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Fatigue: Biomedicine, Health & Behavior

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/rftg20

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To cite this article: Leonard A. Jason, John Kalns, Alicia Richarte, Ben Z. Katz & Chelsea Torres (2021) Saliva fatigue biomarker index as a marker for severe myalgic encephalomyelitis/chronic fatigue syndrome in a community based sample, Fatigue: Biomedicine, Health & Behavior, 9:4, 189-195, DOI: 10.1080/21641846.2021.1994222

To link to this article: https://doi.org/10.1080/21641846.2021.1994222

FATIGUE: BIOMEDICINE, HEALTH & BEHAVIOR
2021, VOL. 9, NO. 4, 189–195
https://doi.org/10.1080/21641846.2021.1994222

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Saliva fatigue biomarker index as a marker for severe myalgic encephalomyelitis/chronic fatigue syndrome in a community based sample

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ABSTRACT

Objective: The prevalence of pediatric Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (ME/CFS) has been estimated from an ethnically and sociodemographically diverse community-based random sample of 10,119 youth aged 5–17. We assessed whether a salivary biomarker of fatigue could identify youth with ME/CFS.

Study design: We examined the ratio of the concentrations of 2 peptide fragments in saliva, referred to as the Fatigue Biomarker Index (FBI), in participants from our study diagnosed with ME/CFS ($n = 59$) and matched controls ($n = 39$).

Results: Significant overall differences were found in the FBI between those participants with severe ME/CFS and those with ME/CFS and the controls.

Conclusions: If confirmed in other populations, the FBI could serve as an objective test to aid in the diagnosis of severe ME/CFS.

Abbreviations: ANOVA: analysis of variance; BSA: bovine serum albumin; FBI: fatigue biomarker index; IM: infectious mononucleosis; ME/CFS: myalgic encephalomyelitis/chronic fatigue syndrome; OF: oral fluid; S-ME/CFS: severe myalgic encephalomyelitis/chronic fatigue syndrome

ARTICLE HISTORY
Received 20 August 2021
Accepted 12 October 2021

KEYWORDS
Fatigue biomarker index; chronc fatigue syndrome; myalgic encephalomyelitis

Introduction

Youth with Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (ME/CFS) have significant problems in physical functioning and school activities [1]. A recent community-based study of a sample of 10,119 youth aged 5–17 from 5622 households in the Chicagoland area was used to estimate the prevalence of ME/CFS among youth [2]. Using a probabilistic, multi-stage formula, the prevalence of pediatric ME/CFS was estimated to be 0.75%. It is important to assess biological samples of youth with ME/CFS.

A possible metabolic marker for ME/CFS could come from saliva, collection of which is noninvasive. For example, John Kalns et al. [3], using reversed-phase liquid chromatography and high-resolution mass spectrometry to separate the various components of

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This article has been corrected with minor changes. These changes do not impact the academic content of the article.

processed saliva, found that fatigue caused the concentration of two peptide fragments found in saliva to change in opposite directions. The ratio of the concentrations of the 2 peptide fragments in saliva is referred to as the Fatigue Biomarker Index (FBI) [4]. Michael et al. [5] studied male recreationally trained cyclists before and after exercise and demonstrated that the FBI decreased with time as relative perceived exertion increased. Kalns et al. [3] found that the level of the salivary FBI was one of the best markers for predicting success or failure in military training candidates. Michael et al. [6] found that this salivary biomarker distinguished 16 sleep-deprived subjects from a control group. Finally, Kuo et al. [7], with a sample of individuals who frequently report severe fatigue, found that the FBI correlated with general fatigue. These studies suggest that this salivary biomarker of physical fatigue holds promise as a measure of fatigue and a possible biomarker for ME/CFS. The current study explored whether this saliva biological marker might differentiate youth with ME/CFS from healthy community controls.

Methods

Procedures

The first stage of this study involved calling households in the greater Chicago area (see Jason et al. [2] for more details). The parent/caretaker respondent indicated the presence or absence of fatigue in each child and adolescent in the household. The *Pediatric ME/CFS Screening Questionnaire* was administered to respondents to screen for ME/CFS-like profiles among children and adolescents [8]. Children and adolescents who screened positive for either significant fatigue or school/learning/memory problems, had substantial reductions in functioning, and three or more ME/CFS Fukuda et al. [9], Institute of Medicine [10], or Carruthers et al. [11] symptoms were considered *screen-positive* and selected for full evaluation in Stages 2 and 3. Additionally, *screen-negative* control participants were brought in for Stages 2 and 3; control individuals were selected based on interest in participating in the study and demographic matching by gender, age, and ethnicity with screen positive participants. For stage 2 of the study, caretakers/parent(s) and youth completed the pediatric version of the DePaul Symptom Questionnaire (DSQ, a self-report measure of ME/CFS symptomatology) [8], the Child Health Questionnaire (CHQ, an instrument that assesses physical and psychosocial well-being) [12], the Autonomic Symptom Checklist [13], and the Fatigue Severity Scale [14]. For Stage 3, the Schedule for Affective Disorders and Schizophrenia for School Aged Children Present and Lifetime Version [15] was administered to both the child and parent/caretaker during the in-person appointment, and the youth were provided with a medical interview and a complete physical examination, the latter of which included comprehensive questioning regarding signs and symptoms of ME/CFS. This study was approved by all relevant the Institutional Review Boards before data collection began and informed consent/assent was obtained from all participants and/or their parents/guardians as appropriate.

Determination of fatigue biomarker index in oral fluid (OF)

Competitive ELISA's were used to determine the concentration of two peptides found in abundance in OF. Peptide 1: GGHPPPP, and peptide 2: GNPQGPSPQGGNKPQGPPPPP GKPQ. All samples were evaluated in a blinded fashion by the laboratory.

Briefly, peptide-bovine serum albumin (BSA) conjugates were allowed to bind to high-protein binding 96-well plates overnight and then blocked with 3% BSA. Standards and OF samples were incubated for 30 minutes at room temperature with primary antibody plus secondary antibody conjugated to Horse Radish Peroxidase (HRP) in 1% BSA in PBS at room temperature. Standards and OF samples conjugated to antibody-HRP complex are added to the wells coated with peptide-BSA conjugates. The binding of antibody-HRP complex to peptide-BSA conjugate coated on the bottom of the well is inversely proportional to the amount of peptide present in standard or sample. Following a 1 h incubation at room temperature, wells are washed four times with 0.05% Tween20 in PBS. HRP substrate 3,3',5,5', tetramethyl benzidine is added, neutralized after incubation by addition of 2N H2SO4 and read on a microtiter plate reader (SpectraMax M2, Molecular Devices) at 450 nm. Concentrations of each peptide present in saliva samples were calculated from standard curves. The Fatigue Biomarker Index was calculated as the log10 (peptide 1/peptide2).

Participants

We screened 5622 households, and survey data were gathered for 10,119 children and adolescents, 298 of whom were reported to suffer from prolonged, unexplained fatigue and additional symptoms that met our criteria for a screen-positive. Of those who screened positive, 165 (55.4%) were able to complete Stages 2 and 3 of the study. Of those who screened negative, 243 were eligible to participate in Stage 2 based on demographic matching protocols, and 44 of the 243 (18.1%) were recruited as control participants.

During the stage 3, in-person visit, saliva was collected between 9 AM and 4 PM via passive drool. At the end of Stage 3, a team of physicians were responsible for making final diagnoses. Two reviewing physicians, aside from the examining physician, had access to all information gathered on each participant during each of the three phases of the study including results from the physical exam and used this information to make a final diagnosis of ME/CFS on the individuals in our study using the Fukuda et al. [9] criteria with revisions recommended by Reeves et al. [16], the pediatric ME/CFS criteria developed by an canadian study group [17] and/or the Institute of Medicine 2015 clinical criteria [10]. Both reviewing physicians had to agree with each diagnosis. A number of studies [18–20] have found that the Fukuda et al. criteria [9] are broader and identify a larger group of patients, with less severity, than those identified by other criteria. We have also shown, in a cohort of college students who developed ME/CFS following infectious mononucleosis that those who met >1 set of criteria of ME/CFS scored significantly worse than those who met only one set of criteria and trended towards worse scores on many other questionnaires as well [21]. In addition, we have shown that those who suffered more severe infectious mononucleosis were more likely to develop ME/CFS that met >1 case definition 6 months later [22]. Similarly, when we compared the participants in our pediatric cohort who were diagnosed with ME/CFS based on meeting a single case definition with those who met >1 case definition, those who met >1 case definition scored higher on the Child Report Form of the DSQ in 3 of 8 domains (post-exertional malaise, neurocognitive symptoms and fatigue) and had worse physical

functioning as measured on the CHQ (data not shown). Thus, participants who met only one set of criteria for ME/CFS, primarily the Fukuda et al. [9] criteria, which are broad and identify a larger group of patients with less severity, were defined as having ME/CFS. Those who met more than one case definition (i.e. the Fukuda and either the international [17] and/or Institute of Medicine [10] criteria) were defined as having *severe* ME/CFS (S-ME/CFS). Finally, those who recovered were labeled controls. The final sample sizes with collected saliva available for analysis were 59 case subjects (19 participants with ME/CFS who met one case definition, and 40 participants who met >1 case definition of ME/CFS [S- ME/CFS]) and 39 controls (C). All samples were coded and analyzed in a blinded fashion.

Statistics

We used analysis of variance (ANOVA) and Tukey planned comparisons to examine the differences (if any) among participant's salivary fatigue biomarker indexes. Tukey uses pairwise post-hoc testing to determine whether there is a difference between all pairs of means using a studentized range distribution [23].

Results

There were no significant differences between the screen-positive subjects and screen-negative controls in terms of gender, race/ethnicity, or age, as expected, as test negative control participants were invited to participate based on a demographic-matching process.

Using ANOVA, significant overall differences were found in the salivary FBI [$F(2,95) = 3.25$, $p < .05$] between the three groups, those participants with S-ME/CFS, those with ME/CFS and the controls. There were also no significant gender, race/ethnicity, or age differences between the S-ME/CFS, ME/CFS and Control groups. Using Tukey planned comparisons, the control group ($M = -1.39$, SD = .46) was significantly different at the $p < .05$ level from the S-ME/CFS group ($M = -1.62$, SD = .34). The Control group was not directionally significantly different from the ME/CFS group ($M = -1.53$, SD = .42), and there were no significant differences between the ME/CFS and the S-ME/CFS groups.

Discussion

The current study found that those with more severe ME/CFS had significantly lower fatigue index scores than controls, whereas those with ME/CFS had only a trend in that direction. This is similar to what we recently reported in another study, where participants with S-ME/CFS following infectious mononucleosis (IM), compared to those with ME/CFS following IM, had more abnormalities prior to IM and more severe IM [21,22].

We have now shown with different analyses on two different populations of participants' with ME/CFS (a youth community sample [present study] and a sample of college students following infectious mononucleosis [21]) that there are two populations of individuals with ME/CFS: those who meet only one set of criteria, primarily the Fukuda criteria and those who meet >1 standard case definition and who are more severely impaired. It is this latter group who have more markers of illness:

in the college student population those who developed S-ME/CFS following infectious mononucleosis had more complaints of fatigue and cytokine abnormalities at baseline [21] and a history of more severe infectious mononucleosis six months' prior [22], and in the current youth community sample those youth with S-ME/CFS had more severe findings on the DSQ and CHQ and had a significantly lower fatigue index.

The fatigue biomarker peptides arise from proline rich proteins found in saliva. Specifically, the numerator peptide GGHPPPP arises from the expression of genes PRH1 and PRH2 giving rise to acidic salivary proline rich proteins 1 and 2. The denominator peptide GNPQGPSPQGGNKPQGPPPPPGKPQ arises from the expression of the gene PRB1 giving rise to basic salivary proline rich protein 1. These proteins are extensively processed into smaller peptides prior to secretion [24]. In previous publications [5], we have used ESPSLIA as the denominator. ESPSLIA arises from the same parent protein as GNPQGPSPQGGNKPQGPPPPPGKPQ and the fatigue index calculated using either of these peptides is similar (data not shown). Analysis of gene expression shows that expression of these proteins occurs in the lung and the parotid glands. The biologically activity of these proteins includes dental carries formation [25] and binding of tannins found in food and wine [26]. Our study has a number of limitations, including a relatively small sample size for the severe ME/CFS group. In addition, as already mentioned, the FBI was only predictive of severe ME/CFS; there was only a trend in the same direction when we examined youth who only met the Fukuda definition of ME/CFS.

Despite these limitations, the FBI might be useful as a biomarker for severe ME/CFS if it can be replicated and validated in other populations and with larger samples. Those who have severe ME/CFS have significant and multiple impairments, and a biomarker could lead to quicker diagnosis and better treatment or prevention strategies in this more affected population.

In conclusion, we have shown that those with more severe ME/CFS had significantly lower fatigue index scores than controls, whereas those with ME/CFS only had a trend in that direction. This study also reinforces our previous work of ME/CFS following infectious mononucleosis in college students [21, 22] in a different population, showing that individuals with severe ME/CFS may have more biological differences when compared with controls than those with ME/CFS [9]).

Acknowledgements

We thank Alex Tomas for expert technical assistance.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

This work was supported by the Eunice Kennedy Shriver National Institute of Child Health and Human Development of the National Institutes of Health [award number R01 HD072208].

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