CEBA

Chronic Fatigue following acute Epstein-Barr Virus Infection in Adolescents

Protocol

INTRODUCTION

Chronic fatigue syndrome (CFS) or *myalgic encephalomyelitis* (ME) is characterized by unexplained, disabling and long lasting fatigue, as well as pain, impaired memory, sleep difficulties and other symptoms [1]. In Norway there are about 600 patients under the age of 18 that suffer from CFS [2]. The disability is substantial, and many patients are absent from school, loose contact with friends and are physically inactive. Family functioning might be severely affected [3]. Treatment options are sparse. Due to the high prevalence, severe disability and limited treatment options, this illness has profound economic impact on society. The Norwegian Labour and Welfare Administration (NAV) estimates the insurance expenses to NOK 500 mill/year. In the US, the total annual cost was estimated to $9 billion in 2004[4].

Epstein-Barr virus (EBV) is a member of the herpes virus family. EBV attacks B-cells and epithelia cells in the pharynx, and spreads through bodily fluids.90% of all adults have undergone EBV infection. The clinical presentation is associated with the age of the patient during the acute infection. Acute infection in small children often goes by unnoticed or with flu-like symptoms. In adolescents and in young adults, approximately 25% develop *infectious mononucleosis* characterized by high fever, sore throat (acute pharyngitis/tonsillitis) and swollen lymphatic glands (lymphadenopathy). Headache and abdominal pain with nausea/vomiting are common. Fatigue is also a major symptom. Previous studies indicate that about 20 % of all adolescents with infectious mononucleosis fulfil narrow diagnostic criteria for CFS after 6 months, whereas approximately 10 % fulfil these criteria after one year [5, 6].

Thus, a study of infectious mononucleosis might provide a "window" on CFS disease mechanism.

BACKGROUND

EBV-infection as a trigger of CFS/ME

The precise role of microorganisms in CFS remains unsettled [7] . However, it is generally accepted that certain infections, such as acute EBV infection, might precipitate the condition [6]. Accordingly, EBV-infection is often characterized by long-lasting fatigue, also in adolescents that do not develop CFS, and there appears to be some similarities regarding autonomic alterations [5]. Thus, acute EBV-infection and CFS might have some pathophysiological features in common. These features have not been thoroughly explored. Furthermore, factors that predispose adolescents with acute EBV-infection to develop CFS remain to be identified.

'Sustained arousal' – a model for disease mechanisms in CFS

The disease mechanisms of CFS remain poorly understood. Previous studies report enhanced sympathetic and attenuated parasympathetic cardiovascular nervous activity [8-18], low-grade systemic inflammation [19], attenuation of the hypothalamus-pituitary-adrenal axis (HPA-axis) [20] and impairment of executive control functions [21]. We have suggested that all these features might be attributed to a persistent stress response or 'sustained arousal' (Figure 1)[13], paralleling the pathophysiology of post-traumatic stress disorder [22]. The 'sustained arousal'-model

complies with other recent CFS models [23, 24] and rests upon contemporary stress theories[25-27]. The model offers a platform for integrated, translational research projects, as demanded by the scientific community[28]. Also, the model is congruent with a 'middle-out' systems biology approach, starting with exploring pathophysiological interaction, which might be further extended 'downwards' to the molecular level and 'upwards' to the clinical/phenotypical level[29].

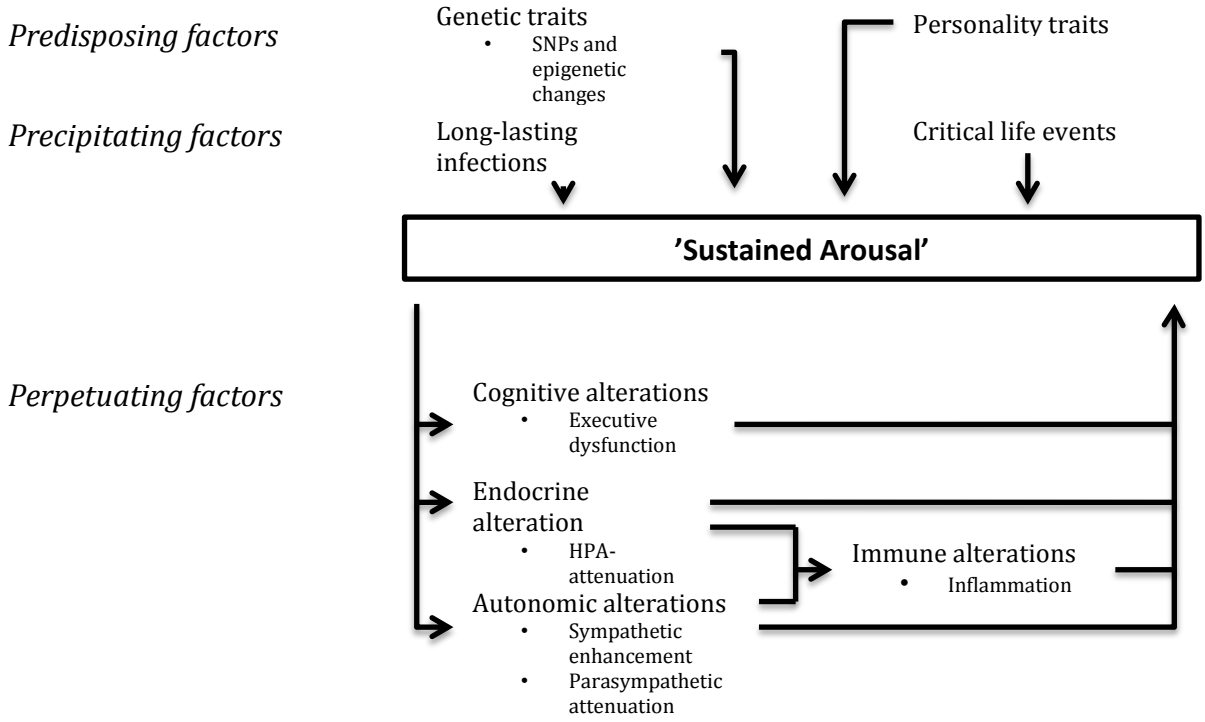

Figure 1. The sustained arousal-model of CFS (adopted from[13] and simplified).

Predisposing factors – personality traits and critical life events

Some studies indicate that personality trait might predispose to CFS [30, 31]. Furthermore, critical life events might precipitate the disorder [32]. The 'sustained arousal'-model allows a more detailed outline of how personality and life events hypothetically contribute to disease development.

Critical life events, as well as infections, normally elicit a similar arousal response characterized by activation of autonomic, endocrine and immune compensatory mechanisms[25]. A long-lasting biological or psychological challenge causes a comparably prolonged arousal response, and in certain cases, the arousal response might be insufficient in solving the initial problem[27]. An attempt of compensation would be to generate a stronger one. As there is no apparent solution to the individual, such attempts might be perceived as inadequate, resulting in negative stimulus and response outcome expectancy. Thus, a vicious circle is established, as the evaluation of the arousal response depends upon expectancies: negative expectations reinforce the arousal response[27]. This inappropriate learning process can be strengthened by attentiveness, corresponding with reports of increased focus on bodily sensations in CFS [33]. Increased worry

about coping abilities is also suggested to be a risk factor[34], complying with personality traits that might be associated with CFS [35].

Predisposing factors – genetic traits

Genealogical studies[36] and twin studies[37] suggest a hereditary predisposition in CFS. As predicted by the sustained arousal-model, several candidate genes are related to catecholaminergic and serotoninergic neural systems, as well as the HPA axis and inflammatory pathways (Table 1) [38-46] .

The rs4680 Single Nucleotide Polymorphism (SNP) in the metabolic enzyme catechol-O-methyltransferase (COMT) is one genetic marker of particular interest; this polymorphism is related to CFS[46], to chronic pain disorders[47, 48], and to altered neural activity in prefrontal cortex (PFC) areas that influences autonomic centers[49]. Interestingly, this SNP increases plasma levels of catecholamines, which is a feature of CFS[14].

Table 1. Candidate genes in CFS

Candidate gene	*Genetic/epigenetic region of particular interest*	*Gene product*	*Biological function*
Catecholaminergic system			
ADRA2A	rs1800544 (pro.)	α2A adrenergic receptor	Transmembrane catecholaminergic receptor
ADRB2	rs1042714 (Gln27Glu)	β2 adrenergic receptor	Transmembrane catecholaminergic receptor
COMT	rs4680 (Val158Met); rs4633 (syn.); rs4818 (syn.); rs6269 (pro.); rs933271 (in.); rs4646312	Catechol-O-methyltransferase	Enzymatic catabolism of catecholamines
MAOA	rs1801291 (syn.); rs979606 (in.); rs979605 (in.)	Monoamine oxidase A	Enzymatic catabolism of catecholamines
MAOB	rs3027452 (in.); rs1799836 (in.)	Monoamine oxidase B	Enzymatic catabolism of catecholamines
Serotoninergic system			
TPH2	rs4565946 (in.); rs2171363 (in.); rs4760816 (in.); rs4760750 (in.); rs1386486 (in.)	Tryptophan hydroxylase 2	Enzymatic synthesis of 5-HT (serotonin)
HTR2A	rs6311 (pro.); rs6313 (syn.); rs17289394 (pro.); CpGs RefSeq –1439, -1420, -1224 (pro.)	5-HT receptor 2A	Transmembrane serotoninergic receptor
5HTT	Polymorphic repetitive elements (pro.)	5-HT transporter	Presynaptic, transmembrane transporter protein
HPA axis			
NR3C1	rs1866388 (in.); rs2918419 (in.); rs860458 (in.); rs852977 (in.); rs6188 (in.); rs258750 (in.); CpGs RefSeq -3220, -3208 (pro.); H3K9 (hist.)	Glucocorticoid receptor	Intracellular steroid receptor
CRHR1	rs7209436 (in.); rs242924 (in.); rs173365 (in.)	CRH receptor 1	Transmembrane receptor for CRH
CRHR2	rs2284217 (in.); rs2267714 /rs1076292 (in.)	CRH receptor 2	Transmembrane receptor for CRH
Inflammatory pathways			
IL6	rs1800795 (pro.)	IL-6	Proinflammatory cytokine
TNFA	rs1799724 (pro.)	TNF-α	Proinflammatory cytokine
IL17F	rs763780 (His161Arg)	IL-17F	Proinflammatory cytokine

syn. = synonymous; pro. = promotor region; in. = intron; hist. = histone modification

Epigenetic modification represents a long-time adaption to stressful environment[50]. The epigenetics of fatigue has hardly been explored [51]. Considering the HPA attenuation in CFS, the glucocorticoid receptor (GR) gene NR3C1 is of interest. In rats as well as in humans, methylation at two CpG-sites in the NR3C1 promotor region is associated with adverse childhood experience and attenuated cortisol response to Dex/CRH stimulation test; one of this CpG-sites is also a binding site for the transcription factor NGFI-A (Egr-1)[52]. Accordingly, chronic stress in newborn rats reduces acetylation of the associated histone site (H3K9), silencing gene expression[52].

Furthermore, a recent CFS study reported enhanced transcription of the gene for the serotoninergic receptor HTR2A [53]. This was partly due to increased frequency of the minor allele A of the promotor SNP rs6311, causing loss of a CpG-site affecting transcriptional GR binding. Thus, in CFS patients, increased frequency of this allele compensates for the tendency towards CpG-methylation and low cortisol levels.

Pathophysiological feature - neuroendocrine alterations

Several previous studies have documented enhanced sympathetic and attenuated parasympathetic cardiovascular nervous activity in CFS [9-18, 54]. Increased plasma norepinephrine is a conspicuous finding among our patient samples [8, 14], consistent with a report of high plasma neuropeptide Y-levels [55].

In addition, the patients tend to have low levels of cortisol in plasma, urine and saliva[56], altered circadian rhythms[57] and weaker responses of the hypothalamus-pituitary-adrenal axis (HPA-axis) during stimuli that normally increase cortisol secretion[58]. Thus, a general HPA-axis attenuation appears to be a characteristic feature of the pathophysiology, as recently confirmed in the NorCAPITAL project [8].

Pathophysiological feature – immunological alterations

Immune function has been extensively studied in CFS. Low levels and attenuated function of NK-cells was recently reported [59]. The NorCAPITAL project suggest low-grade systemic inflammation [8], complying with reports of slightly increased levels of proinflammatory cytokines in other studies [7]. Animal models of chronic stress display similar immune alterations [60]. The source of the cytokines is unknown; a recent report of beneficial effect of rituximab treatment [61] suggests a prominent role for the B-cells, which participates in non-autoimmune inflammatory disorders[62]. Alternatively, autoantibodies might play a role in the pathogenesis; of note, autoantibodies against cellular signal transduction mechanisms were recently documented in a closely related clinical condition (POTS) [63].

B-cells expresses β2AR, but nervous stimulation of this receptor has complex effects[64]. For instance, in PBMC, β2AR agonist causes increased secretion of the proinflammatory cytokine IL-6 via PKA-activation of the GATA1 transcription factor; however, the positive relation between sympathetic activity and IL-6 secretion is dependent on a SNP (rs1800795) in the IL-6 promoter region [65]. Furthermore, the expression of β2AR and the effect of stimulation are dependent upon epigenetic modifications, the differentiation state of the cell, and the total cytokine microenvironment [66].

In CFS, immune alterations might also be a consequence of HPA attenuation. Accordingly, proinflammatory cytokine increment in post-treatment cancer fatigue is related to decreased glucocorticoid transcript and increased NF-κB transcript in PBMC [67]. Also, interaction with sympathetic signaling seems likely. For instance, GR phosphorylation of serine residues by different kinases (such as MAPKs, CDKs and GSK-3β) modulates the DNA transcriptional pattern, explaining differential effects of cortisol [68]. Such phosphorylation might be caused by β2AR stimulation, which has been shown to activate the GSK-3β signaling pathway [69].

Finally, attenuated parasympathetic signaling might contribute to enhanced proinflammatory cytokine levels in CFS. Vagal efferent activity reduces production of TNF-α, IL-1β and IL-6 in spleen macrophages by suppressing NF-ϰB and activating Jak-STAT pathways [70]. Interestingly, activity in the central autonomic network as well as heart rate variability indices of parasympathetic modulation correlates with levels of proinflammatory cytokines in healthy controls [71].

Subgrouping

Although the sustained arousal-model suggests a 'common pathway' of CFS disease mechanisms, sub-groups of patients might have different pathophysiological characteristics. Subgrouping might be based upon diagnostic criteria, such as the criteria from International Chronic Fatigue Syndrome Study Group at the Centers for Disease Control and Prevention[72]; however, recent evidence has questioned their validity[15, 73-75]. Eventually, predisposing or precipitating factors might be used for subgrouping (such as the presence or not of a critical life event in addition to infectious mononucleosis). This study allows exploration of different subgrouping strategies.

AIMS

The general aims of this study are:

- To identify factors that predispose to chronic fatigue among adolescents with acute EBV infection
- To compare pathophysiological features of patients with acute EBV infection with a group of healthy controls.

Possible risk factors for chronic fatigue 6 months after EBV-infection include:

- Severity of the initial infection
- Immune responses characteristics
- Characteristics of the neuroendocrine stress response
- Cognitive functioning
- Emotional disturbances (anxiety/depression)
- Genetics/ epigenetics of candidate genes
- Certain personality traits (perfectionism)
- Critical life events

The primary endpoints are fatigue measured by Chalder fatigue questionnaire, and physical activity as measured by accelerometer (mean steps/day), cf. below.

EXECUTION

Design overview

A total of 200 adolescents with acute EBV infection will be included and followed prospectively for 6 months (Figure 3). A similar investigational program is to be conducted at baseline (0

months) and 6 months. In addition, 70 healthy controls having the same distribution of gender and age as the patients will be included.

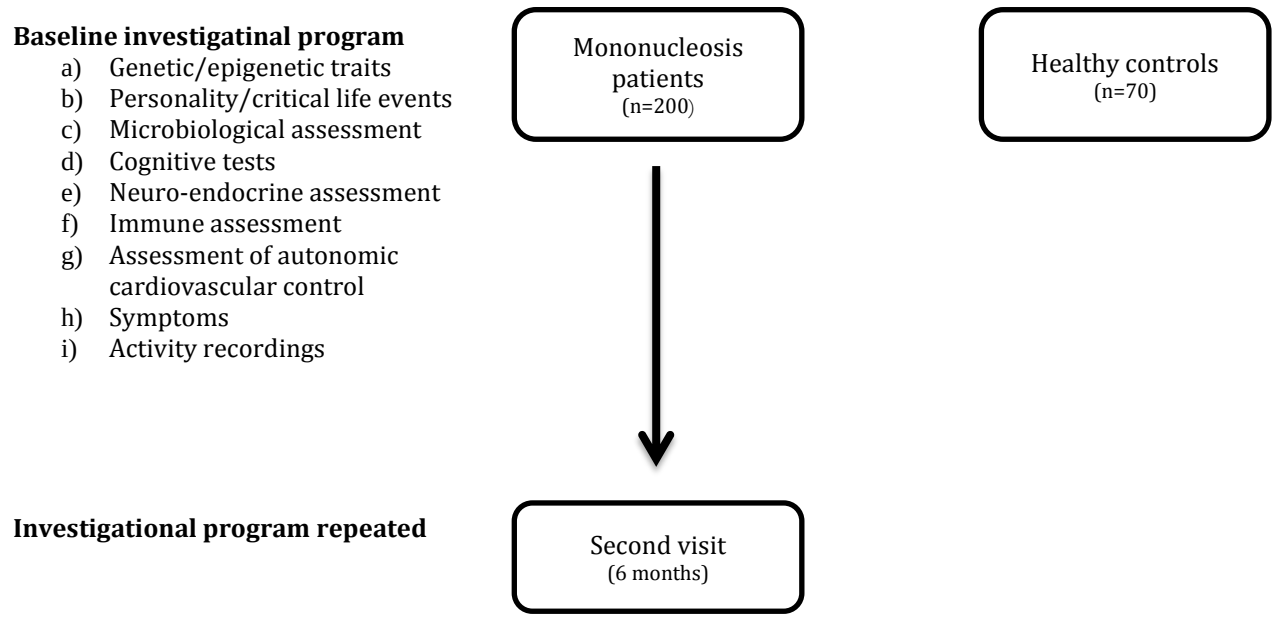

Figure 2. Design overview

Power calculation

In a cross-sectional-design, a total number of 200 EBV patients and 70 healthy controls will give a power of at least 80% to detect mean differences between the two groups of ≥ 0.5 standard deviations (i.e. effect size ≥0.5). Previous studies indicate that the study is thus sufficiently large to detect clinically important differences in pathophysiology[8].

For the prospective part there are two primary endpoints; the total sum score in the Chalder Fatigue Questionnaire (0-33) and the mean number of steps per day measured over 7 consecutive days. The primary statistical analysis will be a linear regression analysis. With 200 EBV patients and significance level 5% the power to detect that a variable explains 5% of the total variance (R^2=0.05) is at least 80%. Correspondingly, the power would be close to 95% to detect R^2=0.075. That implies that the study has sufficient power to detect small to medium effect sizes.

Previous studies indicate that up to 1/3 of all adolescents with EBV-infection might suffer from chronic fatigue after one year (defined as a sum score of dichotomized responses ≥ 4 on the Chalder Fatigue questionnaire[76]). The NorCAPITAL project suggests a drop-out rate of approximately 10 %, leaving 60 patients with a significantly different endpoint score. With 60 chronic fatigue patients followed over time, the power to detect an effect size of ≥0.4 is ≥87%. This effect size is slightly smaller than the change in fatigue score observed in the NorCAPITAL project, thus the sample size is regarded as sufficiently large. For all the measurable risk factor values an effect size of 0.4 seems reasonable (0.4 times the standard deviation).

200 EBV patients will according to the table below give sufficient power to detect effects of different dichotomous (present or not present) risk factors (Table 2).

Table 2. Power calculation. Following table present the power calculation of the bionomic risk factors. n=number of participants in each group, p = stipulated presence of risk factor in each group. Level of significance is set to 0,05.

Risk of chronic fatigue (p)				Risk difference	Relative risk	Power %
Present		Not present				
n	p	n	p			
100	0.4	100	0.2	0.2	2	85
50	0.4	150	0.2	0.2	2	86
100	0.25	100	0.1	0.15	2.5	80
40	0.25	160	0.1	0.15	2.5	76

Recruitment, inclusion and exclusion

The Microbiological Laboratory at AHUS University Hospital and Fürst laboratory provides microbiological analyses for almost all General Practitioners in the hospital's population area. Identification of adolescents with acute EBV infection is based on their antibody response characteristics (Table 3).

Table 3. The possible antibody response characteristics and the interpretation of them. The first two lines represent the two antibody response characteristics included.

Rapid test*	EBV-VCA-IgM	EBV-VCA-IgG	EBNA-IgG		
positive	Positive	negative	negative	Infection (debut 1-3 weeks ago)	Eligible
pos/neg	Positive	positive	negative	Infection (debut 2-8 weeks ago)	
negative	Negative	negative	negative	Not EBV infection	Not eligible
negative	Negative	positive	positive	Earlier infection	
pos/neg	Positive	positive	positive	Reactivation of EBV infection	
negative	Negative	negative	positive	Probably false neg EBV-VCA-IgG	
negative	Positive	negative	negative	Inconclusive	New bloodsample in 2-3 weeks.
positive	Negative	negative	negative	Isolated positive rapid test is inconclusive	
negative	Negative	light positive	negative	IgG sometimes appears before IgM (this could be a newly infected). High IgG is interpreted as an earlier infection	

*The rapid test is only performed where the specific tests alone are inconclusive

Patients with acute EBV infection in the relevant age group will be consecutively identified (cf. Table 4). Information about the study will be provided through telephonic contact with either the patient himself or his or hers parents (depending on the patient's age). This telephonic conversation will be conducted according to a standardized procedure. Thereafter, written

information about the study, including a formal invitation to participate, will be sent to those who agree to receive such information.

A final decision on inclusion will be taken during the initial phase of the clinical encounter (see below). Contraceptive pills and antibiotics against tonsillitis/pharyngitis are accepted. Patients on any other medication will be excluded.

Healthy controls will be recruited among the patients' peers.

Table 4. Criteria for inclusion and exclusion

Criteria for inclusion and exclusion	
Inclusion criteria	*Exclusion criteria*
Patients	
Age ≥ 12 years and < 20 years Serological confirmation of acute EBV infection Lives in one of the following Norwegian counties: Oslo, Akershus, Buskerud, Vestfold, Østfold	Medical treatment for another disease (hormonal conterception and antibiotics against tonsillitis/pharyngitis are accepted) Pregnancy Debut of illness > 6 weeks ago (anamnestic)
Healthy controls	
Age ≥ 12 years and < 20 years Lives in one of the following Norwegian counties: Oslo, Akershus, Buskerud, Vestfold, Østfold	Medical treatment for another disease (hormonal conterception is accecpted) Pregnancy

Investigational program

Eligible participants will be received by a physician at our research unit and subjected to an investigational program. All participants will be instructed to fast overnight and abstain from tobacco products and caffeine at least 48 hours. The total length of the clinical encounter is stipulated to 3.5 hours. The final evaluation according to the inclusion and exclusion criteria takes place during the first minutes of the encounter (and after the pregnancy test for the girls). The participants as well as and parents/next of kin will be thoroughly informed; inclusion is based upon written informed consent.

After decision on inclusion, the investigational program proceeds with the following elements (for details, see below):

- Clinical examination
- Pain threshold assessment
- Cardiovascular assessment
- Cognitive assessment
- Sampling of biological material (blood and urine)
- Questionnaire

Following the in-hospital assessment, daily physical activity will be monitored during seven consecutive days using the *activPAL* accelerometer device (PAL Technologies Ltd, Scotland).

Each participant will receive a gift certificate having the value of NOK 200 after each completed in-hospital assessment.

Clinical examination
The clinical examination includes auscultation of the heart and lungs, palpation of the cervical lymphatic nodes, inspection of the throat and ears and palpation the abdomen. Percentile scores for weight/height and height/age will be recorded. In addition, the examination includes ultrasound measurement of splenic enlargement

Pain threshold assessment
Pain threshold will be assessed by means of an algometer (Algometer Commander, JTECH Medical, Salt Lake City, USA). Anatomically well-defined "trigger-points" are subjected to increasing pressure; the patients alert at the point where the pressure is perceived to be painful [77].

Cardiovascular assessment
At supine rest, participants will be attached to the Task Force Monitor® (Model 3040i, CNSystems Medizintechnic, Graz, Austria); a combined hardware and software device for noninvasive continuous recording of cardiovascular variables [78]. A 5 minute baseline recording will be obtained. Thereafter, the participants are instructed to breathe at a fixed breathing rate of 0.2 Hz (12 breaths per minute) for 5 minutes. Finally, the participants are instructed to stand upright for 3 minutes.

Instantaneous heart rate (HR) is obtained from the R-R interval (RRI) of the electrocardiogram. Photoplethysmography on the right middle finger will be used to obtain a non-invasive, continuous recording of arterial blood pressure [79]. Impedance cardiography will be used to obtain a continuous recording of the temporal derivate of the transthoracic impedance (dZ/dt) [80]. All recorded signals is on-line transferred to the built-in recording computer of the Task Force Monitor®, running software for real-time data acquisition.

Cognitive assessment
Participants will undergo cognitive testing in the following sequence: The digit span test from the Wechsler Intelligence Scale for Children, 4th edition (WISC-IV), [81], the Color-Word Interference test from the Delis-Kaplan Executive Function System (D-KEFS) [82], and the Hopkins Verbal Learning Test-Revised (HVLT-R) [83]. In addition, two subtests form of the Wechsler Abbreviated Scale of Intelligence (WASI) will be used to estimate the patients IQ.

Sampling of biological material
Blood samples will be collected between 08.15 and 09.15 am. An ointment containing the local anaesthetic lidocaine (Emla®) will be applied on the skin in the elbows one hour prior to blood sample collection. After 15 minutes of supine rest in calm surroundings, blood samples for different laboratory assays will be obtained in a fixed sequence from antecubital venous puncture.

As a general routine, plasma samples will be centrifuged (4 °C, 3500 x *g*, 15 min) within 30 minutes and frozen at -80 °C until assayed. Also, participants will be instructed to bring a morning spot urine sample in a sterile container. Finally, a hair sample of 2 cm length and 0.5 cm with is obtained from the base of the scull.

Further analyses of the biological material includes

- Hematology and biochemistry routine assays will be performed at the accredited laboratory at Akershus University Hospital, Norway.
- Blood samples for microbiological analyses will be collected in 4 mL EDTA tubes and gel-containing tubes, respectively. Detection of microbial EBV-DNA will be performed by real-time polymerase chain reaction (PCR) in whole blood using a commercial kit (artus EBV, Qiagen, Hilden, Germany). Specific antibody responses will be assessed using anti-EBV EBNA IgG (Bio-Rad, Dreieich, Germany) and anti-EBV VCA IgG and IgM (Hiss Diagnostics, Freiburg, Germany). Also, antibodies against CMV and *Borrelia burgdorferi* will be assayed.
- Blood samples for analyses of plasma catecholamines will be obtained in vacutainer tubes treated with ethylene glycol tetra acetic acid (EGTA)–glutathione, and thereafter subjected to high-performance liquid chromatography (HPLC) with a reversed-phase column and glassy carbon electrochemical detector (Antec, Leyden Deacade II SCC, Zoeterwoude, The Netherlands) using a commercial kit (Chromsystems, München, Germany) [84, 85].
- For genetic analyses, DNA will be extracted from whole blood; SNPs of candidate genes will be assayed with standard methods (TaqMan). For gene expression analyses, samples will be obtained in PaxGENE tubes and subsequently subjected to quantitative PCR analyses.
- For immune assessment, a broad range of cytokines will be assayed by Luminex microarray in EDTA plasma. Number and cytotoxic function of NK-cells will be assessed applying flow sorting and stimulation of cell cultures. Also, peripheral blood mononuclear cells (PBMC) will be snap frozen, making subsequent molecular analyses feasible.
- Urine samples and hair samples will be subjected to analyses of cortisol.

Questionnaire

A questionnaire is distributed to all participants, being composed of the following validated instruments:

- Autonomic Symptom Profile[86], translated and slightly modified [8]
- Chalder Fatigue Questionnaire[76], translated and validated for a Norwegian population[87]
- PedsQL [88], translated and validated for a Norwegian population[89]
- Functional Disability Inventory[90], translated and slightly modified
- Brief Pain Inventory[91]
- Life Event Checklist (LEC)[92]
- Hospital Anxiety and Depression Scale (HADS)[93]

- Child-Adolescent Perfectionism Scale (CAPS) [94]
- Toronto Alexithymia Scale-20 item (TAS-20) [95]
- Brief Illness Perception Questionnaire(BIPQ)[96]
- Karolinska Sleep Questionnaire[97]
- The Penn State Worry Questionnaire

Furthermore, there are questions specifically related to the different diagnostic criteria of CFS, including the CDC-criteria[72], and the Canadian criteria [98], and simple questions regarding life style and demographics. The questionnaire is completed during the in-hospital investigational day. Chalder Fatigue Questionnaire (CFQ) [76] is regarded a valid outcome measure in CFS research among adults [72, 99] as well as adolescents [100, 101]. In this study, the CFQ total sum score is selected as one of the primary endpoints; ie. the sum across all 11 CFQ items, each of which is scored on a 0-3 Likert scale. Total range is from 0 to 33; higher scores imply more severe fatigue. In addition, dichotomous scores (0 – 0 – 1 – 1) will be used for definition of chronic fatigue caseness; i.e. a sum score of dichotomised responses ≥ 4 (see above).

Physical activity

Accelerometers are widely used devices for accurate measurements of physical activity [102]. They provide reliable and valid data among patients with impaired physical capacity [103], and have been successfully applied in previous CFS studies [104, 105].

In this study, we will use the *activPAL* accelerometer device (PAL Technologies Ltd, Glasgow, Scotland) for monitoring of daily physical activity during seven consecutive days. *ActivPAL* provides reliable and valid data on step number and cadence as well as time spent on walking, standing and sitting/lying during everyday activities [106, 107]. The device has also been validated in an adolescent population [108], and it is sensitive for changes of step number with time [109].

A recording period of seven consecutive days is selected, according to present recommendation [102]. The recording unit (weight: 15 grams, size: 53 x 35 x 7 mm), will be attached midline on the anterior aspect of the thigh by specially designed adhesive strips (PAL*stickies*), according to the manufacturer's instruction. The participants will be instructed to wear the unit permanently (ie, also during the night); however, they will be shown how to remove it during showering/bathing and re-apply it afterwards. After the recording period, the unit will be returned by mail in a pre-stamped envelope.

Data from the recording units is transferred to a computer running producer developed software. For each participant, all recording epochs will be carefully and independently reviewed. If one recording day is considered to contain erroneous or incomplete data, that entire day will be removed from further calculation. Finally, the mean number of steps per day will be calculated for all recording epochs. The mean number of steps per day is one of the primary endpoint in this study.

Statistical analyses

Continuous variables will be reported with parametric (mean/standard deviation) or non-parametric (median, quartiles) descriptive statistics, depending on the distribution.

Ordinal/nominal variables will be reported as frequency tabulation. All statistical tests will be carried out two-sided. A p-value ≤ 0.05 is considered statistically significant.

Patients with acute EBV-infection (full analysis set) will be compared with healthy controls for background variables applying the Student t-test or the Mann-Whitney U test as appropriate. The null hypothesis is no differences between patients and healthy controls.

The changes within the acute EBV-infection patients (full analysis set) over time will be analyzed using (multiple) linear regression analysis. The two endpoints are set as the dependent variables in separate analyses, and all the different potential risk factors as independent (or explanatory) variables. In each analysis the null hypothesis is that the dependent variables not associated with the independent variables (the potential risk factors). The primary endpoints are Chalder fatigue score and mean number of steps/day count during 7 consecutive days at 6 months.

The potential relationship between each risk factor variable and the two end points are first explored in separate linear regression analyses including one risk factor. The distribution of residuals will be assessed for normality. Risk factors with $p<0.1$ will be included in a multivariable linear regression analyses. P-values <0.05 will be regarded as statistically significant.

Ethical considerations

To obtain a large enough number of participants, it is necessary to recruit patients from routine analyses at microbiological departments. This procedure implies a contravention of the rule of professional secrecy for the laboratory workers who provide information about microbiological answers indicating acute EBV infection. To minimalize the contravention, the only information given is the name and personal security number to eligible participants (cf. table 3). No other information will be collected without the participants (or their parents) consent. Furthermore, the PhD student in this project is the only person that is certified to receive this information.

Participation is based upon informed consent, and thorough information will be provided orally as well as in writing to the participants and (if younger than 16 years) to their parents/next-of-kin. All data will be treated and stored without personal identifying information, and in accordance with national directives. Approbation will be sought from the Regional Committee for Ethics in Medical Research and the Norwegian Data Inspectorate. The study will be registered at ClinicalTrials.gov, and will adhere to the STROBE statement.

Venous puncture might be painful; therefore, an ointment containing the local anaesthetic lidocaine (Emla®) is routinely given as a prophylactic. Other methods applied in this study are neither painful nor harmful.

Clinical follow-up

All patients will be examined by a physician at our research unit. The same physician is responsible for evaluation of all laboratory results and clinical findings, and will eventually organize routine clinical follow-up if needed.

Progression and finances
In 2013, the Microbiological Laboratory at AHUS University Hospital identified 67 patients that would have been eligible for this study. In addition, Fürst laboratory identified 296 possible eligible patients in the nearby counties (Oslo, Akershus, Buskerud, Østfold and Vestfold). Thus, inclusion of 200 patients in this study within one year seems feasible. Healthy controls will be included in parallel with patient inclusion. Given 26 weeks of follow-up, 1.5 years will be necessary to obtain a complete data set. A third year will be spent on analyses and publishing. The project will start in 2015; publication of the main results is to be expected in 2017.

This project has received financial support from The Health South-East Hospital Trust for one PhD-student full time for 3 years.

Publishing
Results from this project will be published in international, peer-reviewed medical journal and constitute the basis for one PhD-dissertation. The most important results will be offered to clinical journals of high impact. We will also report negative results. Co-authorship will be granted according to the Vancouver guidelines. In addition, the following means of dissemination will be considered:

- Participation in CFS conferences as well as general scientific conferences
- Review papers in international and national journals
- The activity provided by the recently established Centre of Competance for CFS at OUS (information leaflets, supervision, conferences, etc.)
- Direct contact with all participants in this project.
- Communication with two national patients' organizations for CFS/ME (Norges ME-forening og MENiN).
- Participation in the official CFS network headed by the Norwegian Health Directorate
- Articles and interviews in the mass media and social media

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