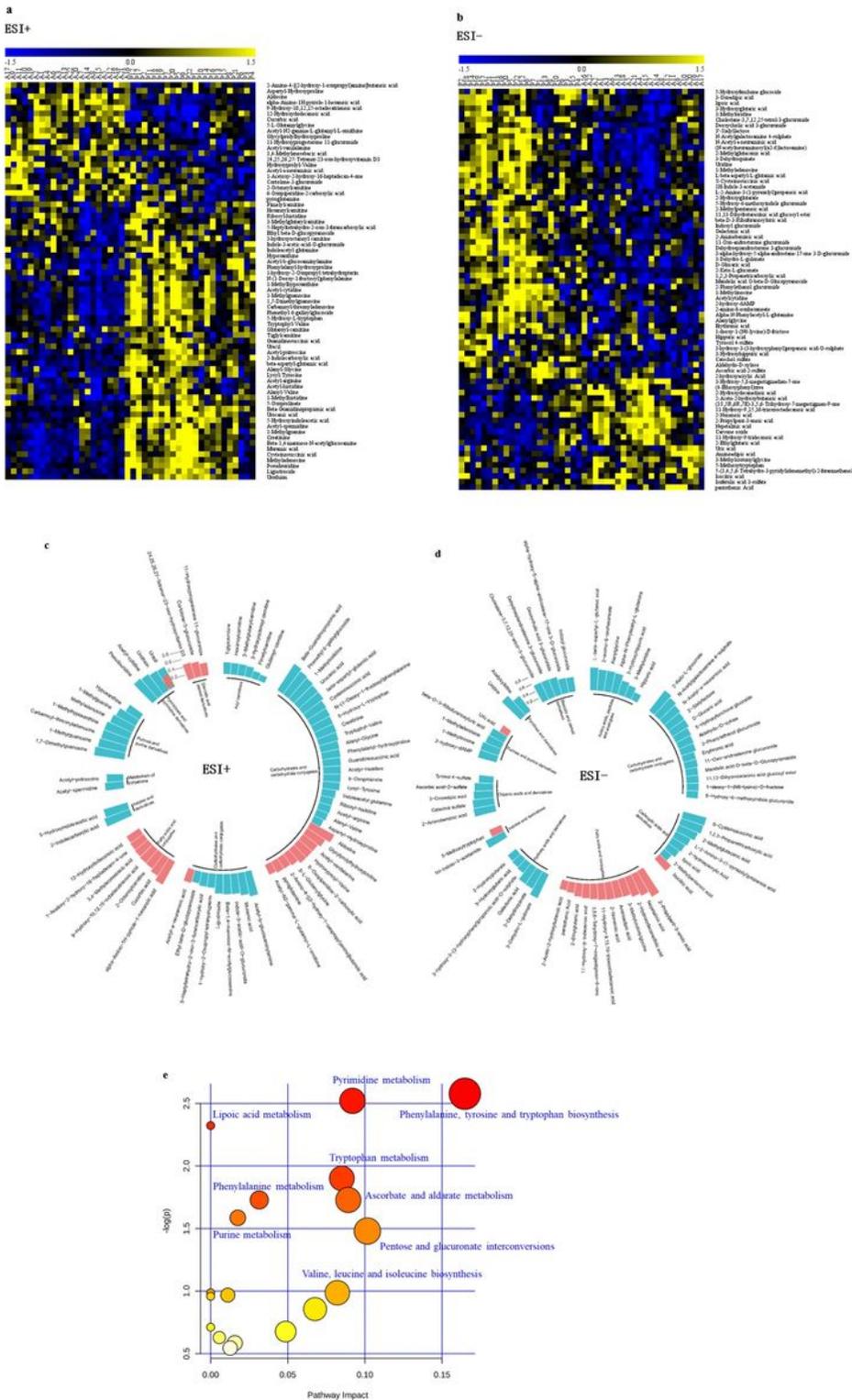


Figure 2

Differential metabolites related to flight fatigue. a and b, the hierarchical cluster analysis of the differential metabolites in ESI+ and ESI- modes. The relative normalized quantities of the identified differential metabolites in AF compared to those in BF group were further visualized in a heat map according to their Pearson correlation coefficients. Coloured squares indicate the changes of metabolite content, and positive and negative values indicate upregulation and downregulation of metabolites,

respectively. c and d, Spearman's correlation coefficients between differential metabolites in ESI+ and ESI- modes and flight fatigue. Positive correlations are marked in red and negative correlations in blue. e, the summary of aberrant pathways caused by flight fatigue. All matched pathways were shown according to p values from the pathway enrichment analysis (y-axis) and pathway impact values from pathway topology analysis (x-axis), with the most impacted pathways colored in red.

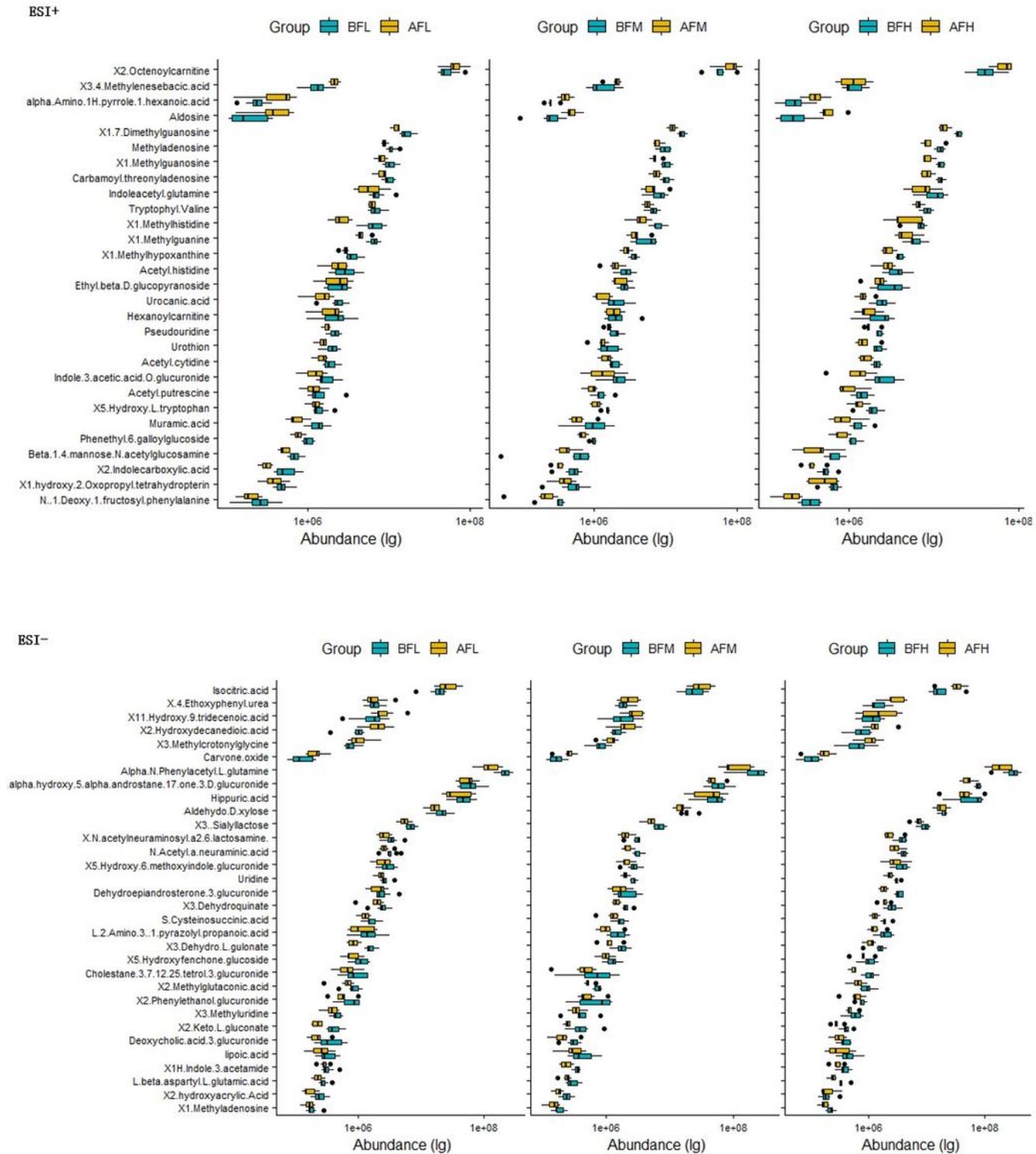


Figure 3

Please see the Manuscript file for the complete figure caption.

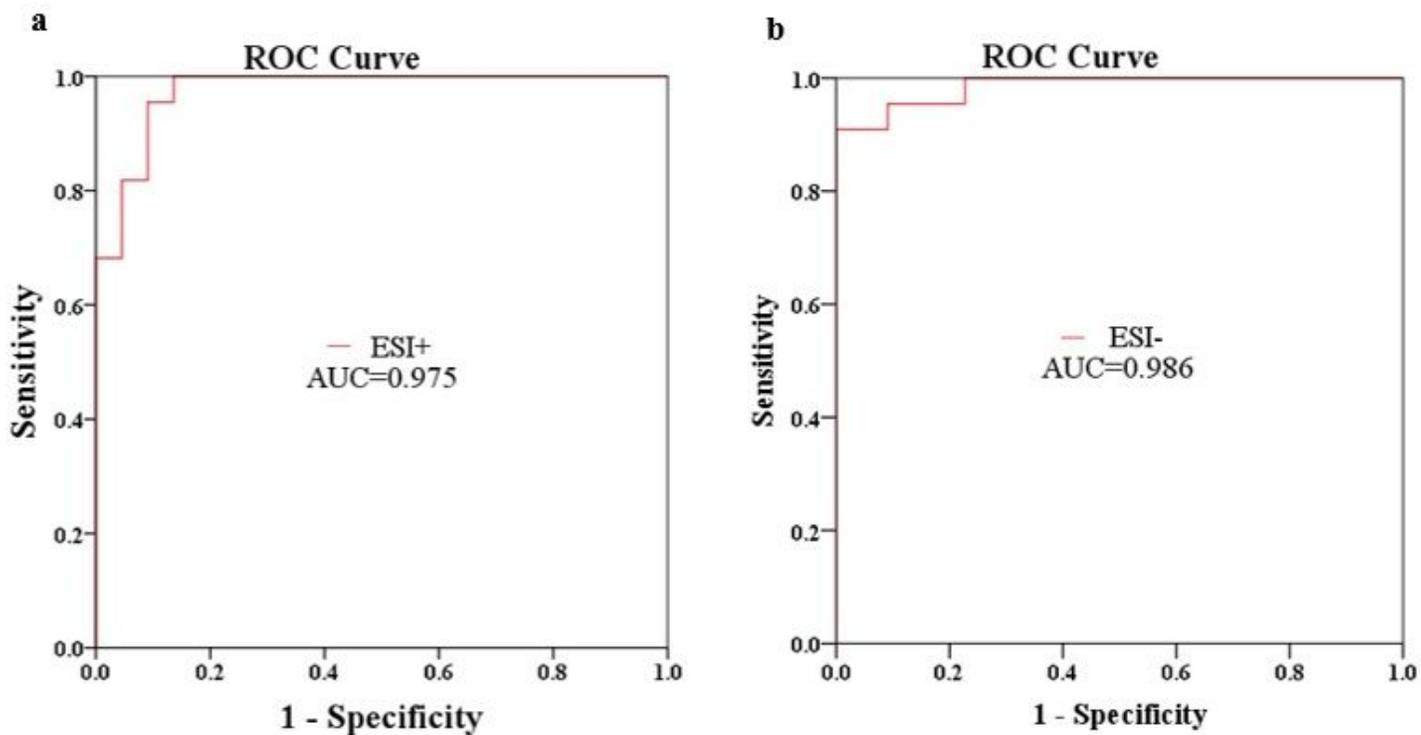


Figure 4

The diagnostic performance of the biomarkers screened out in ESI+(a) and ESI- (b) modes. The area under the curve AUC of the ROC was calculated to evaluate the ability of the potential biomarkers to distinguish the occurring of flight fatigue.

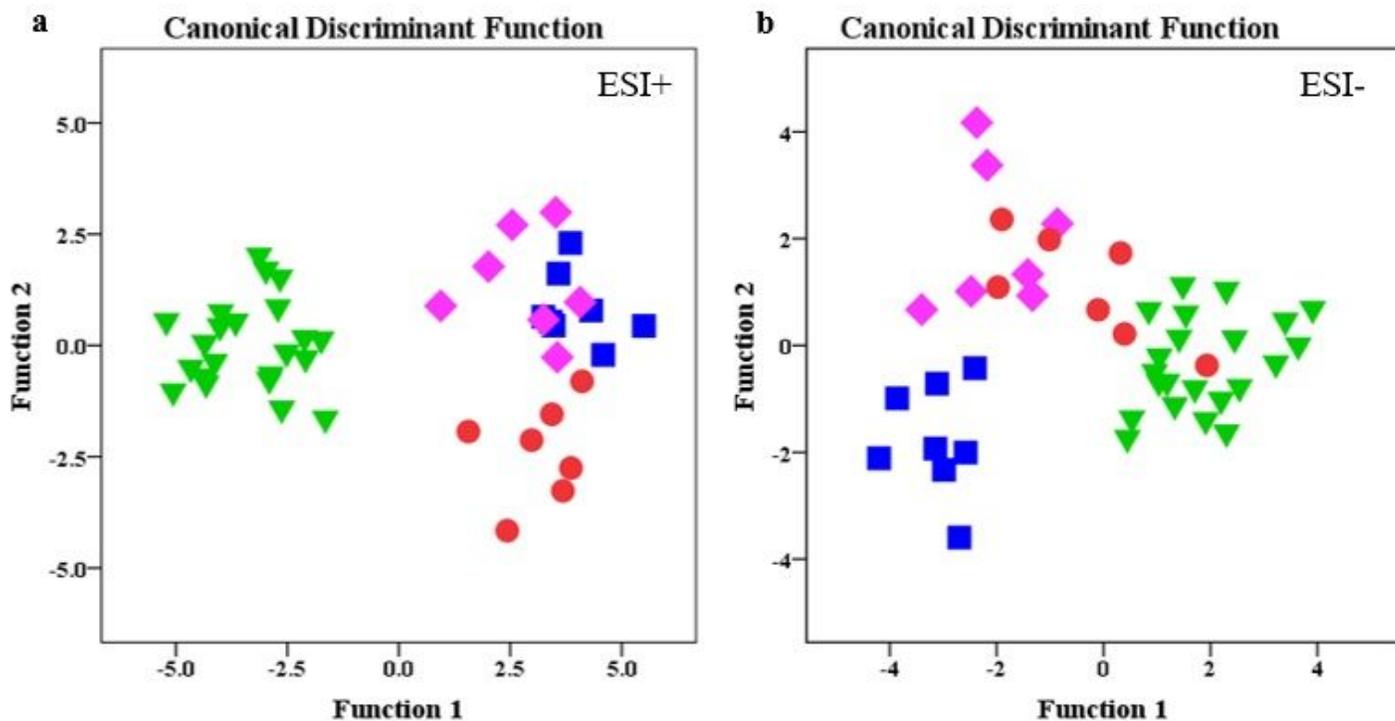


Figure 5

Multiple discriminant analysis of the extent of flight fatigue in ESI+(a) and ESI- (b) modes. The discriminant function models of several metabolites were established based on Bayes' Rule by stepwise discriminant analysis to distinguish the extent of flight fatigue.