



Human herpesvirus 6 and 7 are biomarkers for fatigue, which distinguish between physiological fatigue and pathological fatigue



Ryo Aoki^{a,*}, Nobuyuki Kobayashi^b, Go Suzuki^c, Hirohiko Kuratsune^{d,e}, Kazuya Shimada^b, Naomi Oka^b, Mayumi Takahashi^b, Wataru Yamadera^a, Masayuki Iwashita^a, Shinichi Tokuno^f, Masashi Nibuya^g, Masaaki Tanichi^g, Yasuo Mukai^h, Keiji Mitaniⁱ, Kazuhiro Kondo^b, Hiroshi Ito^a, Kazuhiko Nakayama^a

^a Department of Psychiatry, The Jikei University School of Medicine, 3-25-8 Nishi-Shimbashi, Minato-ku, Tokyo 105-8461, Japan

^b Department of Virology, The Jikei University School of Medicine, 3-25-8 Nishi-Shimbashi, Minato-ku, Tokyo 105-8461, Japan

^c Flight Crew Operations and Technology Unit, Japan Aerospace Exploration Agency Houston Office, 18050 Saturn Lane, Suite 310, Houston, TX 77058, USA

^d Clinical Center for Fatigue Science, Osaka City University Graduate School of Medicine, Osaka, Japan

^e Department of Health Science, Faculty of Health Science for Welfare, Kansai University of Welfare Sciences, Osaka, Japan

^f Department of Defense Medicine, National Defense Medical College, Tokorozawa, Saitama, Japan

^g Department of Psychiatry, National Defense Medical College, Tokorozawa, Saitama, Japan

^h Military Medicine Research Unit, Test and Evaluation Command, Japan Ground Self Defense Force, Setagaya, Tokyo, Japan

ⁱ Department of Internal Medicine, Japan Self-Defense Force Central Hospital, Setagaya, Tokyo, Japan

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ABSTRACT

Fatigue reduces productivity and is a risk factor for lifestyle diseases and mental disorders. Everyone experiences physiological fatigue and recovers with rest. Pathological fatigue, however, greatly reduces quality of life and requires therapeutic interventions. It is therefore necessary to distinguish between the two but there has been no biomarker for this. We report on the measurement of salivary human herpesvirus (HHV-) 6 and HHV-7 as biomarkers for quantifying physiological fatigue. They increased with military training and work and rapidly decreased with rest. Our results suggested that macrophage activation and differentiation were necessary for virus reactivation. However, HHV-6 and HHV-7 did not increase in obstructive sleep apnea syndrome (OSAS), chronic fatigue syndrome (CFS) and major depressive disorder (MDD), which are thought to cause pathological fatigue. Thus, HHV-6 and HHV-7 would be useful biomarkers for distinguishing between physiological and pathological fatigue. Our findings suggest a fundamentally new approach to evaluating fatigue and preventing fatigue-related diseases.

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1. Introduction

Resulting from work-related or other stress or insomnia, fatigue is something that everyone experiences. Long-term fatigue can cause cardiovascular dysfunction, mental disorders such as depression, and occupational sudden death (karoshi) [1–3]. Fatigue is therefore a major social problem.

People recover from physiological fatigue with rest. Pathological fatigue, however, persists for 3 months or more and greatly affects QOL [4]. As the latter requires therapeutic interventions, we must

distinguish between physiological and pathological fatigue. In this study, we investigated potential biomarkers for objectively assessing fatigue.

Fatigue is frequently assessed by self-reporting using the Checklist Individual Strength, Profile of Mood States or visual analog scales [5,6]. However, its perception is influenced by negative or positive work events and compensation practices can motivate workers to distort self-reported fatigue levels [7], which therefore may not be a correct indicator.

We focused on human herpesvirus (HHV-) 6 and HHV-7, which are reactivated by fatigue. These viruses cause exanthem subitum and establish latency in almost all individuals [8]. Frequently reactivated and shed in saliva, they are potentially a useful fatigue biomarker [9]. HHV-6 is reportedly useful for monitoring cognitive

* Corresponding author.

E-mail address: ryo-fcb@jikei.ac.jp (R. Aoki).

function, and adverse reactions in cancer chemotherapy [10,11] and HHV-7 for assessing fatigue in end-stage renal disease [12].

We assessed fatigue prior to, during and after Japan Self-Defense Force (JSDF) military training using HHV-6 and HHV-7 DNA quantities. The JSDF was considered to be a uniform population regarding sleep, diet and other characteristics, with few confounding factors. We also investigated the mechanism of HHV-6 and HHV-7 reactivation in a mouse fatigue model, focusing on changes in inflammatory cytokines and CD14 and CD16 expressions in spleens due to fatigue. Research has shown that HHV-6 latently infects monocytes/macrophages [13] and that inflammatory cytokines play an important role in its reactivation [14]. Inflammatory cytokines are reportedly involved in monocyte activation and differentiation [15] and mature macrophages are positive for CD14 and CD16 [16,17], markers of their differentiation.

We also measured salivary HHV-6 and HHV-7 DNA quantities in patients with obstructive sleep apnea syndrome (OSAS), chronic fatigue syndrome (CFS) and major depressive disorder (MDD), which are thought to cause pathological fatigue, and normal controls (NCs). In OSAS, repeated episodes of upper-airway obstruction during sleep and cause nocturnal awakening, impairment of sound sleep, and other forms of insomnia. Causing daytime sleepiness and fatigue [18,19], OSAS is a major societal problem because sufferers become less efficient at work. CFS is triggered by complex conditions, including infection, and its diagnosis requires 6 months of unexplained fatigue that is not alleviated by rest, with 4 of 8 additional symptoms (e.g. unrefreshing sleep, sore throat, muscle pain) [20]. Depression is a low mental state with loss of interest and feeling of joy, that is frequently accompanied by fatigue [21].

To determine the usefulness of salivary HHV-6 and HHV-7 as fatigue biomarkers, we used subjects with physiological fatigue and those with pathological fatigue.

2. Materials and methods

2.1. Ethics statement

The study was approved by the Ethics Committees of the Jikei University School of Medicine, National Defense Medical College, Osaka City University, Soiken Inc. and Soiken Clinic. Written informed consent was obtained from each subject. Animal experiments were approved by the Institutional Animal Care and Use Committee of the Jikei University.

2.2. Participants

Members of the Japan Self Defense Force (JSDF) 1st Airborne Brigade participating in 9-week ranger training were recruited for the study [22]. It was confirmed that participants had no serious physical diseases or previous psychiatric illnesses from the results of annual physical examinations and pre-training selection examinations. Those who dropped out during the training were excluded. All were male and their average age was 26.6 ± 0.4 [mean \pm s.e.m.] years ($n = 55$). The training program basically consisted of self-training for a week, a day's rest, base training for 4 weeks, a day's rest and then 4 week's of field training. Subjects rated their fatigue level on a visual analog scale (VAS) whose total score ranged from 0 (no fatigue) to 100 (total exhaustion) at four time points: before training (2 days before training), during base-training (2 weeks into training), during field-training (3 weeks into training), and after training (3–5 days after end of the training) [22]. Saliva samples were all collected at the same time.

We also recruited 113 NCs (49 females, 64 males; age 43.5 ± 0.8 [mean \pm s.e.m.]) using an advertisement. Exclusion criteria were history of medical illness, taking chronic medication or

supplemental vitamins, body weight less than 40 kg, and blood donation within one month before the study or blood hemoglobin level less than 12.0 g/dl.

Regarding pathological fatigue, 42 patients aged 20–64 years who were diagnosed with OSAS at Jikei University Hospital (Tokyo) (6 females, 36 males; age 49.8 ± 1.7 [mean \pm s.e.m.]) were enrolled. Diagnosis was by overnight polysomnography (PSG) in accordance with the International Classification of Sleep Disorders (ICSD-2) [23]. Also enrolled were 97 CFS patients (68 females, 29 males; age 37.8 ± 0.8 [mean \pm s.e.m.]) aged between 20 and 64 years who were diagnosed at the Osaka City University Hospital based on the 1994 revised working case definition of Centers for Disease Control and Prevention (CDC) [20]. Symptoms were assessed using the Chalder Fatigue Scale [24]. We additionally enrolled 33 MDD patients (10 females, 23 males; age 45.3 ± 1.9 [mean \pm s.e.m.]) aged between 20 and 64 years who were diagnosed at the Jikei University Hospital or the Jikei University Kashiwa Hospital based on the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-V) [21]. Depressive symptoms were assessed using the Montgomery-Asberg depression rating scale (MADRS) [25,26].

Saliva samples of less than 200 μ L were omitted from the analysis.

2.3. Measurement of salivary HHV-6, HHV-7

Saliva samples were collected in a test tube (Salivette; Sarstedt AG & Co.). Following centrifugation, the flow-through was stored at -80°C until analyzed. Viral DNA was extracted from 400 μ L saliva or phosphate-buffered saline-diluted samples by automatic isolation with the BioRobot EZ1 workstation and EZ1 virus mini kit v2.0 (QIAGEN, Inc.), according to the manufacturer's protocol. DNA was eluted in 90 μ L of elution buffer. Copies of HHV-6 and HHV-7 DNA in the saliva samples were quantified by real-time PCR with an Applied Biosystems 7300 real-time PCR System (Applied Biosystems). Amplifications were performed in duplicate in a total volume of 50 μ L containing 25 μ L of Premix Ex Taq (Perfect Real Time) (Takara Bio Inc.), 0.45 μ L of PCR forward primer (100 μ M), 0.45 μ L of PCR reverse primer (100 μ M), 1.25 μ L of TaqMan probe (10 μ M), 1 μ L of Rox reference dye, 5 μ L of the viral DNA, and 16.85 μ L of PCR-grade water. The primers used for real-time PCR were as follows: HHV-6 forward primer, 5'-GACAATCACATGCCTGGA-TAATG-3'; HHV-6 reverse primer, 5'-TGTAAGCGTGTGGTAATGGAC-TAA-3'; HHV-6 probe, 5'-FAM-AGCAGCTGGCGAAAAGTGCTGTGC-TAMRA-3'; HHV-7 forward primer, 5'-CGGAAGTCACTGGAGTAAT-GAC-3'; HHV-7 reverse primer, 5'-CCAATCCTCCGAAACCGAT-3'; and HHV-7 probe, 5'-FAM-CCTCGCAGATTGCTTGTGGCCATG-TAMRA-3' [27,28]. The thermal profile was 95°C for 30 s, followed by 50 cycles of 95°C for 5 s and 60°C for 31 s. Data analysis used Sequence Detection Software version 1.4 (Applied Biosystems).

2.4. Animals

Six-week-old C57BL/6NCRSlc male mice were purchased from SLC Japan. They were housed in standard cages in a temperature- and humidity-controlled room with a 12-h light/dark cycle (lights on at 8:00) and free access to standard lab chow and water. We modified a rat model of fatigue for application to mice [29]. Briefly, the mice were divided into two groups: control group (Control) with no stress load and 24-h group (Fatigue) in which the mice were placed for 24 h in a cage filled with water to a height of 1 cm. The mouse spleens were harvested immediately after stress loading and preserved at -80°C until analysis.

2.5. Real-time RT-PCR

Total RNA was extracted from spleen samples using the Bio-Robot EZ1 workstation and EZ1 RNA Universal Tissue Kit (QIAGEN, Inc.). cDNA was synthesized from total RNA using the PrimeScript RT reagent Kit (Takara Bio Inc.). Mouse spleen mRNA levels of inflammatory cytokines (IL-1 β and IL-6), CD14 and CD16, and β -actin (ACTB; internal control) were determined by real-time PCR with the Applied Biosystems 7300 real-time PCR System (Applied Biosystems). Amplifications were performed in duplicate in a total volume of 25 μ l containing 12.5 μ l of FastStart TaqMan Probe Master (ROX) (Roche Diagnostics, Indianapolis, IN, USA), 0.225 μ l of PCR forward primer (100 μ M), 0.225 μ l of PCR reverse primer (100 μ M), 0.625 μ l of TaqMan probe (10 μ M), 2 μ l of the cDNA, and 9.425 μ l of PCR-grade water. The primers used for real-time PCR were as follows: IL-1 β forward primer, 5'-CCTGAACCTCAACTGTGAAATGCC- 3'; IL-1 β reverse primer, 5'-CTGCTGCGAGATTTGAAGCTG- 3'; IL-1 β probe, 5'-FAM-TGCTCTCATCAGGACAGCCCAGGTCA-TAMRA-3'; IL-6 forward primer, 5'-CCGGAGAGGAGACTTCACAGA- 3'; IL-6 reverse primer, 5'-GTTGTCATACAATCAGAATTGCCATT- 3'; IL-6 probe, 5'-FAM-ACCACTCCCAACAGACACTGTCTATACCACT-TAMRA-3'; CD14 forward primer, 5'-GCACACTCGTCAACTTTCC- 3'; CD14 reverse primer, 5'-CTTGGAGGTTCGGGAATTG- 3'; CD14 probe, 5'-FAM-TCTTCCTGCCTCTCCACCTTAGACCT-TAMRA-3'; CD16 forward primer, 5'-CAGTCCAAGCTGTCACCATC- 3'; CD16 reverse primer, 5'-GCACATCACTAGGAGAAAGCA- 3'; CD16 probe, 5'-FAM-TGTCCAAGACCCAGCAACTACATCCTCC-TAMRA-3'; β -actin forward primer, 5'-CGCGAGCACAGTCTCTTTG- 3'; β -actin reverse primer: 5'-CGACCAGCGCAGCGATATC- 3'; and β -actin probe, 5'-FAM-CACACCCGCCACCAGTTCGCCATG-TAMRA-3'. The thermal profile was 95 $^{\circ}$ C for 10 min, followed by 45 cycles of 95 $^{\circ}$ C for 10s and 60 $^{\circ}$ C for 31s. Data analysis used Sequence Detection Software version 1.4 (Applied Biosystems).

2.6. Flow cytometric analysis

Mouse spleens were placed into a 60 mm Petri dish and mashed using the plunger end of a syringe. The cell suspension was subjected to erythrocyte lysis using red blood cell lysis solution (Miltenyi Biotech), and the cell pellet resuspended in RPMI 1640 medium supplemented with 10% FBS. Flow cytometric analysis was performed on 1×10^6 spleen cells in 100 μ l PBS supplemented with 0.5% BSA and 2 mM EDTA. FITC-conjugated rat anti-mouse CD14

[clone rmC5-3] (BD Pharmingen) or FITC Rat IgG1, κ isotype control [clone 275003] (BD Pharmingen) was added and incubation conducted for 10 min at 4 $^{\circ}$ C in the dark, followed by addition of allophycocyanin (APC)-conjugated rat anti-mouse Fc gamma RIII/CD16 [clone 275003] (R&D Systems) or APC Rat IgG2A isotype control [clone 54447] (R&D Systems) and further incubation for 30 min at 4 $^{\circ}$ C in the dark. Next, cells were washed twice with PBS supplemented with 0.5% BSA and 2 mM EDTA, and fixed with 0.5% paraformaldehyde. Flow cytometric analysis was performed on a MACSQuant Analyzer using MACSQuantify software (Miltenyi Biotech). Results were verified by performing at least five independent experiments.

2.7. Statistical analysis

Data from the JSDF subjects were analyzed using repeated measures analysis of variance (ANOVA). To compare two different groups, Welch's *t*-test was used. Multigroup comparisons were performed using the Kruskal-Wallis test with Dunn's multiple comparisons test. Correlations between HHV-6 DNA quantities or HHV-7 DNA quantities and clinical data were examined using Spearman's rank correlation coefficients. $P < 0.05$ was considered statistically significant. Statistical analyses were conducted using SPSS Statistics 21 for Windows (IBM Corporation) and Prism 6 for Mac OS X (GraphPad Software, Inc.).

3. Results

3.1. Salivary HHV-6 and HHV-7 DNA copy numbers in JSDF

Feeling of fatigue steadily increased in the base training and field training and decreased with rest. However, after resting, it was greater than before training (Fig. 1A). Salivary HHV-6 DNA increased in the field training and with rest decreased to the same level as before training. (Fig. 1B). The Salivary HHV-7 DNA amount increased in the base training and field training and decreased with rest, but after rest it was greater than before training, similar to feeling of fatigue (Fig. 1C).

3.2. Changes in inflammatory cytokines and myeloid cell differentiation markers in animal model of fatigue

Fatigue loading increased mRNA expression of interleukin 1 β (IL-1 β) in mouse spleens (Fig. 2A), but there was no significant

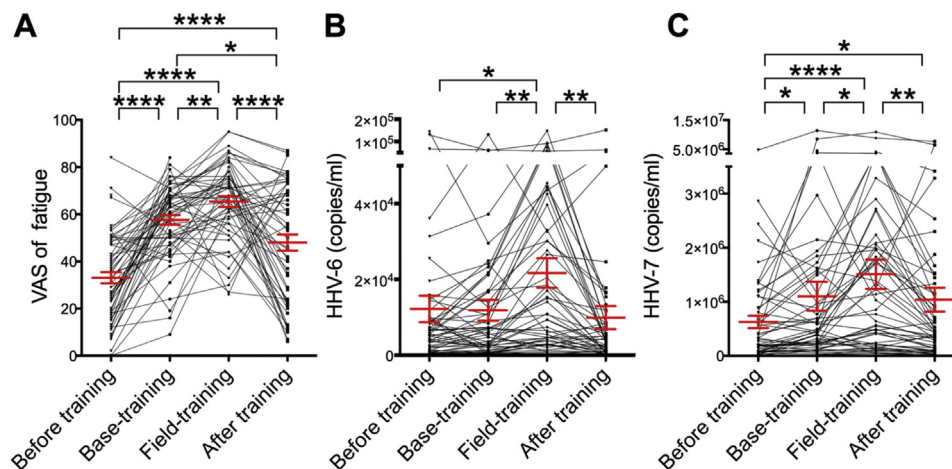


Fig. 1. VAS scores for feeling of fatigue (A), Numbers of HHV-6 (B) and HHV-7 (C) DNA copies are plotted at four points in the ranger training period. Horizontal lines show means and error bars indicate s.e.m. Significant differences between groups are indicated as * $P < 0.05$, ** $P < 0.01$, and **** $P < 0.0001$ using repeated measures ANOVA.

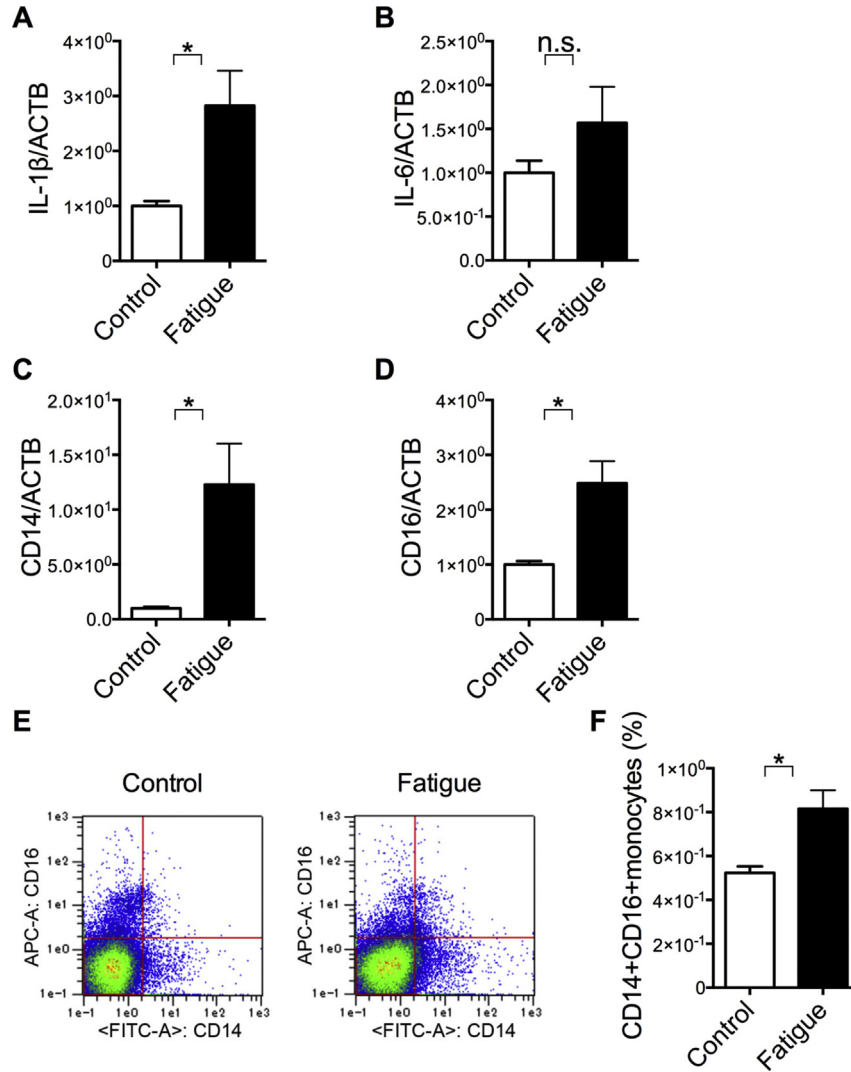


Fig. 2. Changes in inflammatory cytokines, myeloid cell differentiation markers in mouse spleens with fatigue loading. Spleen mRNA levels of IL-1 β (A), IL-6 (B), CD14 (C) and CD16 (D) with fatigue (Fatigue; n = 6) vs. no fatigue (Control; n = 7). CD14 and CD16 positive monocyte levels with fatigue (Fatigue; n = 7) vs. no fatigue (Control; n = 6) (E). Positive rates for CD14 and CD16 together vs. control (F). Horizontal lines indicate means and error bars s.e.m. Significant differences between groups are indicated as * P < 0.05, n.s.: not significant by Welch's t -test.

change in IL-6 mRNA expression (Fig. 2B). It also increased CD14 and CD16 mRNA expressions (Fig. 2C and D) and FACS analysis revealed an increase in CD14 and CD16 positive cells (mature macrophages) (Fig. 2E and F) [16,17].

Salivary HHV-6 and HHV-7 DNA copy numbers in NC, OSAS, CFS and depression.

As compared to the NCs, no significant change in salivary HHV-6 DNA copy numbers was observed in the groups with, OSAS, CFS and depression (Fig. 3A). Copy numbers of HHV-7 DNA were lower in OSAS and CFS than in NCs (Fig. 3B).

3.3. Correlations between HHV-6 and HHV-7 DNA copy numbers and clinical assessments

HHV-6 and HHV-7 DNA copy numbers were weakly correlated with working time per week in the NC group, excluding 3 subjects whose working times were unknown ($\rho = 0.289$, $P = 0.002^{**}$, $\rho = 0.240$, $P = 0.011^*$, $n = 110$, * P < 0.05, ** P < 0.01, Spearman's rank correlation coefficients) (Fig. 4A and B). No significant correlations were observed between HHV-6 and HHV-7 DNA copy numbers and

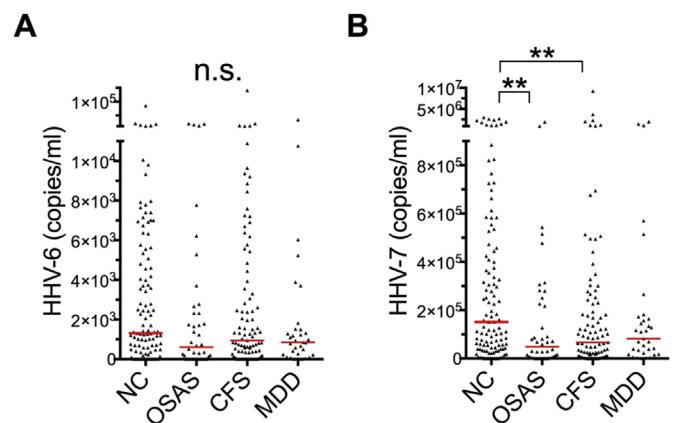


Fig. 3. HHV-6 (A) and HHV-7 (B) DNA copy numbers are plotted for NC, OSAS, CFS and MDD. Horizontal lines indicate median values. ** P < 0.01, n.s.: not significant using Kruskal-Wallis test with Dunn's multiple comparisons.

apnea-hypopnea index (AHI), Chalder Fatigue Scale or MADRS in the NC, OSAS, CFS or MDD groups (Fig. 4C–H).

4. Discussion

We examined salivary HHV-6 and HHV-7 amounts due to training in JSDF personnel. Since VAS fatigue scores increased during training, we considered it provided sufficient physiological fatigue loading (Fig. 1A). Salivary HHV-6 and HHV-7 DNA amounts increased with training and decreased with rest (Fig. 1 B and C), suggesting usefulness as biomarkers of physiological fatigue. HHV-6 DNA amounts quickly returned to the original levels with rest suggesting that changes in HHV-7 DNA levels were more closely matched to those in subjective fatigue.

To explore a mechanism for virus reactivation, we examined fatigue-induced changes in mouse spleens, in whose abundant macrophages latent virus infection is established [13]. There was an

increase in IL-1 β but not IL-6, and CD14 and CD16 expressions increased in myeloid cells. Also, it was reported that physiological fatigue, such as from exercise, induces inflammatory cytokines in humans [30]. Inflammatory cytokines are involved in HHV-6 reactivation [14], myeloid cell differentiation is important in cytomegalovirus reactivation [31] and cytomegalovirus is similar to HHV-6 and HHV-7 in nature. This suggests that a fatigue-induced increase in IL-1 β leads to macrophage activation and differentiation, which in turn causes reactivation of HHV-6 and HHV-7.

To determine whether salivary HHV-6 and HHV-7 DNA amounts could also be biomarkers for pathological fatigue, we measured them in OSAS, CFS and MDD. There were no increases as compared with NCs (Fig. 3A and B). In OSAS and CFS, the HHV-7 DNA amount was actually less than in NCs (Fig. 3B), probably due to reduced physical activity. Disease severity was not correlated with HHV-6 and HHV-7 DNA amounts (Fig. 3 C–H).

However, in NCs, HHV-6 and HHV-7 DNA amounts were

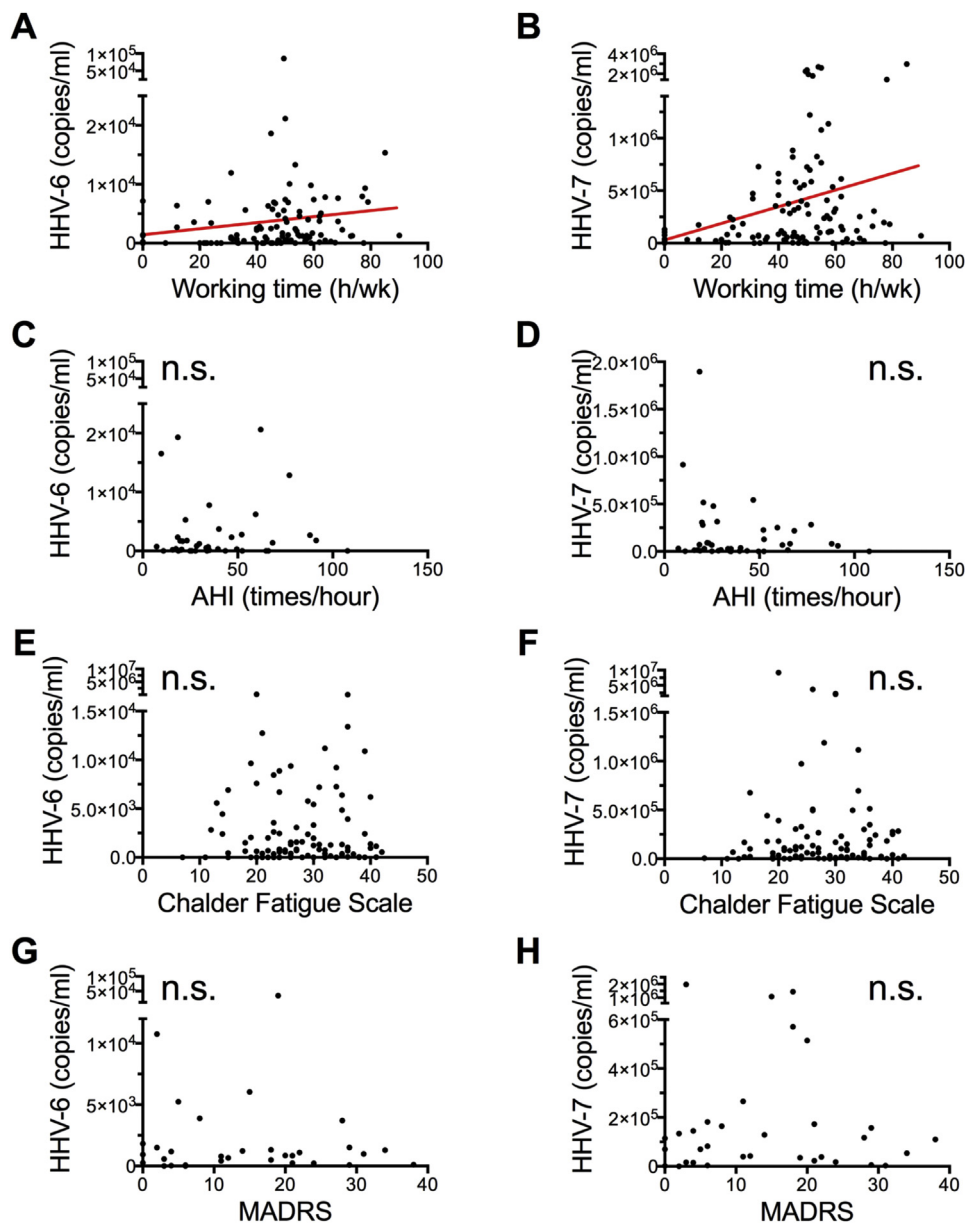


Fig. 4. Correlations between HHV-6 and HHV-7 and working time per week in NCs (A and B), AHI in OSAS (C and D), Chalder Fatigue Scale in CFS (E and F) and MADRS in MDD (G and H). n.s.: not significant by Spearman's rank correlation coefficients.

correlated with working time (Fig. 3A and B), suggesting that HHV-6 and HHV-7 are reactivated by physiological fatigue but not pathological fatigue.

In OSAS, an increase in IL-6 was reported, but no increase in IL-1 β [32]. Various studies have examined associations between inflammatory cytokines and CFS [33]. In severe CFS, there was no increase in IL-1 β or IL-6 [34]. For MDD, similar to OSAS, IL-6 increased but not IL-1 β [35]. The lack of an increase in IL-1 β with pathological fatigue was considered a reason that it did not reactivate the viruses.

Thus, salivary HHV-6 and HHV-7 were thought to be sensitive biomarkers for physiological fatigue since they were not reactivated by pathological fatigue. By distinguishing between the 2 types, they could assist in the diagnosis of patients with fatigue and deciding on their treatment. Furthermore, HHV-6 and HHV-7 assessment would be more useful in preventing overwork than methods relying on subjective feeling of fatigue, which can be masked by motivation and incentives [7]. Also, saliva samples are easy to collect and quantify for HHV-6 and HHV-7 in the primary care setting.

Our findings should be helpful in the objective evaluation of fatigue and answering remaining questions in fatigue science.

As a limitation, for comparisons between NC and OSAS, CFS and MDD, subjects were not matched regarding age and gender due to gender differences in disease prevalence and variation in ages of susceptibility. However, as patterns of salivary HHV-6 and HHV-7 were similar in OSAS, CFS and MDD, the influence of this was probably small.

Salivary HHV-6 and HHV-7 were shown to be useful objective biomarkers for physiological fatigue. As they were not reactivated by pathological fatigue, they could distinguish the 2 types of fatigue.

Competing interests

K.K. has submitted a patent application entitled “Methods For Assessing Fatigue Level and Applications Thereof” US patent application publication 2008/0280283 A1. K.K. has stock in Virus Ikagaku Kenkyusho Inc. The other authors report no competing interests.

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