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Discovery and identification of fatigue-related biomarkers in human saliva

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Abstract. – OBJECTIVE: To identify stable and specific biomarkers/biomarker combinations for fatigue assessment and establish a discriminant model.

PATIENTS AND METHODS: Saliva was collected and electroencephalogram analysis was performed for 47 emergency physicians while awake and after continuoutas duty for 18-24 h. Physicians were divided into the fatigue and non-fatigue groups. Protein spectra of completely quantified saliva specimens were identified before and after long working hours using mass spectrometry. Data were analyzed through Proteome Discoverer software combined with SEQUEST to search protein databases. Proteins were characterized by collision-induced dissociation spectra. A global internal standard (GIS) was added to each group of samples and labeled by tandem mass tags m/z 131.1. All data were compared with GIS, and data between groups were further compared. Qualitative and quantitative data on proteins were exported for fatigue-related proteomic analysis, and a fatigue assessment model was established.

RESULTS: We identified 767 salivary proteins in the fatigue group. The correct rates of the discriminant function of the non-fatigue and fatigue groups were 97.1% and 91.7%, respectively (the total correct rate was 95.7%).

CONCLUSIONS: We identified 30 fatigue-related protein markers from saliva. We also established a fatigue assessment model for emergency physicians using salivary biomarkers.

Key Words
Fatigue identification, Human saliva, Biological markers, Proteomics, Q Exactive Plus, Electroencephalography (EEG) monitoring, Fisher discriminant analysis, Prospective studies.

Introduction

Fatigue is a subjective discomfort that can lead to the loss of normal activity or ability to work, as well as increased susceptibility to infectious or other diseases, resulting in economic losses and additional social costs. Fatigue negatively affects human alertness, coordination, information processing and decision-making. Workers with long working hours such as pilots, drivers and physicians are at the greatest risk, and pose significant risks to others. Fatigue is a major factor in 25-30% of traffic accidents[1]. A survey of 380 physicians in the United States showed that 93.7% of fatigued hospital residents from 2003 to 2009 provided false data, and 39% encountered at least one medical malpractice during this period. In another survey of 890s physicians, 40% reported mistakes at work at least once per month, and 36% claimed to be exploited at work every week, which may lead to misdiagnosis and mistreatment. The degree of exhaustion varies by department and is the highest among first-line physicians, especially emergency medicine physicians. Studies have recommended interventions to reduce medical malpractice and ensure the safety of patients[2,3]. Therefore, an assay to screen for fatigue, similar to a drunk-driving test, would be useful for fatigue assessment and enforcement to reduce iatrogenic and occupational injuries.

Previous studies have focused on the detection of fatigue associated with physiological signals, vision tasks and biomarkers. The detection of physiological signals include changes in electroencephalogram (EEG) theta (θ) waves, high-frequency (HF) EEG, pulse signals, and the ratio

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between low and high-frequency components (LF/HF ratio)[4-6]. Vision tasks include glare conditions and frequencies, oral movements and head poses[7,8]. The detection of physiological markers includes creatine kinase[9,10], blood interleukin (IL)-8[11], α-amylase[12] and cortisol[13]. These measurements have been used for the assessment of fatigue in clinical practice, but most are invasive diagnostic tests requiring blood samples that cannot be used for rapid, on-site and accurate identification of fatigue. For convenient and non-invasive fatigue assessment, some scholars have used saliva specimens to identify fatigue-related biomarkers[14-17]. However, most studies have only focused on monitoring changes in metabolite levels in the saliva of athletes and soldiers[14,17], and have not established definitive standards for fatigue assessment. Moreover, they lack a rigorous research design. Therefore, there is a need for high-quality and rigorous methods to evaluate the impact of fatigue on physician behavior[3].

To establish a saliva assay using fatigue-related biomarkers, we recruited emergency physicians, who have high degrees of exhaustion, as a model of fatigue. To achieve rapid detection, we used biomass spectrometry to identify stable and specific biomarker combinations and to establish a fatigue-related biomarker assessment model. Compared with previous researches that primarily focused on salivary metabolites, proteomics provides relatively stable results. Further systematic development of saliva identification spectra, especially the implementation of convenient and rapid testing methods based on biosensor technology, can be used to improve fatigue assessment.

Patients and Methods

Inclusion and Exclusion Criteria

Inclusion criteria: healthy male and female emergency physicians from 30 to 45 years old who signed informed consent and had no organic diseases or chronic fatigue symptoms. Exclusion criteria were as follows: (1) persistent or recurrent fatigue lasting over 6 months; (2) sore throat; (3) swollen neck or axillary lymph nodes; (4) muscle pain; (5) multiple non-arthritic pain; (6) headache; (7) sleep disorders; (8) discomfort after exhaustion lasting for over 24 h; (9) oral diseases; (10) took any drug or dietary or nutritional supplement within 3 months. Any participants who smoked or had a cavity filled in the past week were also excluded.

Research Subjects

A total of 57 healthy emergency physicians (including eight who were transferred from other departments) who met the inclusion criteria volunteered to participate in this study and worked in the Emergency Department of the Affiliated Hospital of Hebei Engineering University. All volunteers signed informed consent before participating in the study. Saliva specimens were collected from December 2015 to May 2016. For the permission of human specimen, this study was approved by the Ethics Committee of the Affiliated Hospital of Hebei University of Engineering (Hebei, China) and registered with the clinical trial (registration number: ChiCTR-DCD-14005746). Moreover, all methods were performed in accordance with the relevant guidelines and regulations.

Among 57 subjects, saliva samples from 49 could be obtained at the time points before and after over 18 h of continuous work and the rest of 10 were incapable to undergo sample collection at either of the two-time points. Nevertheless, 2 physicians from 49 persons missed EEG examination because they had to work and did not have spare time for examination. Therefore, they could not be defined as fatigue or not and could not be used for the comparison.

Collection of General Information and Definition of the Groups

General information of the participants was collected the day before the saliva specimen collection. All data were recorded by designated personnel. General information included participants' ages ranging from 27 to 41, a male-to-female ratio of 33:16, and 32 out of 57 participants being married.

According to the different criteria, several comparisons were made as follows:

(1) Forty-nine samples from emergency physicians were divided into two groups, before and after over 18 h of continuous work, and the components of saliva were compared between the two groups. As 2 out of 49 objects failed to collect EEG, 47 samples were utilized for further analysis as follows.

(2) Forty-seven samples from emergency physicians were also divided into two groups, fatigue and non-fatigue, based on detectable fatigue waves over 18 h of continuous work and scoring system evaluating fatigue. Briefly, after over 18 h of continuous work, the EEGs were measured while the saliva samples were

collected. The EEG monitoring took over 30 min to detect fatigue waves (theta wave). Meanwhile, the subjects' sense of exhaustion was scored according to ESS and PFS. The subject was considered as "fatigue" if theta wave was detected plus the scores of ESS over 8 and PFS over 6.7.

Twelve (8 males and 5 females) were in the fatigue group with an average age of 32±8 years old; 35 (28 males and 7 females) were in the non-fatigue group with an average age of 31±8 years old.

Saliva Sampling

- Saliva specimen collection
  Stimulated whole saliva accumulated at the sublingual site was collected following three-time mouth-rinse with sterile saline. Briefly, the saliva specimens of the emergency physicians were collected before their shifts and following a long work shift (≥18 h). All participants were required to rinse their mouths three times (1 min each time) to eliminate food residues using 30 mL distilled water before specimen collection. After rinsing, each participant sat in front of a mirror for 5 min and was required to sit up straight with their head tilted slightly forward and their eyes remaining open. The participant was then required to chew to stimulate saliva secretion. Once a certain amount of saliva accumulated in the lower jaw, the participant was required to place their tongue against the palate and open their mouth to naturally tilt their tongue and form a V-shape in the lower lip to allow the saliva to naturally flow into a collection tube (ISO9001 certified, Cryo.s™ Greiner Bio-One GmbH, Maybachstraße 2. D-72636 Frickenhausen, Germany) until 2 mL of pure sublingual saliva was collected (0.5 mL saliva was aliquoted and preserved at -70°C later; the intervals between sampling and the previous time of eating, and the food were recorded).
- Storage of saliva specimens
  All saliva specimens were labeled and preserved at -70°C in the Experimental Center, the Affiliated Hospital of the Hebei University of Engineerin
- Transport of the saliva specimens
  The saliva specimens were placed in a dry-ice container and transported to the State Key Laboratory of Infectious Disease Prevention and Control, Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention, for mass spectrometry.

EEG Data Collection

EEG was carried out in each of the participants after a long shift (≥18 h) and the collection of saliva specimens to detect the presence of fatigue waves (increase of slow waves, i.e., δ and θ waves; decrease of fast waves, i.e., alpha (α) and beta (β) waves). The video EEG monitor was a SOLAR-RTA and SOLAR-BFM brain function monitoring system from Solar Technologies Co., Ltd. (Beijing, China). This system was used to collect EEG data in real-time and comprehensively analyze brain functions, and to carry out relevant analyses of dominant wave amplitude and brain function vital signs, respectively (ISO9001 and ISO13485 certifications of the international quality management system). The electrogram was recorded for at least 30 min. The silver disc-like electrode was placed according to the traditional method and was used as a recording electrode. The scalp record point defaulted as 16. The resistance of each electrode lead was controlled within the sampling filter range, and each record was at least 30 min.

Completion of the Fatigue Subjective Feeling Questionnaire

A revised version of the Piper Fatigue Scale (PFS) (based on the original PFS of Piper et al[18] to remove some entries, including four dimensions and 22 items; five items assess participants' feelings and emotions, and six assess cognition and behavior) was employed to complete the fatigue subjective feeling questionnaire. Each item of the questionnaire was scored from 0 to 10 with a higher score representing a higher degree of exhaustion. The emergency physicians provided the score according to their sense of exhaustion. The averages of the total scores of the four categories were ranked into mild fatigue (score: 1-3.3), moderate fatigue (score: 3.4-6.7), and severe fatigue (score: 6.8-10) for each participant. An additional item was used to assess the duration of exhaustion, with a Cronbach's α coefficient of the scale of 0.91 and a test-retest reliability of 0.98. The physicians completed the fatigue symptom questionnaire immediately after their shift. If the average PFS score ranged from 1-3.3, the saliva specimen of the participant was used as a control sample. Each participant was

asked to redo the fatigue symptom questionnaire following a nightshift. If the average PFS score was > 6.7, the physician was later required to provide a 2 mL saliva specimen for fatigue-related biomarker assessment. Epworth Sleepiness Scale (ESS) was also used to access the severity of sleepiness for fatigue[19].

Q Exactive Plus of Saliva Specimens

The test was completed with Q Exactive Plus (Thermo Fisher Scientific, Waltham, MA, USA) at the national key laboratory in the National Institution for Communicable Disease Control and Prevention of Chinese Center for Disease Control and Prevention (http://www.chinacdc.cn).

- Processing flow (Figure 1)
- Materials and Methods

(A) Sample preparation

Two hundred microliters of each saliva specimen were treated using acetone precipitation. The precipitated sample was then totally dissolved in 0.1% (v/v) formic acid-aqueous solution (Thermo Fisher Scientific, Waltham, MA, USA) and the concentration of the protein was determined using the BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). All samples were mixed as a global internal standard (GIS) for comparison between different groups of samples.

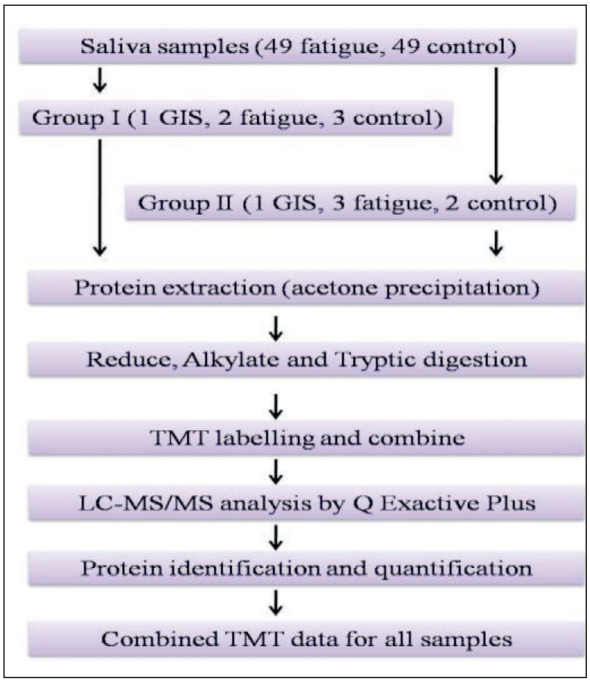

Figure 1. Processing flow.

(B) Reduction, alkylation, digestion and labeling with tandem mass tags (TMT) of the saliva samples

For each TMT-6plex experiment, 100 μg of protein were added to 5 mM DTT (final concentration) (Sigma-Aldrich, St. Louis, MO, USA) and incubated at room temperature for 1 h. Next, 12.5 mM iodoacetamide (final concentration) (Sigma-Aldrich, St. Louis, MO, USA) was added to the mixture to react for 1 h in the dark at room temperature, followed by trypsinization at 37°C overnight. The samples were dissolved in 200 mM TEAB (Thermo Fisher Scientific, Waltham, MA, USA), followed by labeling with TMT (Thermo Fisher Scientific, Waltham, MA, USA) at room temperature for 1 h and subsequent addition of 5% hydroxylamine (w/v) (Thermo Fisher Scientific, Waltham, MA, USA) to stop the reaction by incubating at room temperature for 15 min. All samples were combined and desalted via PepCleanTM C18 spin columns (Pierce Biotechnology, Waltham, MA, USA) and dried under a vacuum.

(C) Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis

Sample measurements were made with the Thermo Scientific™ Q Exactive™ hybrid quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). Samples dissolved in 0.1 % (v/v) formic acid-aqueous solution were used for mass spectrometry. The peptides were passed through a pre-column (Acclaim PepMap 100, 100 μm×2 cm, C18, 5 μm, 300 Å, Thermo Fisher Scientific, Waltham, MA), followed by eluting to the analytical column (Acclaim PepMap 100, 75 μm×15 cm, C18, 3 μm, 200 Å, Thermo Fisher Scientific, Waltham, MA, USA). The mobile phase consisted of buffer A (0.1% formic acid in water) and buffer B (0.1% formic acid in acetonitrile), with a flow rate of 300 nL/min, followed by separation for 120 min. The voltage was set to 1,500 V, and the capillary temperature was 275°C. All mass spectra were acquired in the positive ionization mode with an m/z scan range of 300-2000. After the master scan, three most intense ions were subjected for collision-induced dissociation (CID) fragmentation using an isolation window of 2.0, collision energy of 30, default charge state of 2. The fragmentation of three most intense TMT-reporter-labelled ions was achieved with a higher energy collisional dissociation (HECD) fragmentation at 7500 resolving power using an isolation window of 2, collision energy of 40, default charge state of 2 and activation time of 30 ms.

(D) Protein characterization and quantitation

LC-MS/MS data were analyzed with Proteome Discoverer software (version 1.4). Peptide and protein identification was filtered with the charged state and the filters allowed a 99% confidence level of protein identification with less than 1% false discovery rate. The Reporter Ions Quantitizer in the Proteome Discoverer software was used to quantify the TMT reporter ion intensities at 126.13-131.14 m/z. SEQUEST was interfaced with Proteome Discoverer for a protein database search against the human international protein index (IPI) database (Europan Molecular Biology Laboratory (EMBL) Outstation, The European Bioinformatics Institute, Hinxton, UK).

(E) PANTHER classification system

The protein classification was performed using the PANTHER Classification System based on molecular function, related biological process, cellular component, protein class and related pathway.

Criteria for the Determination of Fatigue and Fatigue Model Establishment

EEG data showing an increase of slow waves and a decrease of fast waves (i.e., increase in δ and θ waves and decrease in α and β waves) were used as the criteria for the determination of fatigue[4], as well as the scores of ESS and PFS described above.

Quality Control

To guarantee precisely following the standard operating procedures, the laboratory staff and subjects involved were trained before the study. A relevant training of researchers was carried out prior to beginning this study, including the collection of saliva specimens and measuring the fatigue status. Also, the details of the tests were provided to each participant, and a volunteer badge was distributed to each of them. This study also employed instructors and supervisors for the saliva specimen collection. Each researcher that conduced saliva sampling received unified saliva sampling training, which was strictly in accordance with the saliva collection flowchart. EEG and sleep deprivation monitoring was performed by designated personnel.

Statistical Analysis

SPSS 19.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical analyses. All data in line with the normal distribution are presented as mean ± standard deviation ($\chi \pm s$). The non-normally distributed data are presented using M (QR) with two decimal places. The comparison of protein contents between the fatigue and non-fatigue groups were performed using the *t*-test if the data were normally distributed with variance homogeneity. The comparison of protein contents before and after long work shifts in the fatigue group were performed using the paired *t*-test. If the data were non-normally distributed, the comparison of protein contents before and after the long work shifts in the fatigue group was performed using the Wilcoxon Signed Ranks Test; the comparison of protein contents between the fatigue and non-fatigue groups was performed using the Mann-Whitney U test. Spearmen correlation analysis was used to test the relationship between the protein contents and fatigue conditions of the emergency physicians. Receiver operating characteristic (ROC) curves of each protein in the fatigue test population were prepared. The above single factor and proteins with statistically significant differences in the related analysis were used as discriminant variables for Fisher's discriminant analysis to establish a fatigue discriminant equation. In addition, the independent variables of the observed values were brought back into the discriminant function, which was used to discriminate the category of the observed value. The classification of the original data was compared, and the correct rates were obtained according to the classification of the discriminant function. $p < 0.05$ was considered to be statistically significant (2-sided test).

Results

In this study, saliva specimens before and after long working hours were collected from 49 out of 57 emergency physicians (20% of specimens were ineligible for analysis or lost as explained in "Research subjects").

PFS Results of the Fatigue Cohort

The average scores of fatigue symptoms of the 49 emergency physicians before and after long working hours were 2.8±0.12 and 8.4±0.03, respectively.

EEG Results of the Fatigue Cohort

Twelve of the 47 emergency physicians were verified the EEG δ (0.5-4 Hz, Fig. 2B) and θ (5–8 Hz) waves as the fatigue model group because δ and θ waves indicate brain inhibition to lose capability and slow activity.

For the non-fatigue group, brain waves were displayed as the basic rhythm of normal EEG ranged from 8-13 Hz (α waves, Figure 2A and β waves).

Q Exactive Plus Results of the Emergency Physicians in the Fatigue Group

A total of 767 proteins could be identified in this study (**Supplementary File 1** includes the names of the 767 proteins). These proteins covered a wide field of cellular functions as a signaling pathway, enzymatic and nuclear-binding activities, molecule carrying and transportation, and so on. The protein classification and pathway analyses of these proteins are shown in Figure 3A and B, respectively.

Protein Analysis

These detectable 767 proteins were further analyzed upon defined groups as follows.

Protein Analysis of Single Factor Analysis

The Wilcoxon Signed Ranks Test was used for the comparison of protein contents before and after long working hours (>18 h) of all emergency physicians. The contents of 12 proteins had significant differences before and after long working hours. See **Supplementary File 2** (1) with Table S1 for details.

The Wilcoxon Signed Ranks Test was used for the comparison of all protein contents of the 12 physicians in the fatigue group that was confirmed with fatigue-related θ waves in their EEGs. Nine proteins were found to be significantly different from each other in the fatigue group. For detailed information, see **Supplementary File 2** (2) with Table S2.

The Mann-Whitney U test was used for the comparison of protein contents after long working hours between the fatigue and non-fatigue groups. The contents of the 36 proteins had significant differences between the two groups. Detailed information is shown in **Supplementary File 2** (3) with Figure S1.

Spearman correlation analysis showed that 34 proteins were significantly associated with fatigue in emergency physicians after long working hours. For detailed information, see **Supplementary File 2** (4) with Figure S2.

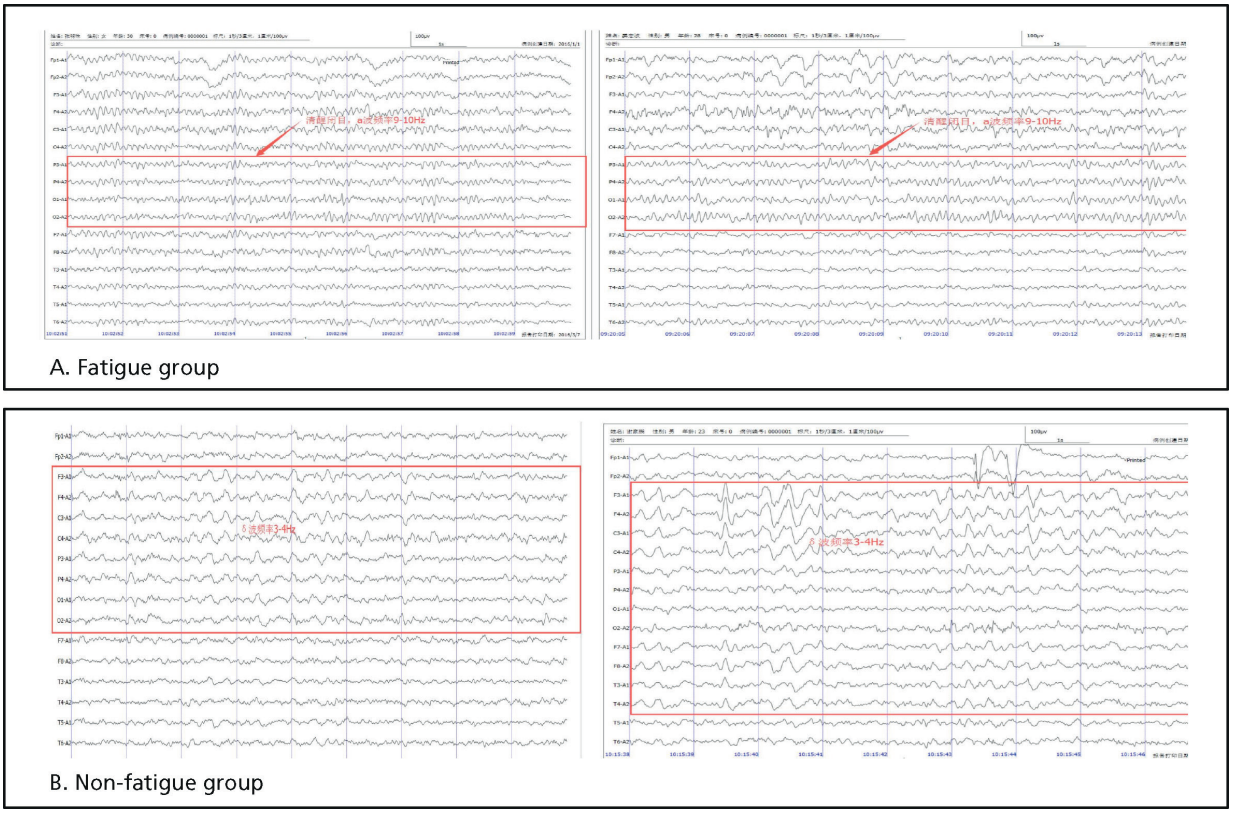

Figure 2. EEGs of emergency physicians. **A**, α fast waves of the non-fatigue group (basic rhythm of normal EEG ranged from 8-13 Hz). **B**, δ slow waves (0.5-4 Hz) of the fatigue group. Slow waves indicate brain inhibition.

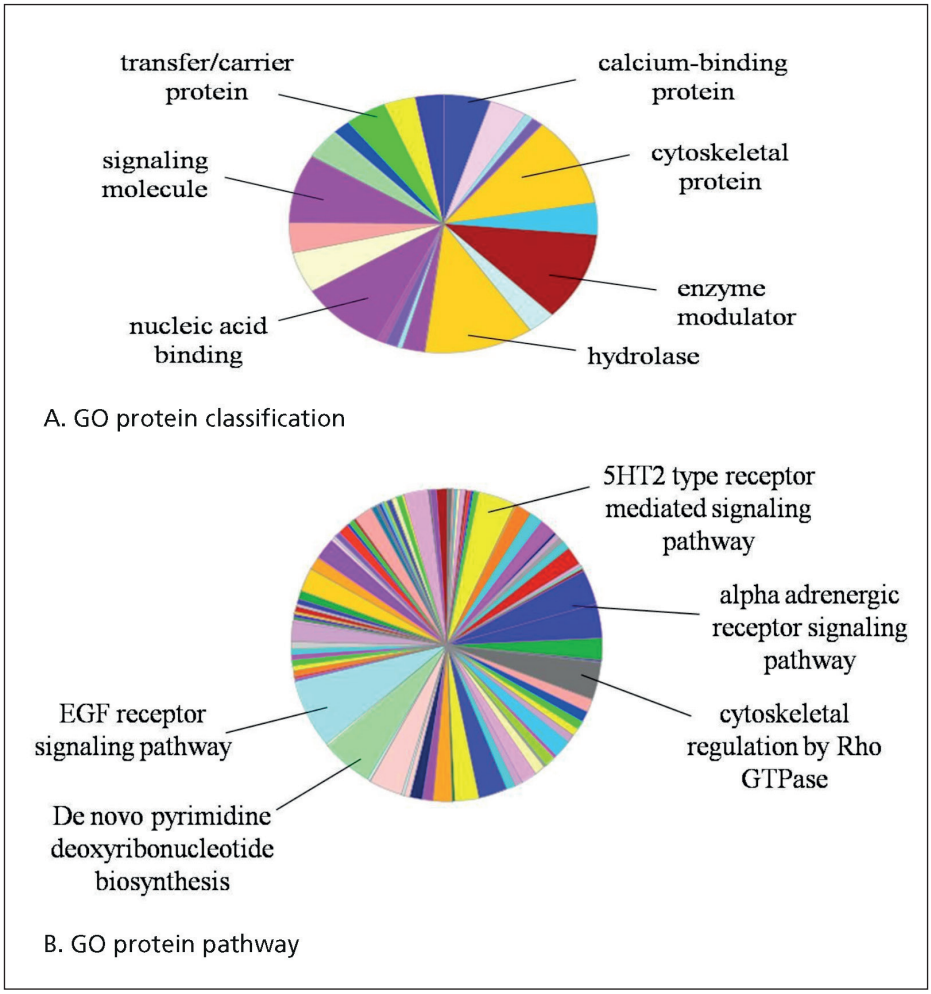

Figure 3. Comparison of protein content ratios between the fatigue and non-fatigue groups. **A**, Gene ontology (GO) protein classification analysis. **B**, GO protein pathway analysis.

Among 34 proteins significantly associated with fatigue, Spearman correlation analysis revealed that 25 proteins had strong significant correlations. For detailed information, see **Supplementary File 2** (5) with Figure S3.

Considering single factor with poorer diagnostic value, all proteins above with significance undergone further multi-factors analysis as follows.

ROC Curves for the Verification of Fatigue-Related Proteins

ROC curves were first used to determine if the aforementioned protein content ratios with significant differences (derived from single factor analysis) were associated with the fatigue of emergency physicians after long hours of work, i.e. the specificity and sensitivity of measurement (Figure 4). The areas under the curve (AUC) of the fatigue-related proteins for the fatigue assessment are as follows:

Ig κ chain V-I region Lay (0.700), Growth factor receptor-bound protein 2 (0.607), ADP-ribosyl cyclase/cyclic ADP-ribose hydrolase 2 (0.650), Lipocalin-1 (0.673), Mitochondrial malate dehydrogenase (0.651), Heat shock cognate 71 kDa protein (0.643), Ig κ chain V-III region SIE (0.651), Golgi membrane protein 1 (0.298), Cystatin-A (0.617), Mucin-19 (0.619), Heat shock protein β-1 (0.679), Ig γ-3 chain C region(0.717), Serpin B13 (0.689), Rab GDP dissociation inhibitor β (0.650), Annexin A1 (0.675), Afamin (0.651), Protein disulfide-isomerase A3 (0.676), Ig κ chain V-III region HAH (0.662), Transmembrane protease serine 11D (0.654), Nucleoside diphosphate kinase B (0.643), Bactericidal permeability-increasing protein (0.612), Ubiquitin-like modifier-activating enzyme 1 (0.649), Macrophage-capping protein

(0.686), Myeloid-derived growth factor (0.669), Pancreatic α-amylase (0.583), Synaptic vesicle membrane protein VAT-1 homolog (0.607), L-lactate dehydrogenase B chain (0.583), Cytoplasmic malate dehydrogenase (0.583), Sialic acid-binding Ig-like lectin 16 (0.583), Mitochondrial peroxiredoxin-5 (0.607), Thioredoxin domain-containing protein 17 (0.612), Prominin-1 (0.633), Elongation factor 1-α 1 (0.583), Macrophage migration inhibitory factor (0.612), Transforming protein RhoA (0.583), Retinol-binding protein 4 (0.612), Purine nucleoside phosphorylase (0.583), SCY1-like protein 2 (0.583), Tissue α-L-fucosidase (0.640), CD9 antigen (0.612), Collagen α-2(VI) chain (0.583), Serum paraoxonase/arylesterase 1 (0.583), Ras-related protein Rap-1b-like protein (0.583), Type II cytoskeletal keratin 4 (0.293), Trefoil factor 3 (0.292), Ig κ chain V-II region RPMI 6410 (0.604), Type I cytoskeletal keratin 13 (0.495), Type II cytoskeletal keratin 5 (0.552), Calpastatin (0.529), and Interleukin-6 receptor subunit β (0.490) (Figure 4).

The data suggested that the measurement had convinced specificity and sensitivity as the AUC of most proteins (44/49, 89.8%) were between 58.3-71.7%.

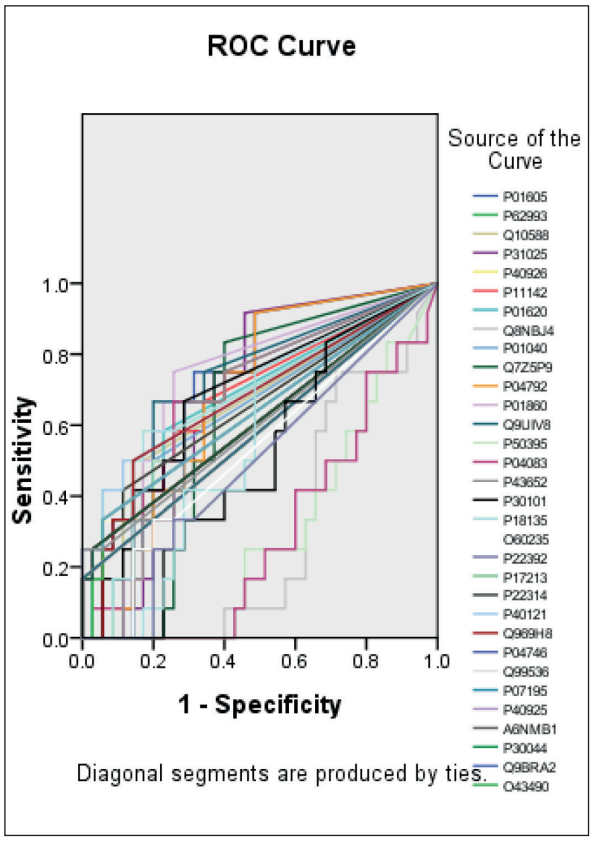

Figure 4. ROC curves for the verification of fatigue-related proteins.

Fisher Discriminant Analysis of the Above Proteins with Significant Differences

To explore diagnostic values, all proteins with clinical significance measured by single factor assessment (univariate analysis and Spearman correlation analysis) were used as the variables in Fisher discriminant analysis. The clinical indicators for the discriminant function equation included 30 following proteins: Ig κ chain V-I region Lay, Growth factor receptor-bound protein 2, ADP-ribosyl cyclase/cyclic ADP-ribose hydrolase 2, Lipocalin-1, Mitochondrial malate dehydrogenase, Heat shock cognate 71 kDa protein, Ig κ chain V-III region SIE, Golgi membrane protein 1, Cystatin-A, Mucin-19, Heat shock protein β-1, Ig γ-3 chain C region, Serpin B13, Rab GDP dissociation inhibitor β, Annexin A1, Afamin, Protein disulfide-isomerase A3, Ig κ chain V-III region HAH, Transmembrane protease serine 11D, Nucleoside diphosphate kinase B, Bactericidal permeability-increasing protein, Ubiquitin-like modifier-activating enzyme 1, Macrophage-capping protein, Myeloid-derived growth factor, Pancreatic α-amylase, L-lactate dehydrogenase B chain, Mitochondrial peroxiredoxin-5, Prominin-1, Type II cytoskeletal keratin 4, and Salivary acidic proline-rich phosphoprotein 1/2.

Stepwise discriminant analysis according to SPSS13.0 was used to analyze the results and establish a Bayes discriminant equation. The specific equations of these 30 fatigue-related proteins are as follows:

Y non-fatigue = −0.972 Ig κ chain V-I region Lay + 2.313 Growth factor receptor-bound protein 2 + −21.550 ADP-ribosyl cyclase/cyclic ADP-ribose hydrolase 2 + −4.315 Lipocalin-1 + −20.009 Mitochondrial malate dehydrogenase + 8.721 Heat shock cognate 71 kDa protein + −23.350 Ig κ chain V-III region SIE + 1.971 Golgi membrane protein 1 + 20.165 Cystatin-A + 4.629 Mucin-19 + 9.640 Heat shock protein β-1 + 22.552 Ig γ-3 chain C region + −8.255 Serpin B13 + −1.195 Rab GDP dissociation inhibitor β + 3.444 Annexin A1 + 5.674 Afamin + −4.240 Protein disulfide-isomerase A3 + 0.001 Ig κ chain V-III region HAH + −15.930 Transmembrane protease serine 11D + 1.026 Nucleoside diphosphate kinase B + 34.242 Bactericidal permeability-increasing protein + 7.223 Ubiquitin-like

modifier-activating enzyme 1 + −0.746 Macrophage-capping protein + −1.435 Myeloid-derived growth factor + 62.069 Pancreatic α-amylase + −143.047 L-lactate dehydrogenase B chain + 25.851 Mitochondrial peroxiredoxin-5 + 1.212 Prominin-1 + 2.124 Type II cytoskeletal keratin 4 + 2.191 Salivary acidic proline-rich phosphoprotein 1/2 + −6.180 (Constant);

Y fatigue = −3.833 Ig κ chain V-I region Lay + 18.428 Growth factor receptor-bound protein 2 + −27.316 ADP-ribosyl cyclase/cyclic ADP-ribose hydrolase 2 + −9.979 Lipocalin-1 + −16.015 Mitochondrial malate dehydrogenase + 5.804 Heat shock cognate 71 kDa protein + −21.123 Ig κ chain V-III region SIE + −0.363 Golgi membrane protein 1 + 25.313 Cystatin-A + 1.479 Mucin-19 + 13.059 Heat shock protein β-1 + 39.326 Ig γ-3 chain C region + 3.158 Serpin B13 + −14.713 Rab GDP dissociation inhibitor β + 8.239 Annexin A1 + 2.730 Afamin + −3.769 Protein disulfide-isomerase A3 + 3.022 Ig κ chain V-III region HAH + −31.311Transmembrane protease serine 11D + 21.347 Nucleoside diphosphate kinase B + 33.216 Bactericidal permeability-increasing protein + −26.402 Ubiquitin-like modifier-activating enzyme 1 + 12.967 Macrophage-capping protein OS + 6.548 Myeloid-derived growth factor + 18.672 Pancreatic α-amylase + −121.731 L-lactate dehydrogenase B chain + 32.379 Mitochondrial peroxiredoxin-5 + 4.061 Prominin-1 + 2.567 Type II cytoskeletal keratin 4 + 3.634 Salivary acidic proline-rich phosphoprotein 1/2 + −14.741 (Constant); See Tables I and II for details.

Based on the classification function coefficients above for non-fatigue and fatigue group, the discriminant equations of the fatigue-related proteins for fatigue assessment exhibited better correct diagnostic rates for overall, non-fatigue and fatigue groups, respectively, than simply combining positive indicators (Table II).

Discussion

This study was the first to identify fatigue-related biomarkers of emergency physicians using salivary proteomics and to demonstrate significant changes in some of the markers after long and consecutive work periods (>18 h). We identified 767 proteins in the saliva of the fatigue group of emergency physicians. Through a series of statistical analyses, we identified the following fatigue-related proteins: Ig κ chain V-I region Lay, Growth factor receptor-bound protein 2, ADP-ribosyl cyclase/cyclic ADP-ribose hydrolase 2, Lipocalin-1, Mitochondrial malate dehydrogenase, Heat shock cognate 71 kDa protein, Ig κ chain V-III region SIE, Golgi membrane protein 1, Cystatin-A, Mucin-19, Heat shock protein β-1, Ig γ-3 chain C region, Serpin B13, Rab GDP dissociation inhibitor β, Annexin A1, Afamin, Protein disulfide-isomerase A3, Ig κ chain V-III region HAH, Transmembrane protease serine 11D, Nucleoside diphosphate kinase B, Bactericidal permeability-increasing protein, Ubiquitin-like modifier-activating enzyme 1, Macrophage-capping protein, Myeloid-derived growth factor, Pancreatic α-amylase, L-lactate dehydrogenase B chain, Mitochondrial peroxiredoxin-5, Prominin-1, Type II cytoskeletal keratin 4, and Salivary acidic proline-rich phosphoprotein 1/2.

Individual diagnostic tests of the above proteins showed that the top eight proteins with relatively high diagnostic values were Ig γ-3 chain C region (AUC = 0.717), Ig κ chain V-I region Lay (AUC = 0.700), Serpin B13 (AUC = 0.689), Macrophage-capping protein (AUC = 0.686), Heat shock protein β-1 (AUC = 0.679), Protein disulfide-isomerase A3 (AUC = 0.676), Annexin A1 (AUC = 0.675), and Lipocalin-1 (AUC = 0.673). Single proteins had poor performance as diagnostic indicators. For this reason, a discriminant equation based on each indicator and its diagnostic correlation was assigned according to the corresponding degree of correlation, taking full account of the comprehensiveness of the tested indicator and avoiding the reduction of specificity caused by simply combining positive indicators[20]. This approach had a higher correct rate than simply combining positive indicators. We used the above proteins to successfully establish discriminant equations of the fatigue-related protein group for fatigue assessment, with an overall correct diagnostic rate of 95.7% and correct diagnostic rates of 97.1% in the non-fatigue group and 91.7% in the fatigue group. The discriminant equations of the fatigue-related protein group significantly improved the positive detection rates compared with using a single indicator that had important clinical value for the diagnosis of fatigue. The identification of proteomic biomarkers associated with fatigue also provides a basis for subsequent multidimensional research and rapid qualitative and quantitative testing of fatigue.

Table I. Discriminant analysis of the classification function coefficient. The Fisher discriminant equation was used to determine the classification results. The rate of the correct diagnosis of the non-fatigue group was 97.1%, and the rate of the correct diagnosis of the fatigue group was 91.7%, with a total correct rate of 95.7% (Table II).

No.	Protein		Non-fatigue	Fatigue	No.	Protein		Non-fatigue	Fatigue
1	P01605	Ig κ chain V-I region Lay	−0.972	−3.833	16	P43652	Afamin	5.674	2.730
2	P62993	Growth factor receptor-bound protein 2	2.313	18.428	17	P30101	Protein disulfide-isomerase A3	−4.240	−3.769
3	Q10588	ADP-ribosyl cyclase/cyclic ADP-ribose hydrolase 2	−21.550	−27.316	18	P18135	Ig kappa chain V-III region HAH	.001	3.022
4	P31025	Lipocalin-1	−4.315	−9.979	19	O60235	Transmembrane protease serine 11D	−15.930	−31.311
5	P40926	Malate dehydrogenase, mitochondrial	−20.009	−16.015	20	P22392	Nucleoside diphosphate kinase B	1.026	21.347
6	P11142	Heat shock cognate 71 kDa protein	8.721	5.804	21	P17213	Bactericidal permeability-increasing protein	34.242	33.216
7	P01620	Ig kappa chain V-III region SIE	−23.350	−21.123	22	P22314	Ubiquitin-like modifier-activating enzyme 1	7.223	−26.402
8	Q8NBJ4	Golgi membrane protein 1	1.971	−.363	23	P40121	Afamin	−0.746	12.967
9	P01040	Cystatin-A	20.165	25.313	24	Q969H8	Macrophage-capping protein	−1.435	6.548
10	Q7Z5P9	Mucin-19	4.629	1.479	25	P04746	Myeloid-derived growth factor	62.069	18.672
11	P04792	Heat shock protein beta¬1	9.640	13.059	26	P07195	Pancreatic alpha-amylase	−143.047	−121.731
12	P01860	Ig gamma-3 chain C region	22.552	39.326	27	P30044	L-lactate dehydrogenase B chain	25.851	32.379
13	Q9UIV8	Serpin B13	−8.255	3.158	28	O43490	Peroxiredoxin-5, mitochondrial	1.212	4.061
14	P50395	Rab GDP dissociation inhibitor beta	−1.195	−14.713	29	P19013	Prominin-1	2.124	2.567
15	P04083	Annexin A1	3.444	8.239	30	P02810 (Constant)	Keratin, type II cytoskeletal 4	2.191 −6.180	3.634 −14.741

Table II. Discriminant analysis results. Overall, 95.7% of the originally grouped cases were correctly classified.

Fatigue			Predicted group membership		Total
			0	1	
Original	Count	0	34	1	35
		1	1	11	12
	%	0	97.1	2.9	100.0
		1	8.3	91.7	100.0

Interpretation of Results, Contribution of the Study to Understanding the Fatigue Mechanism, and Analysis

Among the 767 saliva proteins identified in this study, 30 fatigue-related proteins were found by systematic statistical analysis. In total, 30% of proteins were related to immunoregulation, and 6.67% were related to inflammatory factors and the inflammatory response. 13.33% were related to metabolism, and 16.67% were related to tumors.

Proteins Related to Immunoregulation

Proteins related to immunoregulation identified in this study included cysteine protease inhibitors, heat shock cognate 71 kDa protein, growth factor receptor binding proteins, immunoglobulin K, immunoglobulin G3, albumin, immunoglobulin K V-III, heat shock protein β1 and serine protease inhibitors. Previous studies have repeatedly shown that fatigue can impair immune function, which can serve as an indicator of the degree and classification of fatigue. Many patients with fatigue are found to have abnormal immune cell counts, immune system molecules and cytokines. For example, Bax et al[21] showed that an increase in fatigue results in immune function decline, indicating that fatigue is a major symptom of suboptimal health status, and has negative effects population-wide. Tirelli et al[22] showed that the immunoglobulin levels of patients with chronic fatigue syndrome were elevated. Papacosta et al[23] detected changes in immunoglobulin A concentrations in the saliva of weightlifters before and after high-intensity exercise that were correlated with exercise load, and showed that salivary immunoglobulin A concentrations could be used to monitor weightlifters' training. The circadian rhythm of salivary immunoglobulin A secretion could be combined for analytical evaluation. These results were consistent with our current findings.

In this study, the efficacies of immunoglobulin K and immunoglobulin G3 as independent diagnostic indicators were above 70%, showing promise for future research. Immunoglobulins are antibodies present in the blood and elsewhere that constitute an important *in vivo* defense mechanism. Immunoglobulin G is the main immunoglobulin generated in the secondary humoral immune response. Furthermore, immunoglobulin G combined with an antigen can activate the complement system. Through the affinity of cell surface receptors, it activates the phagocytic activity of macrophages and neutrophils, and stimulates the cell-killing effect of cytotoxic K cells, thereby defending against various viruses. Immunoglobulin A levels are only secondary to those of immunoglobulin G in healthy human serum[24]. The results of this study showed that immunoglobulin K and immunoglobulin C levels were increased in the fatigue group compared with the non-fatigue group, confirming the value of immunoglobulins in detecting fatigue. Immunoglobulins are associated with many diseases, some of which are systemic, such as Down's syndrome[25], diabetes mellitus[26], hepatitis B[27] and primary Sjögren syndrome[28]. Proteomics and genomics can potentially be used with saliva specimens for the diagnosis and prediction of the above diseases.

The independent diagnostic efficacy of serine protease inhibitors was 68.9% in this study. One serine protease inhibitor family member, protease nexin (PN-1), is encoded by the SERPINE2 gene and is a single chain glycoprotein secreted by a variety of cells. PN-1 is rarely expressed in plasma, but is expressed in many organs and cell types, and has important regulatory roles during many biological processes, such as blood clotting, immune response, fibrinolysis, angiogenesis, inflammation and tumor inhibition. Numerous previous studies have shown a correlation between fatigue and immune response as well as inflammation, which is consistent with our current findings. Serine protease inhibitors that are associated with fatigue had relatively high di-

agnostic value in this study. They also indirectly verify that fatigue is associated with the immune response and inflammation, which is consistent with the correlation between fatigue and immune indices in the population with suboptimal health[29].

Another notable protein is heat shock cognate 71 kDa protein, which had a diagnostic efficacy of 64.3%. Heat shock proteins are endogenous protective proteins produced by organisms under stress, with low expression in normal cells and increased expression in stressed cells. It is known that exercise is sufficient to cause heat shock protein expression, which is also associated with exercise intensity. Studies have shown that overexpression of heat shock proteins in the central nervous system (CNS) is associated with exercise-induced elevation of body temperature, oxidative stress, glucose reduction, energy consumption, blood pH reduction and other stress factors. Heat shock proteins are overexpressed in psoriatic lesions, and are associated with inflammation and immunoregulation[30] in the fatigue-related high occurrence of psoriasis in taxi drivers and police officers. Further studies focusing on fatigue intervention and blockade, as well as the development of heat shock cognate 71 kDa protein inhibitors, may be of clinical significance in the prevention and treatment of psoriasis recurrence.

In addition, basic and clinical studies have shown that heat shock proteins are involved in the incidence and development of atherosclerosis and cardiovascular diseases mediated by inflammation and the immune response. Heat shock proteins induce autoimmune responses, leading to inflammation of blood vessel walls and promoting the development of atherosclerosis[31]. A recent study showed that high levels of heat shock protein 70 (HSP70) and low levels of HSP70 antibodies are independent risk factors for the prediction of the acute coronary syndrome, and they have synergistic effects; fatigue is also common in patients with cardiovascular diseases[32]. The convenient and rapid screening method based on the salivary heat shock proteins developed in the present study may also be used for cardiovascular diseases.

Fatigue-Related Inflammatory Factors and Inflammatory Response-Related Proteins

Previous studies have primarily focused on fatigue-related cytokine biomarkers in peripheral blood. Cytokines that cause inflammation and hyperreactivity include tumor necrosis factor alpha (TNF-α), IL-1, polymorphonuclear (PMN)-elastase, lysozyme and neopterin, which is a cell activator secreted by macrophages in patients with chronic fatigue syndrome that can serve as a biomarker. The degree of exhaustion is significantly and positively related to inflammatory cytokines[33]. In this study, we found an association between bactericidal permeability increasing protein (BPI), catalase, macrophage-capping protein and apolipoprotein with fatigue. The inflammatory response was thus associated with fatigue, which is consistent with previous studies. For example, Baum et al[34] showed that 12 weeks of exercise increased the synthesis of plasma IL-1β and IL-6 in trail running athletes, indicating that exercise-induced fatigue increases inflammatory cytokines in peripheral tissues. In addition, a previous study showed that exercise-induced inflammatory responses cause the elevation of inflammatory cytokines in the brain[35]. A subsequent study also showed that exercise-induced inflammatory responses are first mediated by the hypothalamic-pituitary-adrenal axis (HPA axis), which is also stimulated by inflammatory cytokines to elevate glucocorticoid hormones, such as adrenaline, thereby affecting inflammation and immunity[36]. The fatigue-related proteins associated with the inflammatory response identified in this study were different from the previous work, which might be related to differences in the study cohort, sampling and analytical techniques.

Apolipoprotein is notable among proteins associated with inflammatory responses, and had an independent diagnostic efficacy of 67.3%. Apolipoprotein, which is detectable in saliva specimens, is not only an important protein for convenient fatigue assessment, but can also be used to predict the occurrence of related diseases. For example, clinical papers have shown that increased apolipoprotein concentrations in serum and urine are predictive of early acute kidney injury[37,38]. Apolipoprotein in serum and urine from 19 studies with over 2,500 patients predicted the diagnosis of acute kidney injury and clinical outcomes, such as dialysis time and mortality[39]. Thus, the method developed here could be used for the detection and prevention of fatigue-induced acute kidney injury.

Metabolism-Related Proteins Associated with Fatigue

Salivary amylase, mitochondrial malate dehydrogenase, lactate dehydrogenase B chain and catalase are metabolism-related proteins found in saliva specimens that were associated with

fatigue. Among them, salivary amylase has been reported to be increased in saliva during fatigue, which is consistent with our current findings. Saliva assays have been developed to determine the degree of exhaustion, which is simpler and more convenient than measuring EEG. It was reported that when people feel tired, their blood glucocorticoid levels increase, thereby leading to the secretion of α-amylase in saliva. The measurement device contains a portable chip on which a human saliva specimen can be placed to measure the degree of exhaustion[40].

In the present research, hydrogen peroxidase, also known as catalase, was found among metabolism-related proteins to be associated with fatigue. It is a terminal oxidase that is widely distributed and evolutionarily conserved. Catalase is important for the removal of H_2O_2, it is involved in protection against aging, cancer, and hypertension[41], and is an important factor in initiating apoptosis. As an endogenous oxygen scavenger, catalase plays an important role in protecting cell membranes while maintaining the balance of oxygen metabolism, especially in coronary heart disease and atherosclerosis[42]. Our results suggest that catalase is closely related to fatigue. Fatigue is commonly present in the highly educated middle to senior white-collar populations[43]. Physicians have long suffered from fatigue, which seriously weakens the immune system, resulting in higher susceptibility to a variety of infectious diseases. In addition, fatigue increases the risk of cardiovascular diseases, such as induced acute myocardial infarction[44].

Tumor-Related Protein Associated with Fatigue

We found that Annexin, Golgi membrane protein, nucleoside diphosphate kinase, cystatin, cysteine, and growth factor receptor binding protein 2 were associated with fatigue. Bhatti et al[45] studied urine specimens of physicians and compared the results with subjects who had a regular sleep at night. Physicians who work night shifts have a very low degree of DNA damage repair, approximately 20% that of people who have regular sleep at night. These findings indicate that DNA damage in people who work night shifts will accumulate over time, as will their risk of cancers and other diseases. In this report, we found that fatigue-related proteins were also associated with tumor-related proteins, which further confirms the results of Bhatti et al[45]. The results of our work also indirectly explain why cancer patients are more likely to have fatigue symptoms. Previous methods for detecting cancer markers have mostly been invasive. Detecting the above proteins from saliva specimens may provide a noninvasive diagnostic method to detect related tumor markers in the future.

Since Annexin had a single diagnostic performance of 67.5%, it could be used in later studies. Annexin A2 is a well-known calcium-dependent phospholipid-binding protein that affects a variety of cellular and molecular processes. Disorders of Annexin A2 expression or regulation are involved in a range of diseases, including autoimmune diseases, neurodegeneration, antiphospholipid antibody syndrome, inflammation, diabetes and various cancers. Numerous studies have showed abnormal Annexin A2 expression in various tumor specimens. Through different mechanisms, it affects tumor cell adhesion, proliferation, apoptosis, invasion, metastasis and tumor angiogenesis[46]. Annexin is not only tumor-related. Annexin A2 elevation in serum helps to assess coronary heart disease and guide treatment[47].

In summary, among the above 30 fatigue-related proteins, the discovery of immune proteins, inflammatory factors, and metabolism- and tumor-related proteins contributes to our understanding of fatigue mechanisms and supports mechanistic hypotheses such as HPA axis disorder[48]. Exercise-induced fatigue stress activates the HPA axis and sympathetic nervous system, leading to the activation of immune cells in the brain (mainly microglia and astrocytes) and the secretion of many inflammatory factors (e.g., IL-1β and TNF-α), triggering neuritis. We identified inflammation-related proteins such as BPI, catalase, and Annexin A1, which is consistent with these observations. However, the corresponding factors varied. We did not find a correlation between IL and fatigue, which is inconsistent with a recent neuropathological study[34]. This may be related to Baum et al[34], who did not perform EEG analyses, and thus did not verify if the tested subjects were truly fatigued[4]. In the present work, we found that macrophage-capping protein, apolipoprotein, BPI and catalase were all associated with fatigue; the single diagnostic efficacy of apolipoprotein was only 67.3%, which supports the hypothesis that fatigue results in dysfunction of a variety of infection- and stress-induced neuroendocrine-immune system interactions. The association of metabolic factors (i.e., salivary amylase, mitochondrial malate dehydrogenase, lactate dehydrogenase B chain, and catalase) fur-

ther confirmed that fatigue, because of sustained mental activity or prolonged physical activity, is associated with loss of peripheral muscle strength and CNS signaling pathway-mediated fatigue perception. Fatigue caused by lack of sleep is related to the consumption of muscle glycogen reserves, the accumulation of metabolites and the imbalance of intracellular ions[49].

CNS-mediated fatigue perception caused by sustained mental activity has been reported to be associated with cytokines and/or neurotransmitters (e.g., IL-1, IL-6, TNF, serotonin, dopamine, and tyrosine)[50]. Prolonged physical activity also triggers changes in the autonomic nervous system, which are characterized by a decrease in excitability of the parasympathetic nervous system and an increase in excitability of the sympathetic nervous system[51]. All of these physiological changes provide potential targets for objective monitoring of fatigue, thereby eliminating the dependence on self-reporting.

Comparison with Other Studies

Given the benefits of fatigue assessment in law enforcement, the prospects of salivary component analysis are of great interest. To understand the complex mechanisms of fatigue, solely relying on the detection of a single biomarker might not achieve optimal sensitivity and specificity. Therefore, proteomics has the potential to contribute to early diagnosis and prediction. Proteomics not only quantifies a variety of proteins, but also detects intra- and extracellular localization, post-translational modifications, protein-protein interactions, and function. Proteomic techniques can be used to screen for protein differences on a large scale, and identify the diagnostic values of protein marker molecules[52-54]. Hu et al[55] used proteomics to successfully identify 309 proteins in normal human saliva. However, previous studies on saliva mainly focused on analyzing small molecules under 1,000 Da. Previous metabolomics studies have shown that metabolites in the saliva of athletes can be used as markers of fatigue, i.e., 3-methylhistidine, glucose-1-phosphate, glucose-6-phosphate, and some amino acid compounds[14,56]. However, further analysis of the diagnostic efficacy of these markers is not yet available.

Kataoka et al[53] used automatic solid-phase micro-extraction (SPME) and LC-MS/MS, and measured testosterone (TES), cortisol (CRT), and dehydroepiandrosterone (DHEA) simultaneously in 40-μL saliva specimens. The SPME and LC-MS/MS methods have good linear relationship ($R \geq 0.9998$), precision (within-day and inter-day precisions of 4.9% and 8.5%, respectively), and detection sensitivity (quantitative limits of approximately 0.01, 0.03, and 0.29 ng/mL saliva, respectively). These methods were used to analyze changes in TES, CRT, and DHEA levels in saliva under stress and fatigue, demonstrating the advantages of mass spectrometry. Although changes in small-molecule hormones and metabolites detected by LC-MS/MS may be associated with fatigue, these changes are easily affected by dietary and other health conditions. In addition, these small molecular markers often do not have good antigenicity. It is difficult to identify the biomarkers (found by precision instrumental analysis) using simple methods such as immunostaining or biosensors. The above studies did not apply EEG to determine the fatigue standard and establish a fatigue assessment model, perhaps because of it being time-consuming, laborious and expensive. Nevertheless, spontaneous EEG has been proposed as the most promising indicator of fatigue[57], even though a variety of psychophysiological parameters have been used in the assessment of fatigue[58]. In the present paper, we used significant increases in δ and θ waves, which are slow waves representing brain inhibition, as an established standard for fatigue assessment for each healthy volunteer. Among them, θ waves offer a state of somnolence with reduced consciousness, light sleep or extreme relaxation; δ waves are emitted during deep and dreamless sleep when there is unconsciousness. This allowed for the establishment of a fatigue assessment model to compare the proteomic data from the saliva of the emergency physicians. Compared with previous investigations, we identified 767 proteins in the saliva, and based on fatigue-related biomarkers ranging from 2,000 to 15,000 Da determined in the preliminary study, our method had stable tested components, less *in vivo* disturbance, and was easily converted to a common detection system. The fatigue-related biomarkers identified in this paper had more theoretical significance and application prospects.

In addition, the research subjects of this work were emergency physicians who had comprehensive fatigue, which was different from the simple exercise-induced fatigue in athletes and soldiers analyzed in previous studies. Emergency physicians often have long and continuous working hours, high work intensity and pressure. In addition, prolonged adequate rest among emergency physicians to ease their fatigue cannot control the underlying problem.

This merely results in subclinical manifestation of chronic fatigue syndrome, leading to different *in vivo* indicator responses, which is different from the populations in previous studies. Individual differences in inflammatory factors and fatigue-related inflammatory factors also differed greatly from previous studies. In addition, we used Q Exactive Plus mass spectrometry, a highly improved technology compared with previous researches, to identify protein (or peptide) spectra in saliva specimens combined with advanced high-throughput mass spectrometry to identify fatigue-related proteins and verify fatigue-related immune, inflammatory and metabolic mechanisms.

Research Significance and Direction Value of the Study

We showed significant changes in proteomic markers before and after long working hours in emergency physicians, and achieved a simple and convenient assessment of fatigue. This study is the first complete fatigue-related proteomic marker analysis in a cohort of emergency physicians with fatigue using Q Exactive Plus mass spectrometry with saliva specimens.

We identified 30 fatigue-related proteins based on the EEG-defined fatigue standards and established a fatigue-assessment model with good diagnostic efficacy. We found the presence of detectable fatigue-related markers in saliva specimens, which will be important for future studies to develop rapid qualitative and quantitative fatigue assessment.

We also found that immune-, inflammatory factor-, metabolism-, and tumor-related proteins were fatigue-related, which will aid in the study of mechanisms of fatigue. Our findings support the current understanding of fatigue mechanisms, such as the association with HPA axis disorder, neuroendocrine-immune system interaction dysfunction, peripheral muscle strength loss, and the CNS signaling pathway-mediated fatigue perception. This study provides potential targets for the objective monitoring of the degree of exhaustion.

In addition, this work may expand our understanding of various fatigue-related diseases. Among the fatigue-related protein markers in our proteomic analysis, many are associated with stroke, cancer, myocardial infarction and skin diseases. The correlation analyses among the fatigue-related protein markers in saliva and between fatigue factors and stroke, psoriasis and cancer may provide new directions for the prevention and treatment of these diseases.

Further Study

The current proteomics analysis and the discovery of fatigue markers provides a first step toward rapid and convenient fatigue assessment for law enforcement; however, many further studies will be necessary for verification. They should focus on the structural prediction of proteins with potential diagnostic values to assess fatigue marker efficacy and application potential according to the existing linear epitopes and through high-throughput epitope peptide synthesis, antigenicity evaluation and the preparation of salivary immune-micro assays using protein chips. Ultimately, this will guide the development of rapid, convenient, reliable and objective fatigue-detection technology with practical applications.

Conclusions

The results of this study are important for the identification and testing of fatigue-related mechanisms, and have important clinical implications. We verified detectable fatigue markers in saliva and their association with immunity, inflammation and metabolism. This work established a fatigue assessment model and related biomarkers in saliva as a basis for non-invasive, rapid, and convenient assays for fatigue assessment and to assist in law enforcement, thereby reducing iatrogenic and occupational injuries caused by fatigue.

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Author contributions statement

YX and YG participated in the study design, carried out the study, collected important background information, analyzed and interpreted data, wrote and revised the manuscript; DX performed experimental analysis planning and execution; XP performed saliva sample analysis; XG conceived and designed the study, performed literature searches, data acquisition, data analysis and manuscript preparation; CZ, LH, LY, and DM participated in saliva

sample collection, preservation and transportation; AX, XS, FL and JW participated in saliva sample collection, volunteer organization, equipment operation, EEG data collection and analysis of results; LL participated in data analysis; *ZL participated in the organization and implementation of the study; **JZ conceived and designed the experiments and participated in the writing and revision of the manuscript.

Competing financial interest statement

The authors declare there are no competing financial interests.

Data availability statement

All data generated or analyzed during this study are included in this published article (and its supplementary information files).

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