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Clinical symptoms and markers of disease mechanisms in adolescent chronic fatigue following Epstein-Barr virus infection: An exploratory cross-sectional study

Miriam Skjerven Kristiansen[a,b], Julie Stabursvik[a,b], Elise Catriona O'Leary[a,b], Maria Pedersen[a,b], Tarjei Tørre Asprusten[a,b], Truls Leegaard[a,c], Liv Toril Osnes[d], Trygve Tjade[e], Eva Skovlund[f,g], Kristin Godang[h], Vegard Bruun Bratholm Wyller[a,b,*]

[a] Institute of Clinical Medicine, University of Oslo, Oslo, Norway
[b] Dept. of Pediatrics, Akershus University Hospital, Lørenskog, Norway
[c] Dept. of Microbiology and Infection Control, Akershus University Hospital, Lørenskog, Norway
[d] Dept. of Immunology, Oslo University Hospital, Oslo, Norway
[e] Fürst Medical Laboratory, Lørenskog, Norway
[f] Dept. of Public Health and Nursing, Norwegian University of Science and Technology, Trondheim, Norway
[g] Norwegian Institute of Public Health, Norway
[h] Section of Specialized Endocrinology, Dept. of Endocrinology, Oslo University Hospital, Norway

A B S T R A C T

Introduction: Acute Epstein-Barr virus (EBV) infection is a trigger of chronic fatigue (CF) and Chronic Fatigue Syndrome (CFS). The aim of this cross-sectional study was to explore clinical symptoms as well as markers of disease mechanisms in fatigued and non-fatigued adolescents 6 months after EBV-infection, and in healthy controls.

Materials and methods: A total of 200 adolescents (12–20 years old) with acute EBV infection were assessed 6 months after the initial infectious event and divided into fatigued (EBV CF+) and non-fatigued (EBV CF−) cases based on questionnaire score. The EBV CF+ cases were further sub-divided according to case definitions of CFS. In addition, a group of 70 healthy controls with similar distribution of sex and age was included. Symptoms were mapped with a questionnaire. Laboratory assays included EBV PCR and serology; detailed blood leukocyte phenotyping and serum high-sensitive C-reactive protein; and plasma and urine cortisol and catecholamines. Assessment of autonomic activity was performed with continuous, non-invasive monitoring of cardiovascular variables during supine rest, controlled breathing and upright standing. Differences between EBV CF+ and EBV CF− were assessed by simple and multiple linear regression adjusting for sex as well as symptoms of depression and anxiety. A p-value ≤ 0.05 was considered statistically significant. This study is part of the CEBA-project (Chronic fatigue following acute Epstein-Barr virus infection in adolescents).

Results: The EBV CF+ group had significantly higher scores for all clinical symptoms. All markers of infection and most immune, neuroendocrine and autonomic markers were similar across the EBV CF+ and EBV CF− group. However, the EBV CF+ group had slightly higher serum C-reactive protein (0.48 vs 0.43 mg/L, p = 0.031, high-sensitive assay), total T cell (CD3+) count (median 1573 vs 1481 × 10^6 cells/L, p = 0.012), plasma norepinephrine (1420 vs 1113 pmol/L, p = 0.01) and plasma epinephrine (363 vs 237 nmol/L, p = 0.032); lower low-frequency:high frequency (LF/HF) ratio of heart rate variability at supine rest (0.63 vs 0.76, p = 0.008); and an attenuated decline in LF/HF ratio during controlled breathing (−0.11 vs −0.25, p = 0.002). Subgrouping according to different CFS diagnostic criteria did not significantly alter the results. Within the EBV CF+ group, there were no strong correlations between clinical symptoms and markers of disease mechanisms. In a multiple regression analysis, serum CRP levels were independently associated with serum cortisol (B = 4.5 × 10^{-4}, p < 0.001), urine norepinephrine (B = 9.6 × 10^{-2}, p = 0.044) and high-frequency power of heart rate variability (B = –3.7 × 10^{-2}, p = 0.024).

Conclusions: In adolescents, CF and CFS 6 months after acute EBV infection are associated with high symptom burden, but no signs of increased viral load and only subtle alterations of immune, autonomic, and neuroendocrine markers of which no one is strongly correlated with symptom scores. A slight sympathetic over parasympathetic predominance is evident in CF and might explain slightly increased CRP levels.

1. Introduction

Chronic fatigue (CF), defined as substantial fatigue lasting for more than six months, is a common health complaint in Western countries (Pawlikowska et al., 1994). Among adolescents, about 20% of girls and 6.5% of boys report to have been severely fatigued during the last month (Crawley, 2014). If the experience of fatigue is unexplained, long lasting, disabling and accompanied by other symptoms, the patient may

* Corresponding author at: Dept. of Paediatrics, Akershus University Hospital, N-1478 Lørenskog, Norway.
E-mail address: brwylle@online.no (V.B.B. Wyller).

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suffer from Chronic Fatigue Syndrome (CFS) (IOM, 2015). CFS is one of the most significant health problems among adolescents, being associated with decreased quality of life (Eddy & Cruz, 2007), negative development of psychosocial and academic abilities (Kennedy et al., 2010), family problems (Missen et al., 2012), and large societal costs (Reynolds et al., 2004). No diagnostic biomarker for CFS has been established, and there are controversies regarding symptom-based diagnostic criteria; the most commonly applied case definitions are the Fukuda-definition (Fukuda et al., 1994) and the Canada-definition (Carruthers et al., 2003), of which the latter has the most extensive symptom requirements. The prevalence of adolescent CFS is estimated at 0.1–1.9% depending on the applied case definition, and more females than males are affected (Jordan et al., 2006; Werker et al., 2013).

It is well known that certain infections, such as Epstein-Barr virus (EBV) infection, can precipitate CF and CFS in predisposed individuals (Hickie et al., 2006; Katz et al., 2009). However, the mechanisms underlying fatigue development – and the potential involvement of infectious and immunological processes – remain to be understood. Some clinical symptoms are strikingly similar across acute EBV infection, chronic active EBV infection and CFS, such as the experience of fatigue, post-exertional malaise, chills, sore throat and muscle pain (IOM, 2015; Balfour et al., 2015). Still, while strongly increased blood viral replication is commonly found in acute and chronic active EBV infection (Balfour et al., 2015; Kimura et al., 2001), increased viral load has not been demonstrated among CFS patients, neither in CFS following EBV infection (Cameron et al., 2010) nor in CFS generally (Koelle et al., 2002).

Subtle immune alterations have been reported in numerous CFS studies; the most consistent finding appears to be a tendency towards low-grade systemic inflammation, as reflected in elevated serum C-reactive protein (CRP) (Sulheim et al., 2014), elevated pro-inflammatory cytokines (Klimas et al., 2012; Montoya et al., 2017), and increased levels of innate immunity gene products in whole blood gene expression analyses (Nguyen et al., 2017). Although a recent review failed to provide evidence for elevation of pro-inflammatory cytokines in CFS (Blundell et al., 2015), low-grade inflammation has been hypothesized as a common pathophysiological phenomenon across fatigue states in general (Lacourt et al., 2018). In support of this hypothesis, elevated CRP-level during the acute phase of EBV infection was identified as an independent risk factor for fatigue development over time (Pedersen et al., 2019). Regarding alterations in immune cell populations, findings are more inconsistent. For instance, studies have reported increased (Maes et al., 2015) and normal (Mihaylova et al., 2007) number of CD8+ cells, increased (Robertson et al., 2005) and normal (Curriu et al., 2013) number of NK cells, and increased (Bradley et al., 2013) and normal (Mihaylova et al., 2007) number of CD19+ cells.

Furthermore, patients with CF and CFS often report complaints suggesting autonomic and/or neuroendocrine disturbances, such as orthostatic intolerance, palpitations, sudden change in skin colour, excessive sweating, and shivering hands (IOM, 2015; Carruthers et al., 2003; Wyller et al., 2007b). Accordingly, autonomic and hormonal disturbances have been found in several CFS studies, including increased sympathetic cardiovascular activity (Wyller et al., 2008b; Wyller et al., 2007a; Hurum et al., 2011; Martinez et al., 2014), decreased parasympathetic (vagal) heart rate control (Wyller et al., 2008a), altered sympathetic thermoregulatory responses (Wyller et al., 2007b), increased plasma and urine catecholamines (Wyller et al., 2007b; Sulheim et al., 2014), and attenuated hypothalamus-pituitary-adrenal (HPA) axis dynamics (Sulheim et al., 2014; Papadopoulos & Cleare, 2011; Segal et al., 2005; Nijhof et al., 2014).

Nevertheless, although autonomic and neuroendocrine disturbances appears to be more consistently reported than immune disturbances, it is not known to what extent across-group differences are specific for CF/CFS, and a relationship between subjective symptoms and objective findings remains to be firmly established. There might be several reasons for this. For instance, most previous studies have not included a post-infectious recovered (ie, non-fatigued) control group; thus, reported across-group differences might have been due to long-term effects of infection rather than the fatigue condition *per se*. Furthermore, previous results might have been biased by the effects of sedentary behaviour and deconditioning, which exerts a strong influence on autonomic and neuroendocrine systems, and by the possible heterogeneity of underlying disease mechanisms in patients with a CFS diagnosis. Finally, normal aging processes might have confounded results.

Preliminary evidence suggests that both autonomic and neuroendocrine disturbances are caused by functional alterations of central nervous system control (Wyller et al., 2011; Wyller et al., 2014; Wyller et al., 2016), and it has been suggested that a sustained central stress response constitutes a key element of CFS pathophysiology (Wyller et al., 2009). Furthermore, according to this model, the autonomic/neuroendocrine alterations are hypothesized to be the causal driver of the inflammatory enhancement (Wyller et al., 2009), as supported by recent evidence: Innate immunity gene product expression is closely related to markers of neuroendocrine/autonomic activity in CFS (Nguyen et al., 2017; Nguyen et al., 2018), and treatment of CFS adolescents with the sympathetic inhibitor and parasympathetic stimulator *clonidine* significantly lowers serum CRP levels (Sulheim et al., 2014). Also, studies from other fields support a link between neuroendocrine/autonomic alterations and low-grade inflammation. The complex immune influence exerted by glucocorticoids has been recognized for decades (Zen et al., 2011); more recently, it has been shown that both parasympathetic and sympathetic nervous activity promotes immunomodulation (Andersson & Tracey, 2012; Padro & Sanders, 2014; Thayer & Sternberg, 2010), which in turn might be related to psychosocial background factors. For instance, perceived social isolation is shown to modulate immune system activity through altered sympathetic activity to the bone marrow (Cole et al., 2015; Cole, 2014).

The project entitled Chronic Fatigue Following Acute Epstein-Barr Virus Infection in Adolescents (CEBA) was designed to overcome previous methodological constraints, encompassing a strictly defined adolescent prospective post-EBV infection cohort (Pedersen et al., 2019). The aim of the present CEBA sub-study was twofold: (a) To compare symptoms as well as markers of disease mechanisms (i.e., immune, infectious, autonomic and neuroendocrine markers) across three groups: Adolescents with chronic fatigue 6 months after EBV infection (EBV CF+), adolescents without chronic fatigue 6 months after EBV infection (EBV CF−), and healthy controls. (b) To explore associations between symptoms, autonomic/neuroendocrine markers and a marker of inflammation (serum CRP) in the EBV CF+ group.

2. Materials and methods

2.1. Study design

The CEBA project (ClinicalTrial ID: NCT02335437) encompasses a prospective cohort of EBV infected adolescents with a total follow-up time of 21 months. The overall design of the CEBA project has been described elsewhere (Pedersen et al., 2019). In the present paper, results from the follow-up visit six months after the infectious event are reported; in addition, a healthy control group is included for reference.

Patient inclusion was based on informed consent. Approbation was granted from The Norwegian National Committee for Ethics in Medical Research.

2.2. Participants

In the period March 2015 until November 2016, adolescents suffering from acute EBV infection were recruited to the CEBA project in close collaboration with Fürst Medical Laboratory and the Department of Microbiology at Akershus University Hospital. Laboratory staff reported regularly (three times weekly) to the CEBA study center all serologically confirmed acute EBV infections in patients between 12

and 20 years old living in the counties Oslo, Akershus and Buskerud. Two well-defined serological patterns were taken as confirmation of acute EBV infection (Supp Table 1). Exclusion criteria were (a) More than six weeks since onset of symptoms suggesting acute EBV infection; (b) Any chronic disease that needed regular use of medication (based upon self report); (c) Pregnancy.

Healthy controls were mainly recruited by asking the already included EBV-infected individuals to bring a friend to the six months follow-up encounter.

Additional details of the recruitment, screening and inclusion procedures are described elsewhere (Pedersen et al., 2019).

2.3. Investigational program

All included participants underwent a one-day investigational program at the CEBA study centre at Akershus University Hospital, including clinical examination, spleen ultrasound imaging, blood and throat swab sampling, assessment of autonomic cardiovascular control, and questionnaire charting, as well as other investigations not relevant for the present study. All procedures were completed in a fixed sequence by two researchers (MP and TTA). Blood samples were collected between 08:30 and 09:00 from antecubital venous puncture after at least 10 min supine rest in a quiet, warm room. The subjects were kept in supine position, and assessment of autonomic cardiovascular control was routinely performed approximately 20 min later. Prior to the visit, all participants were asked to abstain from tobacco- and caffeine products at least 48 h, and to fast overnight. Furthermore, they were instructed to apply the local anaesthetic ointment (EMLA®, AstraZeneca) on the antecubital skin area one hour before arriving.

The EBV-group was followed prospectively and summoned to a similar investigational program 6 months later, whereas the healthy controls were seen only once.

2.4. Clinical examinations and routine laboratory assays

Tympanic temperature was measured with an infrared digital thermometer (Genius 2, Cardinal Health Inc., Dublin, Ohio, USA). Spleen length was determined using a Vivid I ultrasound device with abdominal probe (GE Healthcare, Chicago, IL, USA). Routine blood samples for hematology and biochemistry were assayed at the accredited laboratory at Akershus University Hospital, Norway

2.5. Microbiological assays

Blood samples for microbiological analysis were collected in 4 ml EDTA tubes and gel-containing tubes, respectively. Specific antibody responses were assessed using anti-EBV VCA IgG and IgM (Liason, DiaSorin, Saluggia, Italy) and the anti EBV EBNA IgG (Liason, DiaSorin, Saluggia, Italy) at the Akershus University Hospital, Norway.

Detection of microbial DNA from EBV was performed by real-time polymerase chain reaction in whole blood and in saliva/epithelial cells collected with a throat swab. The samples were transported daily to Dept. of Microbiology, Oslo University Hospital Rikshospitalet, Norway where the analyses were executed. The analysis (Altona Real Star EBV PCR Kit, Altona Diagnostics GmbH, Hamburg, Germany) had a detection limit of 110 IU/mL sample, and quantification range between 1000 and 1000,000 IU/mL.

2.6. High-sensitive C-reactive protein (hsCRP)

Frozen serum samples were used for the high-sensitive C-reactive protein (hsCRP) assay (Cobas c702, Roche Diagnostics, Indianapolis, IN, USA). The test principle is a particle-enhanced immunoturbidimetric assay (CRP Latex HS), where anti-CRP antibodies coupled to latex microparticles react with antigen in the sample; the following agglutination is measured turbidimetrically (Eda et al., 1998). The lower detection limit was 0.15 mg/L; the functional sensitivity was 0.3 mg/L. Intraassay CV was 6.0% for low levels (1.2 mg/L), and 2.0% for high levels (6.1 mg/L).

2.7. Immune cell phenotyping

Samples for immune cell phenotyping were obtained in 4 ml EDTA tubes and analysed consecutively at the Institute of Immunology, Oslo University Hospital Rikshospitalet, Norway. B- and T-cell subpopulations were analysed by flow cytometry. For B-cell analysis, the blood samples were washed twice before incubation with antibodies. T-cell analysis was performed in unwashed blood sample. Briefly, EDTA-blood was incubated with optimally titrated antibodies for 15 min at room temperature, followed by erythrocyte lysis (BD FACSLysing Solution, Beckman Dickinson, Franklin lakes, New Jersey, USA). Data acquisition was performed on a Gallios flow cytometer (Beckman Coulter, Brea, CA, USA). For T-cells 1×10^5 cells was acquired, and for B-cells 1×10^6 if possible.

B-cells were gated as $CD19^+$ and further sub-classified as naïve (IgD^+, $CD27^-$), IgM memory ($CD27^+$, IgD^+, IgM^+), class switched ($CD27^+$, IgM^-, IgD^-), plasmablasts ($CD19^{+dim}$, $CD27^{++}$, $CD38^{++}$), transitional (IgM^{++}, $CD38^{++}$, $CD24^+$) and CD21 low B cells ($CD38^{low}$, $CD21^{low}$). T-cells were gated as $CD3^+$ and further sub-classified as naive $CD4^+$ ($CD4^+$, $CD45^{RA+}$), recent thymic emigrants ($CD4^+$, $CD45^{RA+}$, $CD31^+$), $CD4^+$ memory ($CD4^+$, $CD45^{RO+}$), follicular like $CD4^+$ ($CD4^+$, $CD45^{RO+}$, $CCR5^+$), regulatory T-cells ($CD4^+$, $CD25^{++}$, $CD127^-$), naive $CD8^+$ ($CD8^+$, $CD27^+$, $CD28^+$), $CD8^+$ early effector memory ($CD8^+$, $CD27^+$, $CD28^-$), $CD8^+$ late effector memory ($CD8^+$, $CD27^-$, $CD28^-$).

2.8. NK-cell functionality

Degranulation of NK-cells was measured as expression of $CD107^a$ on the surface of NK-cells after 2 h incubation with the K562 cells (Bryceson et al., 2012). Briefly, PBMC were isolated and incubated overnight in medium (RPMI, 8% FCS, 1% Streptomycin/penicillin) at 37 °C, 5% CO_2. The next day, monoclonal antibody to $CD107^{a-PE}$ was added to each well to be used in a 96-well V-bottom plate. 100 µl PBMC (2×10^6 cells/ml) was added to the wells, and then 100 µl K562 (2×10^6 cells/ml) added in half of the wells for stimulation and only medium (100 µl) to the rest of the wells as unstimulated control. The plate was centrifuged at 300*g* for 3 min and then incubated for 2 h at 37 °C, 5% CO_2. After incubation, the cells were harvested and stained with anti-CD3, anti-CD56 and anti-CD45 for 20 min, re-suspended in PBS-EDTA-BSA, washed and re-suspended again in PBS-EDTA-BSA before acquisition on a Canto II flowcytometer (Beckman Dickinson, Franklin lakes, New Jersey, USA). Datafiles were analyzed using Kaluza software (Beckman Coulter, Brea, CA, USA). NK cells were gated as $CD45^+$, $CD3^-$, $CD56^+$ lymphocytes. Surface expression of $CD107^a$ on NK-cells was measured as the difference ($\Delta CD107^a$) between $CD107^{a+}$ NK-cells in K562 stimulated wells and unstimulated wells (medium alone).

2.9. Neuroendocrine assays

Blood samples for catecholamine analysis were obtained in vacutainer tubes treated with ethylene glycol tetra acetic acid and glutathione from Sigma-Aldrich (St. Louis, MO, USA) covered with aluminium foil. They were immediately put on ice and centrifuged (2500 rpm, ten minutes, 4 °C) within 15 min. Plasma was then transferred into cryotubes and frozen at −80 °C, all within 30 min from sampling. The plasma was transported on ice for analyses at the laboratory at the Section for Specialized Endocrinology, Department of Endocrinology, Oslo University Hospital Rikshospitalet, Norway. Both epinephrine and norepinephrine were analyzed by high-performance liquid chromography (HPLC) (Agilent Technologies, Santa Clara, CA,

USA) with a reversed-phase C-18 column (Chromsystem, München, Germany) and electrochemical detector (Antec, Leyden Decade II SCC, Zoeterwoude, The Netherlands) using a commercial kit from Chromsystems. The intra- and inter-assay CV were 3.9% and 10.8% respectively. All samples from an individual subject were measured in one run to minimize the interassay variability (Tsunoda, 2006; Hjemdahl, 1984).

Urine samples for catecholamine analysis were acidified to pH ~ 2.5 after collections, and thereafter short time stored at minus 18 °C. These urine samples were consecutively analyzed during the study period. The same HPLC system as for plasma measurement was used for the analysis of urine catecholamines. The intra- and inter assay coefficient of variation (CV) for urine were 3.9 and 5.2%, respectively.

Blood samples for assays of other neuroendocrine markers were collected in 4 ml serum tubes. Thyroidea Stimulating Hormone (TSH), thyroxine, and cortisol, were assayed at Akershus University Hospital, Norway (Cobas 8000, Roche Diagnostics, Mannheim, Germany). Urine cortisol was assayed at the Endocrinological Laboratory at Oslo University Hospital, Norway (Immulite 2000xpi, Siemens Healthineers, Erlangen, Germany). Plasma adrenocorticotropic hormone (ACTH) was determined by routine assays at the accredited laboratory at Oslo University Hospital, Norway.

2.10. Assessment of autonomic cardiovascular control

The Task Force Monitor® (Model 3040i, CNSystems Medizintechnic, Graz, Austria) is a combined hardware and software device for non-invasive continuous recording of cardiovascular variables (Fortin et al., 2006). In the present study, recordings were performed (1) During five minutes supine rest; (2) During five minutes supine rest combined with controlled breathing (15 breaths/minute), applying a beeper signal; (3) During three minutes upright standing.

Instantaneous RR intervals and heart rate (HR) was obtained from the electrocardiogram. Continuous arterial blood pressure was measured noninvasively beat-to-beat by finger plethysmography (Parati et al., 1989). The finger blood pressure values were automatically calibrated every third minute against conventional oscillometric upper arm measurements of arterial blood pressure. Impedance cardiography with electrodes placed on the neck and upper abdomen was used to obtain a continuous recording of the temporal derivate of the transthoracic impedance (dZ/dt) (Denniston et al., 1976).

Beat-to-beat stroke volume (SV) was calculated from the impedance signal. Cardiac output (CO) was calculated as SV times HR, and total peripheral resistance index (TPRI) was calculated as mean blood pressure divided by CO indexed for body surface area. Analyses of heart rate variability (HRV) provide information on autonomic cardiac modulation (Eckberg, 1997; Malpas, 2002). In the present study, power spectral analysis (frequency-domain method) of HRV was automatically provided by the TFM, using an adaptive autoregressive model, and calculated in the Low Frequency (LF) range (0.05–0.17 Hz), and High Frequency (HF) range (0.17–0.4 Hz) (Bianchi et al., 1997). Vagal (parasympathetic) activity is the main contributor to HF variability, whereas both vagal and sympathetic activity contributes to LF variability. The low frequency:high frequency (LF/HF) ratio is considered an index of sympathovagal balance (Eckberg, 1997; Malpas, 2002).

2.11. Questionnaire

A questionnaire distributed to all participants included the Chalder Fatigue Questionnaire (CFQ) (Chalder et al., 1993) and the Hospital Anxiety and Depression Scale (HADS) (Zigmond & Snaith, 1983). CFQ consists of 11 items scored on four-point Likert scales; in the present study, fatigue cases were defined as a CFQ total dichotomous score of 4 or higher (each item scored 0-0-1-1) (White et al., 2011). Also, the CFQ total linear score (each item scored on a zero to three Likert scale) is reported. HADS consists of 14 items scored 0–3 on Likert scales; total sum score is reported.

The questionnaire also included a Chronic Fatigue Syndrome (CFS) inventory for adolescents consisting of 24 common symptoms graded on five-point Likert scales from “never/rarely present” to “present all the time” (Wagner et al., 2005; Sulheim et al., 2014). In the present study, scores for infectious symptoms (six items) and post-exertional malaise (one item: “How often do you experience more fatigue the day after an exertion?”) are reported; total range is from one to five, and higher scores imply more severe symptom burden. Also, symptoms pertaining to different case definitions of CFS were used to subgroup the chronically fatigued individuals.

Finally, the questionnaire included the Autonomic Symptom Profile (ASP) (Suarez et al., 1999), which was slightly modified in a previous study to fit our age group (Fagermoen et al., 2015). A composite score reflecting orthostatic symptoms was constructed from 8 single items from the ASP, addressing experiences of dizziness in specific situations (such as rising suddenly from supine position, taking a shower, etc.). The total sum score is from 0 to 8; higher values reflect more pronounced orthostatic problems. In addition, other symptoms related to autonomic cardiovascular control, such as palpitations and feeling alternating hot and cold, were charted on a 1–5 Likert scale.

2.12. Statistical analyses

Variables are reported with mean (standard deviation) or median (interquartile range) depending on the distribution, and with 95% confidence intervals. As a general analytic strategy, we first investigated differences across all three groups (EBV CF+, EBV CF-, and Healthy Controls) applying one-way ANOVA, its nonparametric equivalent Kruskal-Wallis’ test or Chi-Square test, as appropriate. For p-values < 0.1, *post-hoc* comparison between the EBV CF+ and the EBV CF− group was carried out, applying Student t- or Mann-Whitney U tests as appropriate. Finally, simple and multiple linear regression analyses adjusting for possible confounding effects of sex and HADS score were conducted; when feasible, variables were ln-transformed prior to modelling in order to obtain an approximate normal distribution. Possible interaction effects of sex were explored.

Differences between the entire EBV CF+ group and subgroups of CFS patients as defined from, respectively, the Fukuda (Fukuda 1994) and Canada (Carruthers 2003) diagnostic criteria, were analysed by comparing confidence intervals of the central estimates.

Variables that differed significantly between the EBV CF+ and the EBV CF− groups were selected for exploration of associations between symptom scores and markers of disease mechanisms within the EBV CF + group using non-parametric correlation analyses (Kendall’s Tau). As for associations between autonomic/neuroendocrine markers and serum CRP, variables were ln-transformed in order to obtain approximate normal distributions. Thereafter, bivariate linear regression analyses were carried out with CRP as dependent variable; as independent variables, autonomic/neuroendocrine markers that differed significantly between the EBV CF+ and the EBV CF− groups were included, as well as certain other markers based on theoretical considerations. Associations with a p-value < 0.1 were further selected for multivariate modelling. The final multiple linear regression model in the EBV CF+ group was also tested in the EBV CF− group and the healthy control group, in order to display group differences in associations.

All statistical analyses were carried using the SPSS statistical software (IBM SPSS 25 inc., Chicago, IL, USA). Generally, 5% level of significance was applied. As this was an exploratory study, we did not adjust p-values for test multiplicity. We had few missing data; thus, imputation was not considered necessary.

3. Results

A total of 895 adolescents with a serological pattern suggesting

Table 1
Background characteristics.

	EBV-group (n = 195), 6 months after acute infection			Healthy controls (n = 70)
	EBV (CF+) (n = 91)	EBV (CF–) (n = 104)	p-values (EBV (CF+) vs EBV (CF-)	
Sex – no. (%)				
Male	24 (26.4)	44 (42.3)	**0.020**	26 (37.1)
Female	67 (73.6)	60 (57.7)		44 (62.9)
Age, years – mean (SD)	17.4 (1.5)	17.4 (1.7)	0.780	17.0 (1.8)
BMI, $kg/m^{2\ -}$ mean (SD)	22.1 (2.8)	22.2 (2.5)	0.666	21.5 (3.1)
HADS total score – mean (SD)	13.4 (6.3)	8.0 (5.3)	< **0.001**	10.6 (4.6)
Steps per day, number – mean (SD)	8710 (3872)	9329 (3019)	0.239	10,094 (4149)

P-values are based on Chi-Square test, or Student *t*-test, as appropriate. EBV = Epstein-Barr Virus, SD = standard deviation, BMI = body mass index, HADS = Hospital Anxiety and Depression Scale.

acute EBV infection were assessed for eligibility in the CEBA project; 355 were not available, 215 declined participation and 125 met exclusion criteria (102 did not meet the time limit regarding onset of symptoms, and 23 had a chronic co-morbid disorder), resulting in a total of 200 included participants at baseline (Pedersen et al., 2019). Additional five participants were lost to follow-up during the first 6 months, leaving 195 cases for analyses in the present sub-study; 91 (47%) of them were classified as chronic fatigue cases (EBV CF+), whereas 104 (53%) were classified as non-fatigue cases (EBV CF−). The EBV CF+ group contained more females and had higher HADS score than the EBV CF− group (Table 1). A total of 26 (29%) adhered to the Fukuda-definition of CFS (Fukuda et al., 1994), whereas 19 (21%) adhered to the Canada-definition (Carruthers et al., 2003). In addition, 70 healthy controls with similar distribution of sex and age as the EBV groups were included.

The EBV CF+ group had significantly higher scores than the EBV CF− group for all clinical symptoms, including infectious symptoms, post-exertional malaise, orthostatic intolerance, palpitations and feeling alternating hot and cold (Table 2). However, markers of infection (EBV viral load in blood and throat, EBV serology) and other clinical markers (spleen length, tympanic temperature) did not differ between the two groups (Table 2).

As for immune markers, the EBV CF+ group was characterized by significantly higher levels of CRP (median 0.48 vs 0.43 mg/L, p = 0.031) and total T cell (CD3+) count (median 1573 vs 1481 × 10^6 cells/L, p = 0.012); also, there was a trend towards slightly higher total T cell (CD3+) fraction (median 79.9 vs 77.5%, p = 0.065), and lower B cell ($CD19^+$) fraction (median 12.4 vs 13.2%, p = 0.123) as compared to the EBV CF− group. Both EBV groups differed from healthy controls on several other immune markers; for instance, the healthy controls had significantly lower number and fraction of cytotoxic T cells ($CD8^+$), as well as lower NK cell functionality, as compared to both the EBV CF+ and the EBV CF− group.

As for neuroendocrine markers, the EBV CF+ group had significantly higher levels of plasma norepinephrine (1420 vs 1113 pmol/L, p = 0.01) and plasma epinephrine (363 vs 237 nmol/L, p = 0.032) as compared with the EBV CF− group. Regarding autonomic cardiovascular control, the EBV CF+ group had slightly lower LF/HF-ratio at supine rest (0.63 vs 0.76, p = 0.008), and attenuated decline in the LF/HF ratio during controlled breathing (−0.11 vs −0.25, p = 0.002). All responses to orthostatic challenges were similar between the two groups.

Participants classified as CFS cases according to the Fukuda-definition or the Canada-definition had higher symptoms scores but were otherwise similar to the entire EBV CF+ group for the great majority of variables (Supp Table 2). No interaction effect of sex was found (results not shown).

Within the EBV CF+ group, there were weak positive correlations between blood T cell count and the clinical symptoms of fatigue and infection, and weak negative correlations between the LF/HF ratio response to controlled breathing and the symptoms of fatigue and palpitations (Table 3). No other correlations between clinical symptoms and disease markers were found. Serum CRP was significantly and independently associated with serum cortisol, urine norepinephrine and HF-power of heart rate variability within the EBV CF+ group, but with serum cortisol only within the EBV CF− and the healthy control groups (Table 4, Fig. 1, Supp Table 3).

4. Discussion

This study shows that CF and CFS after acute EBV infection is associated with several bodily symptoms including symptoms of persistent infection, despite no signs of increased viral load, and only subtle alterations of immune, autonomic and neuroendocrine markers. Thus, the main finding of the present study is a discrepancy between the subjective experiences and the objective measurements in the fatigued group as well as in the subgroups adhering to different CFS diagnostic criteria. In addition, the study shows (a) That post-infectious CF is associated with a slight predominance of sympathetic nervous activity; (b) That aberrant autonomic activity might be the cause of slightly increased CRP-levels in CF; and (c) That the discriminant validity of CFS diagnostic criteria are questionable.

The persistence of clinical symptoms in the EBV CF+ group corroborates common findings from the CFS literature (Hickie et al., 2006; Fukuda et al., 1994, Carruthers et al., 2003), suggesting that fatigue and other bodily complaints are closely associated. Accordingly, symptom load was even higher in the subgroups fulfilling case definitions of CFS.

The lack of difference in viral load between the EBV CF+ and the EBV CF− group goes well with previous findings (Cameron et al., 2010; Koelle et al., 2002). Of note, individuals fulfilling the CDC or Canada case definitions of CFS tended to have even lower levels of EBV copies in blood as compared to the rest of the EBV CF+ group. Viral load in the acute phase of EBV infection does not predict later fatigue development either, as shown in a previous publication from the CEBA project (Pedersen et al., 2019). While viral infection might still play a role in the pathogenesis of fatigue and CFS (Rasa et al., 2018), the findings of the present study add to the body of negative findings, suggesting that viral replication does not have a central role in perpetuating patients' symptoms.

Some previous studies of cytokine patterns (Klimas et al., 2012; Montoya et al., 2017), CRP-levels (Sulheim et al., 2014), and blood gene expression (Nguyen et al., 2017) suggest the presence of low-grade systemic inflammation in CFS. Also, in a recent report from the CEBA project, CRP-level during the acute phase of EBV infection was identified as an independent risk factor of fatigue development at 6 months follow-up (Pedersen et al., 2019). Furthermore, increased CRP levels have been reported in irritable bowel syndrome (Hod et al., 2016), a disorder which often co-occurs and displays some phenotypical similarities with CFS; accordingly, low-grade inflammation has been hypothesized as a common pathophysiological phenomenon across fatigue

Table 2
Cross-sectional comparison of clinical symptoms, clinical findings and disease markers across all groups.

	EBV (CF+) (n = 91)	EBV (CF–) (n = 104)	Healthy controls (n = 70)	p-value (across all groups)	p-value EBV (CF+) vs EBV (CF-)	Adjusted p-value EBV (CF+) vs EBV (CF-)*
Clinical symptoms						
Chalder Fatigue Questionnaire (CFQ), total score – median (IQR)	19.0 (5.0)	11.0 (2.0)	11.0 (5.0)	< 0.001	< 0.001	< **0.001**
Confidence interval (CI)	18.0 to 20.0	11.0 to 12.0	11.0 to 12.0			
Infectious Symptoms, total score – median (IQR)	2.0 (0.8)	1.3 (0.5)	1.3 (0.5)	< 0.001	< 0.001	< **0.001**
Confidence interval (CI)	1.8 to 2.2	1.3 to 1.5	1.2 to 1.5			
Postexertional Malaise, score – mean (SD)	2.9 (1.1)	1.6 (0.6)	1.7 (0.7)	< 0.001	< 0.001	< **0.001**
Confidence interval (CI)	2.6 to 3.1	1.5 to 1.8	1.6 to 1.9			
Orthostatic symptoms, total score – mean (SD)	2.6 (2.0)	1.0 (1.3)	1.3 (1.4)	< 0.001	< 0.001	**0.001**
Confidence interval (CI)	2.2 to 3.1	0.7 to 1.2	1.0 to 1.7			
Palpitations, score – mean (SD)	1.7 (0.9)	1.2 (0.4)	1.3 (0.5)	< 0.001	< 0.001	**0.003**
Confidence interval (CI)	1.5 to 1.9	1.1 to 1.2	1.1 to 1.4			
Feeling alternating hot and cold, score – mean (SD)	2.3 (1.3)	1.4 (0.7)	1.5 (0.8)	< 0.001	< 0.001	**0.001**
Confidence interval (CI)	2.0 to 2.6	1.3 to 1.6	1.4 to 1.7			
Clinical findings						
Tympanic temperature, ˚C – mean (SD)	36.2 (0.5)	36.2 (0.6)	36.3 (0.4)	0.096	0.424	
Confidence interval (CI)	36.1 to 36.3	36.1 to 36.3	36.2 to 36.4			
Maximum spleen length, cm – median (IQR)	11.6 (1.6)	11.7 (1.9)	11.4 (1.6)	0.253		
Confidence interval (CI)	11.4 to 11.9	11.4 to 12.0	10.8 to 11.8			
Infection markers						
Epstein-Barr Virus (EBV) load, copies in blood – no. (%)						
Negative (< 160)	44 (51.2)	38.0 (37.3)	60 (85.7)	< 0.001	0.123	
Low (1600–2000)	26 (30.2)	35.0 (34.3)	8 (11.4)			
Moderate/high (> 2000)	16 (18.6)	29.0 (28.4)	2 (2.9)			
EBV virus load, cycle threshold (CT) cycle in PCR – no. (%)						
Negative	13 (15.3)	15.0 (15.0)	50 (74.6)	< 0.001	0.723	
Low (CT values > 32)	19 (22.4)	16.0 (16.0)	5 (7.5)			
Moderate (CT values 28 to 32)	52 (61.2)	68.0 (68.0)	11 (16.4)			
High (CT values < 28)	1 (1.2)	1.0 (1.0)	1 (1.5)			
EBV Viral Capsid Antigen (VCA) IgM, titer – median (IQR)	23.0 (51.0)	0.0 (40.0)	0 (0)	< 0.001	0.178	
Confidence interval (CI)	0.0 to 33.0	0.0 to 22.0	0.0 to 0.0			
EBV-VCA-IgG, titer – median (IQR)	175.5 (185.0)	161.0 (150.0)	51.0 (195.0)	< 0.001	0.755	
Confidence interval (CI)	143.0 to 204.0	143.0 to 188.0	0.0 to 115.0			
EBV Nuclear Antigen (EBNA) IgG, titer – median (IQR)	93.0 (271.0)	103.0 (182.0)	57 (349)	0.478		
Confidence interval (CI)	66.0 to 166.0	69.0 to 141.0	0.0 to 158.0			
Immune markers						
Serum high sensitive CRP, mg/L – median (IQR)	0.48 (1.25)	0.43 (0.62)	0.56 (0.41)	0.010	0.043	**0.031**
Confidence interval (CI)	0.39 to 0.76	0.32 to 0.53	0.48 to 0.63			
Serum total IgG, g/L – median (IQR)	9.8 (2.2)	10.0 (1.9)	9.3 (1.7)	0.034	0.303	
Confidence interval (CI)	9.3 to 10.1	9.5 to 10.4	8.8 to 9.7			
Serum total IgM, g/L – median (IQR)	1.0 (0.6)	0.9 (0.6)	0.9 (0.7)	0.156		
Confidence interval (CI)	0.9 to 1.1	0.8 to 1.0	0.8 to 1.1			
Serum total IgA, g/L – mean (SD)	1.4 (0.6)	1.5 (0.6)	1.4 (0.6)	0.271		
Confidence interval (CI)	1.2 to 1.5	1.4 to 1.6	1.3 to 1.6			
Blood Leukocyte total count, 10^9 cells/L – median (IQR)	5.7 (2.3)	5.3 (1.8)	5.3 (2.1)	0.437		
Confidence interval (CI)	5.3 to 6.2	5.1 to 5.6	5.1 to 6.1			
Blood Lymphocyte count, 10^9 cells/L – median (IQR)	2.0 (0.8)	1.8 (0.6)	1.9 (0.6)	0.057		
Confidence interval (CI)	1.9 to 2.1	1.8 to 1.9	1.7 to 2.0			
Blood Monocyte count, 10^9 cells/L – median (IQR)	0.5 (0.2)	0.5 (0.2)	0.5 (0.2)	0.730		
Confidence interval (CI)	0.5 to 0.5	0.5 to 0.5	0.4 to 0.5			
Blood Neutrophil count, 10^9 cells/L – median (IQR)	2.9 (1.7)	2.7 (1.2)	2.9 (1.9)	0.292		
Confidence interval (CI)	2.8 to 3.2	2.5 to 3.0	2.6 to 3.5			
Blood Eosinophil count, 10^9 cells/L – median (IQR)	0.1 (0.1)	0.1 (0.1)	0.1 (0.1)	0.144		
Confidence interval (CI)	0.1 to 0.1	0.1 to 0.1	0.1 to 0.2			
Blood T cell ($CD3^+$) total count, 10^6 cells/L – median (IQR)	1573.0 (643.0)	1481.0 (537.0)	1331.0 (503.0)	0.003	0.066	**0.012**
Confidence interval (CI)	1402.0 to 1676.0	1348.0 to 1533.0	1237.0 to 1453.0			
Blood T cell ($CD3^+$) fraction (of lymfocyte count), % - median (IQR)	79.9 (6.8)	77.5 (8.0)	75.7 (8.2)	< 0.001	0.011	0.065
Confidence interval (CI)	77.8 to 81.4	76.0 to 78.9	73.1 to 76.9			
Blood double negative T cell ($CD4^-CD8^-$) subset (of $CD3^+$ count), % – median (IQR)	0.8 (0.5)	0.8 (0.5)	0.9 (0.6)	0.597		
Confidence interval (CI)	0.7 to 0.9	0.7 to 0.9	0.8 to 1.0			
Blood cytotoxic T cell ($CD8^+$) count, 10^6 cells/L- median (IQR)	599.0 (351.0)	574.0 (261.0)	506.0 (203.0)	< 0.001	0.266	
Confidence interval (CI)	526.0 to 669.0	517.0 to 622.0	436.0 to 535.0			
Blood cytotoxic T cell ($CD8^+$) fraction (of lymfocyte count), % – median (IQR)	31.0 (8.7)	31.1 (7.0)	27 (7.8)	< 0.001	0.784	

(*continued on next page*)

Table 2 (*continued*)

	EBV (CF+) (n = 91)	EBV (CF–) (n = 104)	Healthy controls (n = 70)	p-value (across all groups)	p-value EBV (CF+) vs EBV (CF-)	Adjusted p-value EBV (CF+) vs EBV (CF-)*
Confidence interval (CI)	29.4 to 32.7	29.3 to 32.0	24.7 to 30.2			
Blood naïve T cell subset (of $CD8^+$ count), % – median (IQR)	71.0 (16.9)	71.7 (16.9)	78.7 (20.4)	0.019	0.842	
Confidence interval (CI)	67.4 to 76.0	67.9 to 74.4	70.0 to 82.0			
Blood early effector memory T cell subset (of $CD8^+$ count), % – median (IQR)	18.0 (10.8)	18.7 (8.0)	10.1 (5.2)	< 0.001	0.960	
Confidence interval (CI)	15.9 to 20.9	17.5 to 19.9	8.8 to 11.3			
Blood late effector memory T cell subset (of $CD8^+$ count), % – median (IQR)	5.6 (10.3)	4.3 (12.1)	7.0 (18.1)	0.031	0.693	
Confidence interval (CI)	3.8 to 8.7	3.1 to 6.9	5.8 to 13.0			
Blood helper T cell ($CD4^+$) count, 10^6 cells/L – median (IQR)	804.0 (343.0)	739.0 (293.0)	780.0 (334.0)	0.135		
Confidence interval (CI)	746.0 to 886.0	704.0 to 818.0	674.0 to 869.0			
Blood helper T cell ($CD4^+$) fraction (of lymfocyte count), % – mean (SD)	42.0 (7.3)	44.4 (39.1)	43.7 (6)	0.011	0.141	
Confidence interval (CI)	40.5 to 43.6	36.7 to 52.0	42.2 to 45.1			
Blood recent thymic emigrant T cell subset (of $CD4^+$ $CD45^{RA+}$ T cell count), % – median (IQR)	70.9 (11.7)	69.0 (15.2)	70.1 (10.3)	0.555		
Confidence interval (CI)	68.5 to 73.2	65.6 to 72.6	67.8 to 72.6			
Blood naïve T cell subset (of $CD4^+$ count), % – median (IQR)	62.5 (12.0)	62.2 (12.5)	66.7 (13.9)	0.919		
Confidence interval (CI)	60.6 to 65.9	60.0 to 64.6	61.4 to 68.4			
Blood follicular T cell subset (of $CD4^+$ count), % – median (IQR)	7.6 (3.8)	7.9 (3.6)	7.8 (3.8)	0.919		
Confidence interval (CI)	6.9 to 8.4	7.3 to 8.6	7.0 to 8.9			
Blood regulatory T cell subset (of $CD4^+$ count), % – mean (SD)	5.9 (1.5)	6.2 (1.3)	6.5 (1.5)	0.068		
Confidence interval (CI)	5.6 to 6.3	6.0 to 6.5	6.1 to 6.9			
Blood memory T cell subset (of $CD4^+$ count), % – mean (SD)	50.0 (11.0)	50.0 (10.0)	49.1 (9.8)	0.630		
Confidence interval (CI)	47.6 to 52.2	48.1 to 51.9	46.8 to 51.5			
Blood B cell ($CD19^+$) total count, 10^6 cells/L – median (IQR)	230.0 (140.0)	247.0 (160.0)	251.0 (109.0)	0.853		
Confidence interval (CI)	214.0 to 261.0	220.0 to 281.0	231.0 to 272.0			
Blood B cell ($CD19^+$) fraction (of lymfocyte count), % – median (IQR)	12.4 (5.8)	13.2 (5.8)	14.4 (4.7)	0.015	0.093	0.123
Confidence interval (CI)	11.1 to 13.4	12.2 to 13.9	12.9 to 15.2			
Blood naïve B cell subset (of $CD19^+$ count), % – median (IQR)	80.8 (10.6)	81.0 (10.4)	80.0 (10.3)	0.522		
Confidence interval (CI)	79.0 to 82.2	78.6 to 81.8	77.0 to 81.7			
Blood transitoric B cell subset (of $CD19^+$ count), % – median (IQR)	2.3 (1.8)	2.7 (1.9)	2.4 (1.7)	0.376		
Confidence interval (CI)	2.0 to 2.6	2.1 to 3.0	1.9 to 2.8			
Blood class switch B cell subset (of $CD19^+$ count), % – median (IQR)	5.5 (3.6)	5.5 (4.1)	6.2 (4.7)	0.279		
Confidence interval (CI)	4.9 to 6.8	4.8 to 6.1	5.4 to 6.9			
Blood IgM memory B cell subset (of $CD19^+$ count), % – median (IQR)	9.3 (5.0)	8.5 (6.1)	9.7 (6.6)	0.124		
Confidence interval (CI)	8.0 to 10.4	7.5 to 9.4	8.4 to 11.6			
Blood plasmablast subset (of $CD19^+$ count), % – median (IQR)	0.4 (0.9)	0.3 (0.6)	0.4 (0.8)	0.149		
Confidence interval (CI)	0.3 to 0.6	0.2 to 0.4	0.3 to 0.6			
Blood $CD21^{low}$ B cell subset (of CD19+ count), % – median (IQR)	1.9 (1.8)	1.7 (1.4)	1.6 (1.5)	0.156		
Confidence interval (CI)	1.5 to 2.2	1.5 to 2.0	1.3 to 2.0			
Blood NK cells ($CD16^+CD56^+CD3^-$) count, 10^6 cells/L – median(IQR))	133.0 (116.0)	132.0 (105.0)	159.0 (94.0)	0.513		
Confidence interval (CI)	114.0 to 163.0	115.0 to 155.0	131.0 to 177.0			
NK cell function fraction (degranulated NK cells of total NK cell count), % – mean (SD)	0.27 (0.07)	0.27 (0.09)	0.22 (0.07)	< 0.001	0.517	
Confidence interval (CI)	0.25 to 0.28	0.26 to 0.29	0.20 to 0.24			
Neuroendocrine markers						
Serum TSH, mIE/L – mean (SD)	2.2 (0.9)	2.2 (1.1)	2.2 (1.1)	0.98		
Confidence interval (CI)	2.0 to 2.4	2.0 to 2.4	2.0 to 2.5			
Serum free T4, pmol/L – mean (SD)	12.3 (2.2)	11.8 (2.0)	13.4 (2.5)	< 0.001	0.086	0.069
Confidence interval (CI)	11.9 to 12.8	11.4 to 12.2	12.7 to 14.0			
Plasma ACTH, pmol/L – mean (SD)	4.9 (2.3)	5.5 (3.0)	5.5 (2.6)	0.221		
Confidence interval (CI)	4.4 to 5.4	4.9 to 6.1	4.8 to 6.1			
Serum Cortisol, mmol/L- mean (SD)	411 (158)	373 (177)	357 (125)	0.078	0.114	
Confidence interval (CI)	378 to 444	339 to 407	328 to 387			
Urin Cortisol:Creatinine ratio, nmol/mmol – median (IQR)	3.0 (2.6)	3.2 (3.1)	3.3 (4.6)	0.560		
Confidence interval (CI)	2.6 to 3.5	2.8 to 3.8	2.5 to 4.2			

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Table 2 (*continued*)

	EBV (CF+) (n = 91)	EBV (CF–) (n = 104)	Healthy controls (n = 70)	p-value (across all groups)	p-value EBV (CF+) vs EBV (CF-)	Adjusted p-value EBV (CF+) vs EBV (CF-)*
Plasma Norepinephrine, pmol/L – mean (SD)	1420 (692)	1113 (659)	1252 (567)	0.005	0.002	**0.010**
Confidence interval (CI)	1279 to 1566	985 to 1241	1117 to 1387			
Urine Norepinephrine:Creatinine ratio, pmol/mmol – mean (SD)	12.1 (5.7)	11.3 (4.4)	11.5 (4.4)	0.577		
Confidence interval (CI)	10.8 to 13.3	10.5 to 12.2	10.5 to 12.6			
Plasma Epinephrine, nmol/L – median (IQR)	363 (228)	237 (204)	282 (149)	< 0.001	< 0.001	**0.032**
Confidence interval (CI)	315 to 401	204 to 279	258 to 297			
Urine Epinephrine:Creatinine ratio, nmol/mmol – mean (SD)	1.9 (1.1)	1.9 (2.0)	1.9 (1.3)	0.938		
Confidence interval (CI)	1.6 to 2.1	1.6 to 2.3	1.6 to 2.1			
Markers of autonomic cardiovascular control						
Supine rest						
HR, beats/min – mean (SD)	62.2 (10.1)	60.8 (9.2)	60.3 (8.4)	0.391		
Confidence interval (CI)	60.1 to 64.3	59.0 to 62.6	58.3 to 62.3			
SBP, mmHg – mean (SD)	97.2 (8.1)	99.2 (7.4)	100.4 (8.1)	0.035	0.080	0.755
Confidence interval (CI)	95.5 to 98.9	97.7 to 100.6	98.4 to 102.4			
DPB, mmHg – mean (SD)	59.5 (6.3)	59.3 (6.3)	59.5 (6.3)	0.969		
Confidence interval (CI)	58.2 to 60.8	58.1 to 60.5	58.0 to 61.1			
TPRI, mmHg/L/min/m^2 – mean (SD)	7.5 (1.5)	7.6 (1.7)	7.8 (2.6)	0.592		
Confidence interval (CI)	7.2 to 7.8	7.3 to 8.0	7.2 to 8.4			
LF-RRI, ms^2 – median (IQR)	848 (1138)	986 (1236)	779 (871)	0.270		
Confidence interval (CI)	667 to 1079	805 to 1183	631 to 980			
HF-RRI, ms^2 – median (IQR)	1552 (1877)	1270 (1924)	1255 (1492)	0.987		
Confidence interval (CI)	1100 to 1794	1086 to 1678	1139 to 1563			
LF-RRI:HF-RRI ratio – median (IQR)	0.63 (0.61)	0.76 (0.61)	0.54 (0.58)	0.029	0.023	**0.008**
Confidence interval (CI)	0.5 to 0.7	0.6 to 0.9	0.4 to 0.8			
Controlled breathing response (delta values)						
HR response, beats/min – mean (SD)	0.4 (3.0)	0.2 (2.5)	1.7 (2.8)	0.001	0.586	
Confidence interval (CI)	−0.2 to 1.0	−0.3 to 0.7	1.1 to 2.4			
SBP response, mmHg – mean (SD)	1.0 (4.1)	0.5 (4.1)	0.8 (4.5)	0.805		
Confidence interval (CI)	0.1 to 1.8	−0.3 to 1.4	0.2 to 1.3			
DPB response, mmHg – mean (SD)	−0.5 (4.0)	−0.6 (3.6)	0.2 (4.6)	0.364		
Confidence interval (CI)	−1.4 to 0.3	−1.3 to 0.08	−0.9 to 1.3			
TPRI response, mmHg/L/min/m^2 – mean (SD)	−0.1 (0.5)	−0.2 (0.6)	−0.2 (0.7)	0.275		
Confidence interval (CI)	−0.2 to 0.01	−0.3 to −0.1	−0.4 to −0.08			
LF-RRI response, ms^2 – median (IQR)	−81 (425)	–232 (671)	−259 (559)	0.058	0.061	0.863
Confidence interval (CI)	−181 to −19	−346 to −134	−377 to −128			
HF-RRI response, ms^2 – median (IQR)	57 (637)	75 (755)	39 (847)	0.561		
Confidence interval (CI)	−9 to 201	−16 to 176	−165 to 211			
LF-RRI:HF-RRI ratio response – median (IQR)	−0.11 (0.33)	−0.25 (0.49)	−0.18 (0.40)	0.049	0.016	**0.002**
Confidence interval (CI)	−0.2 to 0.1	−0.3 to −0.2	−0.3 to −0.1			
Orthostatic challenge response (delta values)						
HR response, beats/min – mean (SD)	28.9 (12.3)	26.9 (10.0)	26.9 (14.1)	0.443		
Confidence interval (CI)	26.3 to 31.5	24.9 to 28.9	23.4 to 30.4			
SBP response, mmHg – mean (SD)	3.7 (13.9)	1.9 (9.4)	3.2 (25.7)	0.737		
Confidence interval (CI)	0.7 to 6.8	−0.1 to 3.8	−3.2 to 9.7			
DPB response, mmHg – mean (SD)	11.4 (10.3)	9.4 (7.0)	10.4 (15.9)	0.468		
Confidence interval (CI)	9.2 to 13.6	7.9 to 10.8	6.4 to 14.4			
TPRI response, mmHg/L/min/m^2 – mean (SD)	1.4 (1.6)	1.3 (1.4)	1.5 (3.2)	0.813		
Confidence interval (CI)	1.0 to 1.7	1.0 to 1.5	0.6 to 2.3			
LF-RRI response, ms^2 – median (IQR)	73 (883)	125 (1024)	62 (873)	0.878		
Confidence interval (CI)	−133 to 187	−20 to 222	−111 to 393			
HF-RRI response, ms2 – median (IQR)	−883 (1538)	−744 (1480)	−898 (1514)	0.749		
Confidence interval (CI)	−1338 to −604	−1072 to −497	−1177 to 562			
LF-RRI:HF-RRI ratio response – median (IQR)	1.5 (1.5)	1.4 (2.0)	2.1 (1.8)	0.090	0.761	
Confidence interval (CI)	1.2 to 1.8	1.2 to 2.0	1.4 to 2.7			

P-values ≤ 0.05 in the two right columns are shown in bold for clarity. Statistical tests across the EBV (CF−) and EBV (CF+) groups (second right column) were only carried out if the p-value across all groups were ≤0.1. A total of 113 statistical tests were performed. According to a Bonferroni correction, the level of significance should be set at p = 0.05/113 = 0.0004. TSH = Thyreoidea Stumulating Hormone, T4 = Thyroxine, ACTH = Adrenocorticotropic Hormone, HR = Heart Rate, SBP = Systolic Blood Pressure, DBP = Diastolic Blood Pressure, TPRI = Total Peripheral Resistance Index, HF-RRI = High-Frequency Variability of RR-Interval, LF-RRI = Low-Frequency Variability of the RR-Interval.

* Adjusted for sex and HADS score using multiple linear regression modelling; if feasible, non-normally distributed variables were ln-transformed prior to modelling.

states in general (Lacourt et al., 2018). Our finding of slightly elevated CRP levels in the EBV CF+ group adheres with these previous reports. However, CRP did not correlate with any clinical symptoms in the EBV CF+ group; thus, it seems unlikely that a systemic low-grade inflammation is the underlying cause of patients' experiences. It should also be noted that the CRP level in the EBV CF+ group did not differ

Table 3
Non-parametric correlation (Kendall's Tau) between clinical symptoms and selected immune, neuroendocrine and autonomic markers in the EBV (CF+) group.

	Chalder Fatigue Questionnaire (CFQ), total score	Infectious symptoms, total score	Postexertional Malaise, score	Orthostatic symptoms, total score	Palpitations, score	Feeling alternating hot and cold, score
Blood T cell ($CD3^+$) count						
Kendall's τ	0.13	0.15	0.07	0.10	0.08	0.09
p-value	**0.013**	**0.008**	0.240	0100	0.192	0149
Serum high sensitive CRP						
Kendall's τ	0.03	0.07	0.03	0.03	0.03	0.11
p-value	0.648	0.399	0.702	0.726	0.766	0.204
Plasma Norepinephrine						
Kendall's τ	0.07	−0.11	0.00	−0.09	0.00	0.03
p-value	0.339	0.185	0.988	0.311	0.986	0.739
Plasma Epinephrine						
Kendall's τ	0.12	0.07	0.05	−0.05	0.03	0.05
p-value	0.117	0.375	0.537	0.579	0.722	0.594
LF-RRI:HF-RRI ratio, supine						
Kendall's τ	0.08	0.02	0.06	0.06	0.14	0.02
p-value	0.290	0.815	0.529	0.472	0.139	0.785
LF-RRI:HF-RRI ratio, response to controlled breathing						
Kendall's τ	−0.21	−0.14	−0.09	−0.12	−0.31	−0.12
p-value	**0.005**	0.096	0.296	0.155	**0.001**	0.176

P-values ≤ 0.05 are shown in bold for clarity. A total of 36 statistical tests are performed in the present table. According to a Bonferroni correction, the level of significance should be set at p = 0.05/36 = 0.0013. HF-RRI = High-Frequency Variability of RR-Interval, LF-RRI = Low-Frequency Variability of the RR-Interval.

significantly from healthy controls, further questioning an important role of inflammation in the pathophysiology of CF and CFS.

Differences in lymphocyte subsets between individuals with CF/CFS and healthy controls have been reported in several previous papers, but the findings are somewhat inconsistent. For instance, Maes and co-workers reported increased levels of CD3+ cells in CFS (Maes et al., 2015), in adherence with the results of the present study, whereas Mihaylova and co-workers reported equal levels of CD3+ cells across CFS patients and healthy controls (Mihaylova et al., 2007). In the present study, the increased number and fraction of CD3+ cells in the EBV CF+ group seem to be mainly a consequence of an increment in the CD4+ cell population. This finding is in line with one other study reporting increased CD4+ cells in post-viral CFS (Porter et al., 2010), whereas other studies fail to find any differences in the levels of CD4+ cells (Maes et al., 2015; Mihaylova et al., 2007). Also, in the present study, the fraction (while not the number) of CD19+ cells were (non-significantly) reduced. Again, this finding adheres with some previous cell phenotyping reports (Maes et al., 2015; Bradley et al., 2013) as well with whole blood gene transcription analyses (Nguyen et al., 2017). Other studies, however, find equal levels of CD19+ cells across CFS patients and healthy controls (Mihaylova et al., 2007; Robertson et al., 2005). Replication of the present findings should be scrutinized in future research.

There are several previous reports of altered NK cell count and functional properties in CFS (Robertson et al., 2005; Curriu et al., 2013; Brenu et al., 2014; Brenu et al., 2013; Rivas et al., 2018). Also, altered level of CD8+ cells are a common finding across several previous studies (Maes et al., 2015; Curriu et al., 2013; Porter et al., 2010). In the present study, on the other hand, neither the level and functionality of NK cells, nor the level of CD8+ cells, differed across the EBV CF+ and CF− groups; however, both groups differed significantly from healthy controls. These results suggest that NK cells and CD8+ cells alterations are secondary to the EBV infection itself, and not associated to the degree of fatigue (Balfour et al., 2015). Furthermore, these findings underline the importance of including a post-infective, recovered group in cross-sectional analyses of fatigue biomarkers in order to avoid confounding, as was recently shown by a study of CFS following Giardia-infection (Hanevik et al., 2017).

The increased plasma levels of norepinephrine and epinephrine in the EBV CF+ group directly suggests increased sympathetic nervous activity. Controlled breathing normally stimulates parasympathetic (vagal) nervous activity, as reflected in increased HF-power and decreased LF-power of heart rate variability (Brown et al., 1985). Thus, the attenuated LF/HF-response to controlled breathing in the EBV CF+ group suggests a lack of parasympathetic activation. This given, the slightly reduced LF/HF ratio at supine rest in the EBV CF+ group appears somewhat contradictory, implying a stronger resting sympathetic over parasympathetic heart rate control in the EBV CF− group. This finding is not directly explicable; it should be noted, however, that the group differences are rather small, suggesting the possibility of a false positive result.

The autonomic and neuroendocrine observations in the present study fit well with other CFS studies, and suggest that the previously reported sympathetic predominance is a central feature of being chronically fatigued, not just an epiphenomenon of the complex CFS condition (Wyller et al., 2007a; Wyller et al., 2007b; Wyller et al., 2008a; Wyller et al., 2008b; Hurum et al., 2011; Martinez et al., 2014; Sulheim et al., 2014). Furthermore, the present results are in line with the hypothesized causal relationship between altered autonomic activity and slight inflammatory enhancement in CF/CFS: Whereas the cortisol-to-CRP association was approximately equal across all three groups, a marker of sympathetic nervous activity (urine norepinephrine) and a marker of parasympathetic nervous activity (HF-power of heart rate variability) were independently associated with CRP level in the EBV CF+ group only, possibly explaining the slight CRP increment in this group. The direction of the associations suggest a proinflammatory effect of sympathetic activity and an anti-inflammatory effect of parasympathetic activity, in line with established knowledge on autonomic nervous system control of innate immunity (Andersson & Tracey, 2012; Padro & Sanders, 2014; Thayer & Sternberg, 2010; Cole et al., 2015; Cole, 2014). In particular, the results comply neatly with a large study on the associations between CRP and neuroendocrine/autonomic markers in healthy adults (Thayer & Fisher, 2009). Also, the findings corroborate two previous CFS studies: Nguyen and co-workers showed a relationship between sympathetic to parasympathetic predominance and increased expression of inflammatory related gene products in blood (Nguyen et al., 2017). Sulheim and co-workers demonstrated an anti-inflammatory effect of the sympathetic

Table 4
Multiple linear regression models of associations between C-reactive protein and neuroendocrine/autonomic markers.

	EBV (CF+)				EBV (CF−)				Healthy controls			
	Linear regression coefficient B	Confidence interval	p-value	Δ R^2	Linear regression coefficient B	Confidence interval	p-value	Δ R^2	Linear regression coefficient B	Confidence interval	p-value	Δ R^2
Urine Norepinephrine: Creatinine ratio	9.6×10^{-2}	(0.25 to 18.9×10^{-2})	**0.044**	0.07	5.4×10^{-2}	(-2.3 to 13.1×10^{-2})	0.165	0.02	-1.7×10^{-2}	(-8.3 to 4.9×10^{-2})	0.608	0.02
Serum Cortisol	4.5×10^{-4}	(2.2 to 6.9×10^{-4})	**< 0.001**	0.14	3.0×10^{-4}	(1.4 to 4.7×10^{-4})	**< 0.001**	0.12	3.7×10^{-4}	(1.5 to 5.9×10^{-4})	**0.001**	0.16
HF-RRI, supine	-3.7×10^{-2}	(-6.9 to -0.50×10^{-2})	**0.024**	0.05	-0.48×10^{-2}	(-3.2 to 2.2×10^{-2})	0.725	0.00	0.59×10^{-2}	(-2.0 to 3.2×10^{-2})	0.649	0.00
Explained variance (R^2)	*0.14*				*0.23*				*0.17*			

Bold numbers indicate a significant association ($p < 0.05$). Of note, the majority of the continuous variables were ln-transformed prior to analyses in order to obtain an approximate normal distribution. Thus, the linear regression coefficient B is not immediately informative of the linear associations between non-transformed variables. Δ R^2: The change in total explained variance (R^2) of the model when one variable is removed. HF-RRI = High-Frequency Variability of RR-Interval.

inhibitor and parasympathetic stimulator drug *clonidine* (Sulheim et al., 2014).

The present study failed to replicate previous findings of differences in autonomic cardiovascular responses to orthostatic manoeuvre between chronically fatigued individuals and healthy controls (Wyller et al., 2007a; Wyller et al., 2008a; Wyller et al., 2008b). This result adheres to one previous finding from our group, indicating that differential responses to orthostatic challenge are more related to expectancies than to the gravity *per se* (Wyller et al., 2014). It is possible that an abnormal orthostatic response is a late characteristic of the CFS condition itself, developing over time. However, we speculate that previous findings might have been confounded by deconditioning due to sedentary behaviour, as several previous studies have included CFS patients with a marked reduction in activity levels. In contrast, in the present study, activity level (as measured by steps per day) was not significantly different between the EBV CF+ and the EBV CF− group. It should be noted, though, that most previous studies have been conducted with standardized methods of orthostatic challenge (head-up tilt-test or lower body negative pressure), whereas in the present study, participants were just asked to stand upright. Thus, a discrepancy of findings might be partly due to methodological differences as well.

Also, the present study did not replicate previous findings of attenuated HPA axis in patients suffering from CF and CFS (Sulheim et al., 2014; Papadopoulos & Cleare, 2011; Segal et al., 2005; Nijhof et al., 2014). In particular, urine cortisol was almost identical between the EBV CF+ and EBV CF− group, contrasting the almost 50% reduction reported in a previous paper comparing CFS patients with healthy controls (Sulheim et al., 2014). Again, the underlying explanation might be changes in CFS pathophysiology over time, or the effects of sedentary behaviour. Also, HPA axis dynamics were not directly assessed in the present report, and more precise and comprehensive methods might have yielded different results.

The lack of specific findings pertaining to individuals fulfilling a diagnosis of CFS is also in line with several previous reports (Sulheim et al., 2014; Wyller et al., 2016; Pedersen et al., 2019). An important assumption behind commonly applied CFS case definitions, – the Canada definition in particular (Carruthers et al., 2003) – is that a specific constellation of symptoms is related to certain differences in underlying pathophysiology between patients that adheres to the definition, and patients that do not adhere to it. Thus, the present results question the discriminant validity of these case definitions, corroborating previous findings from another cohort of adolescent CFS sufferers (Asprusten et al., 2015).

Taken together, the differences in disease markers across the EBV CF+ and EBV CF− groups in the present study are rather subtle, and very few remain statistically significant if p-values were to be adjusted for test multiplicity. However, the differences in symptom scores – including infectious symptoms – are large, and clinically as well as statistically highly significant. Thus, there appears to be a striking discrepancy between subjective experiences and objective measurements; a phenomenon that has been observed among CFS patients in other domains as well (Cvejic et al., 2016). Accordingly, we suggest that further research on the CF and CFS phenotype should be integrated with neurobiological concepts that may explain differences in interoception, such as, for instance, sensitization of sensory processing (Brosschot, 2002), in line with recent empirical findings from pain studies in CFS (Nijs et al., 2012; Wyller, 2019).

5. Strengths and limitations

Strengths of this study include the well-defined EBV cohort allowing comparison of a post-viral fatigue group with a post-viral non-fatigued group, the large number of participants, and the low number of drop-outs and missing data. Exclusion of otherwise eligible participants was mainly due to the time limits regarding onset of symptoms; thus, a strong selection bias does not seem likely, supporting generalizability of

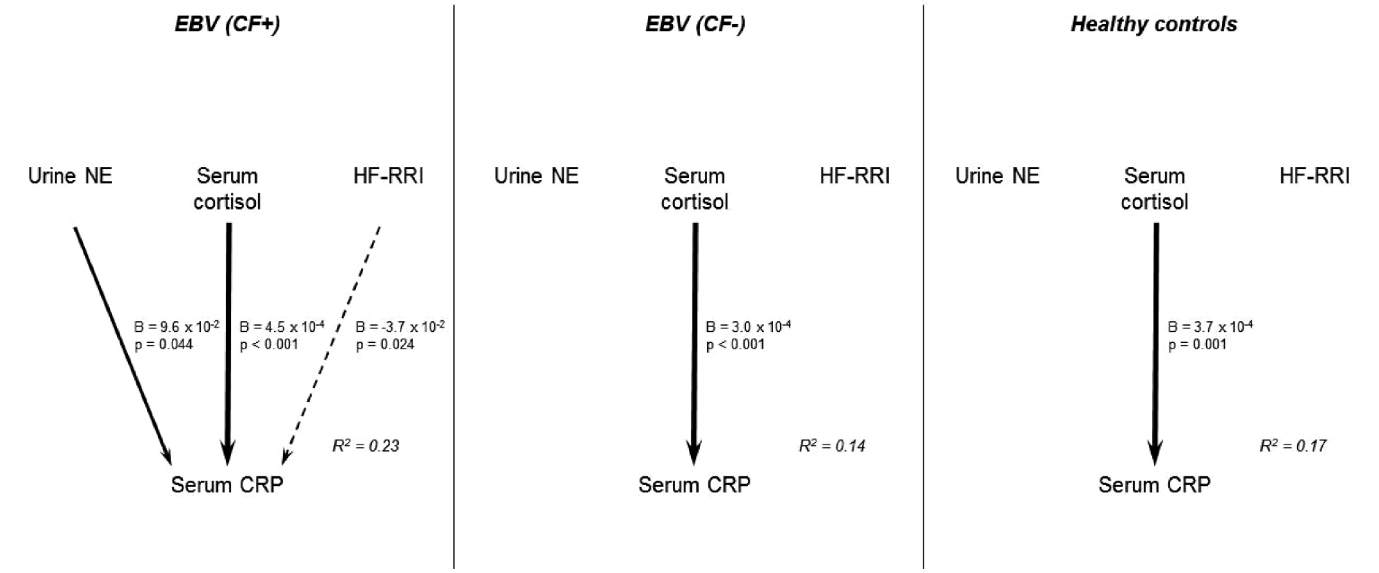

Fig. 1. Multiple linear regression models of associations between serum CRP (dependent variable) and neuroendocrine/autonomic variables (explanatory variables) at 6 months follow-up in the EBV CF+ group, the EBV CF− group and in healthy controls. Continuous lines represent positive associations, whereas dotted lines represent negative associations. The thickness of the arrows corresponds to the impact of the association as reflected in the ΔR^2 – value (Table 4). If feasible, variables were ln-transformed prior to modelling in order to obtain an approximate normal distribution.

the results. In order to scrutinize details of the infectious processes and immune responses, more extensive analyses such as cytokine assays and functional *in vitro*-assessment of lymphocyte subsets would have been beneficial. Also, as most analyses were confined to blood samples only, the results are not necessarily generalizable to other tissues and organ systems, such as the central nervous system. Additional weaknesses include the lack of a standardized method for providing orthostatic challenge, and no direct measurements of HPA dynamics.

As this was an exploratory study, p-values were not adjusted for test multiplicity, leaving a risk for type 1-errors in the cross-sectional comparisons. However, this potential limitation indeed reduces the risk of type 2-errors, and does not seem to weaken our main conclusion of a striking discrepancy between the subjective experiences and the objective measurements in the fatigued group.

6. Conclusion

In adolescents, CF and CFS 6 months after acute EBV infection are associated with high symptom burden, but no signs of increased viral load and only subtle alterations of immune, autonomic, and neuroendocrine markers of which no one is strongly correlated with symptom scores. A slight sympathetic over parasympathetic predominance is evident in CF and might explain slightly increased CRP levels.

Competing interests

None of the authors have conflict of interest or financial relationships relevant to this article to disclose.

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Authors' contribution

Miriam Skjerven Kristiansen, Julie Stabursvik and Elise Catriona O'Leary carried out data analyses and contributed equally to the manuscript. Maria Pedersen and Tarjei Tørre Asprusten collected clinical data, contributed to study design and participated in data analyses. Kristin Godang, Truls Leegaard, Liv Toril Osnes, and Trygve Tjade carried out laboratory analyses and contributed to study design. Eva Skovlund supervised statistical analyses. Vegard Bruun Bratholm Wyller conceived of the study, contributed to study design and participated in data analyses. All authors contributed to data interpretation and drafting of the manuscript.

The principal investigator (VBBW) had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis and had final responsibility for the decision to submit it for publication. No one of the authors has any conflicts of interests relevant to this study.

All authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

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Appendix A. Supplementary material

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