Title: Cytokine responses to exercise and activity in patients with chronic fatigue syndrome: case control study

Short title: Cytokine responses to exercise in CFS

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Abbreviations:

ASB acute sickness behaviour, BMI body mass index, CAMs complementary and alternative medicines, CDC Centers for Disease Control, cDNA copy deoxyribonucleic acid, CFS chronic fatigue syndrome, CNS central nervous system, DSM IV diagnostic statistical manual 4th edition, HADS Hospital Anxiety and Depression Scale, HR heart rate, IFN interferon, IL interleukin, IPAQ International Physical Activity Questionnaire, LOQ lowest level of quantification, mRNA, messenger RNA, ME myalgic encephalomyelitis, PA physical activity, PCR polymerase chain reaction, RNA ribonucleic acid, RPE rate of perceived exertions, SCID Standardised Clinical Interview for DSM IV, SD standard deviation, SDS Sequence Detection System (SDS), SF36 Short Form 36 question version, SSRI selective serotonin reuptake inhibitors, TCAs tricyclic antidepressant, TGF transforming growth factor, TNF tumour necrosis factor, TLR4 toll-like receptor 4 reuptake inhibitors, VO_2 volume of oxygen.

SUMMARY

Chronic fatigue syndrome (CFS) is characterized by fatigue after exertion. A systematic review suggested that transforming growth factor beta (TGF-β) concentrations are often elevated in cases of CFS when compared to healthy controls. This study attempted to replicate this finding, and investigate whether post-exertional symptoms were associated with altered cytokine protein concentrations and their RNA in CFS patients.

Twenty-four patients fulfilling Centers for Disease Control criteria for CFS, but with no comorbid psychiatric disorders, were recruited from two CFS clinics in London, UK. Twenty-one healthy, sedentary controls were matched by gender, age, and other variables. Circulating proteins and RNA were measured for TGF-β, TNF, IL-8, IL-6 and IL-1β. We measured six further cytokine protein concentrations (IL-2, IL-4, IL-5, IL-10, IL-12p70, and IFN-γ). Measures were taken at rest, and before and after both commuting and aerobic exercise.

CFS cases had higher TGF-β protein levels compared to controls at rest (median (quartiles) = 43.9 (19.2, 61.8) versus 18.9 (16.1, 30.0) ng/ml) ($p = 0.003$), and consistently so over a nine-day period. However, this was a spurious finding due to variation between different assay batches.

There were no differences between groups in changes to TGF-β protein concentrations after either commuting or exercise. All other cytokine protein and RNA levels were similar between cases and controls. Post-exertional symptoms and perceived effort were not associated with any increased cytokines.

We were unable to replicate previously found elevations in circulating cytokine concentrations, suggesting that elevated circulating cytokines are not important in the pathophysiology of CFS.

Introduction

Chronic fatigue syndrome (CFS) is an established condition of chronic and disabling fatigue and associated symptoms, which is not caused by an obvious medical disease or psychiatric illness [1,2]. Some authors believe that myalgic encephalomyelitis (ME) is a different illness, whereas others believe that ME is synonymous with CFS. The prevalence is between 0.2 and 2.6%, depending on definition [2]. The most characteristic symptoms of sufferers are fatigue and malaise that are brought on by minimal exertion [1]. The aetiology and pathophysiology of CFS are essentially unknown [2]. The illness can be precipitated by certain infections, but we do not know why only a small proportion of people go on to develop CFS [2].

The symptoms of CFS are similar to those of acute sickness behaviour (ASB) [3]. ASB has been found to be associated with pro-inflammatory cytokine concentrations, which are themselves associated with the individual symptoms of ASB [4]. Changes in certain cytokines, such as TGF-β, may induce symptoms or behaviour similar to CFS [5]. The similarity between the symptoms of CFS and ASB and the effects of some cytokines, along with the commonly observed onset after infections, has led to the hypothesis that abnormal cytokine regulation may be important in CFS pathophysiology [5, 6, 7]. There have been three systematic reviews of the role of the immune system in CFS [8, 9, 10]. The cytokines that have most often been measured in patients at rest include IL-1β, TNF and IL-6 [10]. No significant differences between cases and controls in protein levels of these cytokines were found in between 75% and 80% of studies [10]. The most consistent finding in five of eight case controls studies was that circulating TGF-β was elevated at rest [10].

Apart from the criticisms of using different methods and not considering confounders [8, 9, 10], such as body mass index (BMI), the other problem with measuring baseline cytokine levels without behavioural stimulation is that this does not test whether abnormal cytokine release is associated with the characteristic symptom of post-exertional malaise. Our pilot study showed that ordinary physical activity (commuting) was associated with significantly elevated plasma TGF-β protein concentrations, and that aerobic exercise was associated with significantly elevated serum tumour necrosis factor (TNF) protein concentrations in CFS cases compared to healthy controls [11].

One systematic review, of 23 case control studies of immune changes with exercise in CFS, found no evidence to support cytokine changes with exercise [9]. They did, however, find that gene expression (mRNA) was elevated for IL-10 and toll-like receptor 4 (TLR4), as well as evidence to support complement 4a split product activation [9]. One study, which was included in this review, also found an association between the level of mRNA, measured as the area under the curve, for both IL-10 and TLR4 and post-exercise symptom exacerbation [12]. A case control study, not included in this review, found no differences in IL-10 receptor mRNA between groups [13].

Blundell and colleagues systematically reviewed 38 case control studies, which included studies specifically examining evidence for cytokine changes with exercise [10]. Only three cytokines had been measured in five or more studies, (IL-6, IL-1β, and TNF), with the large majority of studies showing no differences between cases and controls. Two out of two studies found elevated IL-2 after exercise, and a further

two out of two studies found elevated TGF-β after exercise, but no association with post-exertional symptoms [10].

This research, highlighting the possible relevance of TGF-β, TNF, IL-6 and IL-1β, as well as the literature on exercise and cytokine response in the healthy population, in which TNF, IL-1, IL-6, IL-10 and IL-8 seem to be involved [14], informed our choice of hypothesised cytokine proteins (IL-6, IL-8, TNF and TGF-β) for this study. By also measuring the RNA of these four hypothesised cytokines (along with IL-1β as a correlation control for detectable protein vs RNA) we were able to explore their biological pathway. It is possible that the complex temporal regulation of cytokine expression during physical activity may not be fully reflected in simple blood concentrations [15]. Although there is little evidence for this [16], it is possible that some cytokines may remain cell surface bound so bio-active and peripheral levels may not be fully representative [17, 18]. Measuring RNA alongside proteins allows us to determine cytokine gene expression, allowing a greater understanding of cytokine involvement in pathology and physiological processes.

The aim of this study was to test the hypothesis that cytokines are excessively expressed as a response to physical activity in patients suffering from CFS. Our hypotheses were as follows:

1. Aerobic exercise and commuting to hospital induce greater increases in serum protein concentrations of transforming growth factor (TGF-β), tumour necrosis factor (TNF), interleukin 6 (IL-6), interleukin 8 (IL-8) and their RNA in cases of CFS compared to healthy sedentary controls.

2. Perceived effort with exercise and post-exercise increases in malaise, fatigue and pain are associated with increased serum protein concentrations of pro-inflammatory cytokines including TNF, IL-8, IL-6, and their RNA in cases but not controls.

3. Increased post-commuting malaise, fatigue and pain are associated with an increased serum protein concentration of TGF-β, and its RNA in cases, but not controls.

4. Depressed and anxious mood, physical deconditioning and sleep disturbance are mediating factors in the increased protein concentrations of the cytokines TNF, IL-6, IL-8, and TGF-β in cases.

Materials and Method

CFS patients

Consecutive new adult (aged 16-65) outpatient attenders at the CFS clinics at St Bartholomew's and the Royal Free hospitals in London, UK, were invited to take part in this study if they met inclusion criteria. No patient from our original pilot study was recruited into this study [11]. Patients with CFS diagnosed by Centers for Disease Control (CDC) (international) criteria [1], with the modification of not having *any* concurrent co-morbid psychiatric disorder, were studied. We excluded those with comorbid psychiatric disorders (with the exception of simple phobias) in order to reduce the heterogeneity of the patient sample and reduce confounders, since certain (non-excluding) comorbid psychiatric disorders such as non-melancholic depressive disorder may also be associated with abnormal cytokine concentrations [19]. We

used the Standardised Clinical Interview for DSM IV (SCID) to screen patients and controls for psychiatric disorders [20]. We excluded participants (patients and controls) if they had regularly been taking any prescribed medications in the past two weeks that might affect the immune system or exercise challenge. We allowed the use of selective serotonin reuptake inhibitors, tricyclic antidepressants and paracetamol. Participants could have certain co-morbid medical conditions if in remission (e.g. hypothyroidism if biochemically euthyroid on thyroxine replacement therapy) [1]. Medications taken in the previous week were recorded and examined in the analysis as potential confounders. We chose to recruit from secondary care, since our pilot data were from this population and because it allowed for ease of recruitment to test our primary hypotheses.

Healthy controls

The comparison group were 21 healthy but sedentary volunteers (engaging in moderate-intensity exercise or physical activity, for up to 20 minutes in a single episode, less than once a week). We group matched controls by age (+/- 5 years), sex, body mass index (+/- 2.5) and distance they lived from the hospital (+/- 2 miles). We recruited the healthy volunteers through the CFS patients and patient groups (healthy but sedentary friends or relatives that were not blood relatives), the participating hospitals and medical schools (employees or their friends or family), and people living or working locally. We used emails, posters in waiting rooms, local advertising in bulletins, newsletters and newspapers to target potential healthy volunteers. Health status was assessed with a brief interview by the researcher, with

standard questions, excluding all those with a current infection, chronic disease, taking medicines, or psychiatric disorder as above.

Study design and ethics

This was a 16-day study whereby participants (both patients and controls) attended the hospital on four occasions, with blood samples taken over a 9 day period from day 7 to day 16. A baseline assessment on day 1 mainly comprised obtaining written informed consent, self-completion of questionnaires and the SCID [20]. A "commuting challenge" was completed on day 7, which included the participant having blood drawn at home after a night's sleep/rest, before they rose from their bed. Another blood sample was taken when they arrived at the hospital on the same day after commuting from their home to the hospital. An aerobic exercise challenge was completed on day 14. On this day blood was drawn before, immediately after, and 3 hours after exercise. On day 16 (two days after the exercise) a final blood sample was taken at the hospital and follow-up questionnaires were administered. Participants were studied 48 hours after the exercise, rather than 72 hours afterwards as in the pilot study [11], since our clinical impression suggested that CFS patients perceive most post-exertional symptoms at this time.

Participants' blood samples were drawn between 9am and 2pm, except for the sample taken whilst resting at home before commuting (day 7) which was often earlier, and a three-hour post-exercise sample (day 14) which was often later. Samples were obtained at a fixed time after arrival at the hospital (15 minutes), and in a fixed sequence (protein assays followed by RNA) from ante-cubital venous

puncture. All participants were required to have eaten breakfast prior to their arrival at each of their hospital appointments, but to have abstained from caffeinated drinks that morning. Menstrual phase was recorded in premenopausal female subjects. Since appointments spanned 16 days it was not possible to co-ordinate testing during one (luteal) phase of the cycle.

Normal activities at home were resumed between appointments, with seven days considered as giving sufficient time for participants to re-establish baseline cytokine concentrations after each hospital visit; gene expression usually returns to normal 24 hours after exercise in healthy people [21]. Symptom exacerbations were rare a week after hospital travel in the pilot study [11].

To maximise participant retention without influencing the validity of the results we allowed some flexibility with appointment days. If a participant was unable to attend an appointment on the scheduled day, they were allowed to attend on the day before or the day after. However, the final appointment always took place two days after the exercise challenge, and the home visit always occurred on the same day as the commuting challenge.

This study was approved by the London Bridge Research Ethics committee (11/LO/1572). Written informed consent was obtained from all participants who were paid travel expenses. Healthy volunteers were also paid £10 for each of their four hospital visits.

Questionnaires

On days 1 and 16 of the study, fatigue was measured with the 11 item Chalder fatigue questionnaire [22], perceived physical disability was measured using the Short Form (SF-36) physical function sub-scale [23], self-rated mood was measured with the Hospital Anxiety and Depression Scale (HADS) [24] and sleep disturbance was measured using the Jenkins sleep scale [25]. The International Physical Activity Questionnaire (IPAQ) was used on Day 1 to measure physical activity participation in the past week [26]. The McGill present pain intensity scale was used to measure pain intensity on day 1, before and after travelling on day 7, before and after exercise on day 14 and on day 16 [27]. Ad hoc self-rated five item Likert scales were used to measure the effect of activity/exercise on delayed fatigue, pain and malaise (unwell) at the same times. Response options ranged from 'strongly disagree' through 'neither agree nor disagree' to 'strongly agree'. Fear of exercise was determined on day 14 using the Tampa scale for kinesiophobia for fatigue [28], with healthy volunteers being asked to recall the last time they felt extreme tiredness that was not related to a medical condition, being pregnant, or dehydration.

Exercise challenge

The aerobic exercise challenge used a friction-loaded cycle ergometer (Monark 808, Sweden). The cycle ergometer was chosen in preference to a treadmill because it requires limited familiarisation, was used in our pilot study, and is generally perceived by participants as less distressing. All subjects completed a five-minute familiarisation period of unloaded cycling at a cadence of 60 $rev.min^{-1}$. After the familiarisation period, the subjects fulfilled a sub-maximal exercise protocol. We chose this protocol

because in our pilot study we found no statistically significant differences in either subjective reactions or immune variables between the 70% sub-maximal test and the 100% maximal test [11]. The workload was increased by 30 Watts every 3 minutes until a heart rate (HR) equal to 70% of age predicted maximum HR (192 – 0.007 x age in years^2) was achieved [29], to within 10 beats per minute. After this point, the participants continued to exercise at this workload for 20 minutes or until volitional exhaustion. Volitional exhaustion was considered to have occurred if the subject was no longer able to maintain cadence within 10 rev.min^{-1} of the required 60 rev.min^{-1}, in spite of encouragement.

During the exercise challenge participants were asked to breathe through a mouthpiece connected to a system of open circuit spirometry with a nose-clip preventing air escaping. Expired gases were analysed breath-by-breath throughout the exercise by a cardiorespiratory analyser (CPX/D, Medical Graphics Inc., USA), calibrated according to the manufacturer's instructions prior to each test. The oxygen consumption ($\dot{V}O_2$ ml.kg.min^{-1}) was averaged every 30 seconds with peak $\dot{V}O_2$ determined as the highest $\dot{V}O_2$ value obtained. Heart rate (HR) was also recorded every five seconds using a HR monitor (POLAR Electro, Finland). The highest 30-second average HR (b.min^{-1}) was deemed to be the peak HR. The exercise challenge duration (seconds) was also recorded. The original Borg scale of rating of perceived exertion (RPE) was self-rated throughout the exercise test and immediately after the exercise was completed [30]. The RPE score at 70% of peak HR was used as the split-point for the analysis of perceived effort in hypothesis 2, since all participants reached this point.

Laboratory assays

At each bleed, we took 15mls of blood in three tubes: 10ml for serum proteins and 5ml for RNA extraction. The samples were sent to the laboratory and would have arrived at the latest the following morning (if taken in the afternoon). One tube was centrifuged immediately for storage, the second was stored at ambient room temperature for 24 hour and then centrifuged. All samples were then frozen and stored at -70 ^{0}C until analysed in three batches. The RNA tube was immediately stored as it was at -80^{o}C, in line with the manufacturers guidance. All laboratory work was completed by laboratory staff blind to group membership. Eleven cytokines (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, TGF-β, TNF and IFN-γ) were measured on the basis that they would either be expected to be abnormally regulated in CFS after exertion and reflect a broad range of cell types, or that they had been previously shown to be elevated in CFS in some studies [9,10]. Serum protein concentrations of TGF-β were analysed by ELISA methodology according to manufacturer's instructions (R&D systems). The TGF-β samples were analysed in three batches by two laboratory technicians, with one technician analysing the first batch of samples in 2009 and the other technician analysing the second and third batches of the samples in 2011. As part of the standard operating procedure, the serum was centrifuged at 2,500 rpm for 10 minutes. This is important in that TGF-β is released from platelets in greater quantities with faster centrifuging speeds. The remaining cytokines were analysed in three batches, separately from TGF-β, and were determined using multiplex cytokine bead array as per the manufacturer's instructions (BenderMed Systems). We measured all the cytokines at all time points and have reported values as picograms per millilitre (pg/ml). Lowest levels of quantification (LOQ), below which

each cytokine in the assay were considered undetectable are shown in Table 1, and these values are used where a value was undetectable. For quality assurance, in the first batch, where an analyte looked to be a possible outlier we re-analysed the sample and took the mean of the two assays. These values did not alter the results so we did not repeat this with the remaining batches. We analysed RNA for five of the cytokines (IL-1β, IL-6, IL-8, TGF- β and TNF), for which we report relative expression values.

In our pilot study [11] we analysed platelet poor plasma when investigating the circulating levels of TGF-β. TGF-β are synthesised as precursor forms which are biologically latent; the precursor form is cleaved by serine proteases and following dissociation from the latency peptide becomes biologically active [31]. Since the latent pool is bioavailable following protease cleavage, TGF-β assessment should be of active and latent acid-activated subsets. The use of platelet poor plasma has been largely superseded by serum based assays, following an understanding that platelet derived (and usually non-bioavailable) TGF-β is sequestered in the matrix of the clot in serum [32]. There is less experimental variation in the coagulation of serum in serum separator tubes than in producing platelet poor plasma [33]. TGF-β protein levels in serum and plasma are the same (when plasma is optimally prepared) and so serum was employed as the preferred substrate in this study [32].

Sample quality control, cDNA synthesis, endogenous control selection & qPCR

RNA purity and concentration were measured by the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc), and RNA integrity was

evaluated using the Agilent Bioanalyzer 2100 (Agilent Technologies). Initial quality control showed significant DNA contamination of the RNA samples, characterised by masking of the 28s peak on the Bioanalyzer traces. Contaminating DNA was removed using an in-solution DNase treatment, followed by concentration and clean-up using Qiagen RNEasy MinElute columns (Qiagen). RNA concentration and integrity were re-measured post-DNAse treatment. cDNA was synthesised using 200ng of total RNA in a reaction volume of 20uL using the High-Capacity RNA-to-cDNA Master Mix kit (Life Technologies). Resulting cDNA was diluted 1:10 for use in subsequent qPCR reactions.

The most appropriate endogenous control assays from a panel of 6 reference genes (18S, β2M, ATP5β, EIFA42, GAPDH and UBC) were selected after qPCR analysis of 10 representative RNA samples from the study using all 6 assays. The geNorm software package was used to assess the stability of the 6 genes in this sample set. The two most stable genes, ATP5β and GAPDH, were selected to run alongside the assays of interest in order to evaluate relative expression of the candidate genes [34]. Primers and probes used for qPCR were available as inventoried Taqman® assays from Life Technologies (Table 2) and PCR reactions were carried out using the Taqman Universal PCR Master Mix (Life Technologies) in a 10uL reaction volume. Thermal cycling was performed using the 7900HT Sequence Detection System (SDS) (Applied Biosystems), and PCR program 50°C for 2 mins, 95°C for 10 mins followed by 40 cycles of 95°C for 10s and 60°C for 1 min. Each reaction included a no-template control as a negative sample, and each cDNA sample was run in triplicate.

Relative expression compared with GAPDH and ATP5B was generated using the $2^{-\Delta\Delta Ct}$ method, using cycle threshold values (Ct) generated automatically by the 7900HT SDS software. Standard deviations and coefficients of variation were calculated for each set of triplicate data, and any outliers were removed.

Statistical Analysis

Not detectable cytokine protein concentrations were approximated as the lowest level of quantification (LOQ) value (Table 1) for the purpose of the analyses. Testing the hypotheses required non-parametric statistical testing using the Mann-Whitney test as the majority of data were not normally distributed. For hypothesis 1 we first compared absolute cytokine protein values, and their RNA at each time point between groups and then the difference between groups in any relative change (delta) in the values from baseline to post commuting/exercise. We also used the Wilcoxon signed rank test to determine any differences over time within groups.

For the across-group analyses for responses to commuting in hypothesis 1, we compared post-commuting to pre-commuting cytokine protein concentrations and their RNA, and for the response to aerobic exercise we compared pre-exercise to: immediately post-exercise, 3-hours post-exercise and 2-days post-exercise. Where necessary we adjusted the analyses for multiplicity using a Bonferroni adjustment.

Analysing the patients and controls separately, we tested hypothesis 2 using Mann-Whitney tests to determine any differences in the relative change in cytokine protein and RNA levels between those reporting an RPE during the exercise test that was

above or below the group median value. We compared: pre-exercise to: immediately post-exercise, 3-hours post-exercise and 2-days post-exercise. We used the same analyses to determine any differences in cytokine protein and RNA levels for those reporting increased fatigue, pain and malaise after exercise and to determine any differences in TGF-β protein and RNA levels for those reporting increased fatigue, pain and malaise after commuting compared to those who reported no change (hypothesis 3).

Analyzing only the patients, we tested hypothesis 4 using Spearman's rank correlation coefficient to determine the relationships between cytokine protein level (when significantly elevated) and potential mediating factors of depression, anxiety, sleep disturbance and physical deconditioning (using time spent active during a week, time exercising during the exercise test and maximum power output achieved within the exercise test). We used the cytokine protein values two days after exercise since our clinical impression suggested CFS patients perceive most post-exertional symptoms at this time. As a post-hoc analysis we examined the distribution of TGF-β protein in the cases and found it to be bimodal in its distribution.

As an exploratory analysis, we examined all additional seven cytokine protein levels (IL-1β, IL-2, IL-4, IL-5, IL-10, IL-12p70 & IFN-γ) using the Mann-Whitney test. We first compared absolute cytokine protein values at each time point between groups. If there were sufficient detectable data we then looked at the difference in the relative change (delta) scores from pre to post commuting and from pre-exercise to immediately, 3 hours, and two days, after the exercise between groups. Since all cytokine proteins were measured six times, we corrected when necessary for multiple measures using a Bonferroni adjustment.

Confounders

To further determine whether the results were influenced by possible confounders, we adjusted significant findings for assay batch technician, gender, contraceptive pill use, whether taking TCAs, smoking status, use of antihistamines, homeopathy, vitamins and minerals, or other complementary or alternative medicines (CAMs), age, BMI, day of menstrual cycle at time of blood test, duration of CFS (> or < 3 years) [35], time spent walking in one week, time spent in bed in a week, perceived effort of the exercise test (RPE at 70% peak HR), fear of having blood taken, sleep disturbance [25], and levels of anxiety and depression [24]. We only explored cytokines protein and RNA that showed significant differences between groups and we used cytokine values 2-days after the exercise challenge. We modelled significantly associated ($p \leq 0.10$) confounders using appropriate regression models.

Power analyses

We used our pilot data to guide our calculation and took the significance level as 0.05 and the power of the study at 80%. In order to detect a difference in baseline TGF-β protein between cases and controls of 0.35 ng/ml with an estimated standard deviation (SD) of 0.31 we needed 14 cases in each group. Due to non-normality of the data we upwardly adjusted the sample size to 16 in each group [36]. When we repeated the power calculations using the pilot data for TGF-β protein three days after exercise and the mean (SD) difference in TGF-β protein before and after travelling to hospital, the number of samples required was similar. We increased the sample sizes

to 24 in each group in order to allow our hypothesis-generating work. Since there have been no previous studies of the associations between cytokine protein levels and dependent variables such as post-exertional symptoms or activity, this part of the study was exploratory.

Results

A total of 24 patients (cases) and 21 controls were recruited into the study. Gender, age, smoking status, distance they lived from the hospital, amount of physical activity they undertook and time spent sitting were not significantly different across groups (Table 3). There were no statistically significant differences in the use of antidepressants, antihistamines, the oral contraceptive pill and thyroxine, but cases were generally more likely to be taking vitamins, minerals or other complementary and alternative medicines (CAMs) and spent more time in bed each night (Table 3).

The median (quartiles) duration of illness in the cases was 61 (37, 122) months. As expected, cases had significantly worse scores for fatigue, physical functioning, mood (anxiety and depression), pain and sleep measures than controls (table 4).

Median (quartiles) rating of perceived exertion (RPE) measured at 70% of predicted peak HR, during the exercise challenge, was significantly higher in cases (15 (14, 17)) compared to controls (13 (12.5, 13)) ($p = 0.002$) (table 5). Furthermore, four times as many cases had an RPE >14 (median for all subjects) at 70% of their predicted peak HR during the exercise test (16 vs. 4; $p = 0.001$). Cases spent significantly less time cycling during the exercise test ($p = 0.027$), but showed a similar peak $\dot{V}O2$ (ml.kg.min-1) and peak heart rate (bpm).

Table 6 shows baseline levels of all cytokines protein and RNA measured at rest in bed, before rising, on day 7. There was no significant difference between groups in the number of detectable versus undetectable levels in any of the cytokines (in analysis of the protein or RNA results).

Hypothesis 1 – Increases in cytokines in cases compared to controls

After a Bonferroni adjustment for multiple-testing (x6), TGF-β protein levels were significantly elevated in cases compared to controls at rest (median = 43.9ng/ml vs 18.9 ng/ml; p = 0.003), after commuting (median = 56.9ng/ml vs 21.4ng/ml; p = 0.002) and 2-days post-exercise (median = 49.1ng/ml vs 21.9ng/ml; p = 0.007) (Fig. 1). The changes in TGF-β protein concentrations due to either commuting or exercise were not significantly different between the groups although there was a significant increase pre to post commuting in the cases (p = 0.003) but not pre- to post-exercise.

After a Bonferroni adjustment for multiple-testing, IL-8 protein levels were not significantly higher in the cases compared to controls at any time point. Values for IL-6 and TNF proteins were not significantly higher in the cases compared to controls at any time point.

No RNA values were significantly different between cases and controls at any time point from pre-commuting to 2-days post exercise. Neither commuting nor exercise induced greater relative increases in concentrations of RNA of TGF-β, TNF, IL-8 or IL-6, in cases compared to controls.

Hypothesis 2a – perceived effort with exercise

Cases that reported an effort (RPE) during the exercise above the median did not demonstrate significantly greater increases in values for protein or RNA for TNF, IL-8 or IL-6 either immediately, 3-hours or 2-days after the exercise compared to those reporting a lower effort with exercise.

Hypothesis 2b – pain, malaise and fatigue with exercise

Significantly more cases, compared to controls, agreed or strongly agreed that they were more fatigued (19; 83% vs 1; 5%; $p < 0.001$), in more pain (13; 57% vs 1; 5%; $p < 0.001$), or felt more malaise (15; 65% vs 0; 0%; $p < 0.001$), 2-days after the exercise.

Cases who agreed that their *fatigue, malaise or pain* increased after the exercise test did not have significantly greater relative changes in TNF, IL8 or IL-6 protein or RNA either immediately, 3 hours or 2 days after the exercise, compared to those who disagreed.

Hypothesis 3 – post-commuting symptoms related to TGF-β

After commuting to the hospital, neither TGF-β protein nor RNA were significantly increased in patients who agreed they felt more *fatigue*, *malaise* or *pain* after commuting, compared to those who disagreed. In the control group there were not enough data to analyse because only two participants reported feeling more fatigued and none reported more malaise or pain after commuting.

Hypothesis 4 - Potential mediators in cases

When analysing TGF-β protein levels in cases alone, we noticed a clearly bimodal pattern to the data with 12 (50%) cases having consistently high values and the other 12 having consistently low values (Fig. 2). The cut-off we used was 37ng/ml. The mean values for TGF-β protein levels in the 'high TGF-β' group were significantly higher than the values for the 'low TGF-β' group ($p < 0.001$) when compared after exercise.

In cases, concentrations of TGF-β protein after the exercise challenge were not significantly associated with depressed or anxious mood, with physical deconditioning or sleep disturbance (data not shown).

Exploratory work

The exploratory work did not show any statistically significant differences in cytokine protein levels between cases and controls at any time point, and there were no significantly different relative changes in any of the exploratory cytokine levels between cases and controls after commuting or the exercise challenge.

Confounders in cases and controls

On deeper analysis of confounders, we examined TGF-β values by both assay batches and by the two laboratory technicians who ran the assays. We show these data for one of the times where there was a significant difference between groups (table 7). This shows that batch one TGF-β concentrations (both for cases and

controls) were significantly higher than the concentrations for batches 2 and 3. Comparing the results of the two technicians shows that technician 1 found significantly higher concentrations than technician 2 (patient versus patient $p < 0.001$).

Analysing cases and controls together, menstrual cycle phase, allowable medications and smoking status did not influence TGF-β protein levels. However, taking the oral contraceptive pill (OCP) ($p = 0.02$) having a higher depression score ($p = 0.03$), and having a sample analysed by operator 1 ($p < 0.001$) predicted higher levels of TGF-β protein two days after exercise. We undertook a multiple regression analysis which included laboratory technician, group, OCP and depression, plus body mass index ($p = 0.07$). We used the stepwise method using transformed TGF-β protein (log e) on day 16 and found that laboratory technician (1 vs 2) was the only significant predictor, which accounted for 79% of the variance in TGF-β protein (adjusted $R^2 = 0.79$; $p < 0.001$).

Summary of findings

After correcting for multiple analyses, TGF-β protein was the only cytokine protein or RNA that showed significantly different values between CFS and control groups. This was found to be a spurious finding, explained by assay variation between laboratory technicians. Neither protein nor RNA concentrations of any other cytokines were significantly higher in cases after commuting or an exercise test.

Discussion

In this study we investigated circulating cytokines and their RNA in patients with CFS at rest, after commuting, and after an acute bout of physical activity. We found no evidence to support our hypothesis that these activities increased cytokine levels or their RNA, or that they were associated with post-exertional symptoms and higher perceived effort of exercise that are commonly experienced by people with CFS. In contrast, our data showed a significant elevation in circulating TGF-β at rest and afterwards over the nine days of testing in patients compared to controls. Further analysis showed a clear bimodal distribution to TGF-β, particularly in cases. This was found to be an artefact, explained by a laboratory anomaly, with technician 1 finding consistently higher values for TGF-β in both cases and controls, compared to technician 2. Since there were 13 (54%) cases and only 2 (10%) controls assayed by technician 1, it therefore appeared as though cases were more likely to have elevated concentrations when analysed by summarised group. Changes in TGF-β protein levels due to commuting or exercise were similar in patients and controls.

Previous studies have been criticised for not accounting for confounding factors that can affect cytokine concentrations [9, 10]. We measured a number of possible confounders and found that only laboratory variation influenced TGF-β protein levels. We were unable to replicate finding a difference by duration of illness [35], although this study had smaller numbers of patients. Contrary to some previous studies, including our own pilot study [11], we found no differences in concentrations of TNF between patients and controls. Our finding that any change in TGF-β after exercise was not significantly different from any change in the control group replicates previous work [37].

We cannot know whether the same laboratory variation explains the previous case control findings for TGF-β, but case control studies can start subject recruitment with cases, in order to ascertain accurate matching of controls, so any inter-batch assay variation might explain why it appeared that cases had different concentrations from controls. We were unable to ascertain the differences in laboratory processing that led to the differences between batches, but assume that the difference was due to using different centrifuge times, which might affect TGF-β release from platelets, leading to differences in TGF-β concentrations. Great care needs to be taken in any case control study where matters such as centrifuge speed and timing can determine release of cytokines such as TGF-β.

This study does not rule out a role for local release of cytokines in the central nervous system (CNS). There is a two way relationship between the immune system and the CNS [3, 4]. Cytokines can cross the blood brain barrier, particularly when the blood brain barrier is modified by factors such as intercurrent infections or distress [38]. Cytokines are locally released by glial cells in the CNS, and TGF-β can cause central motor fatigue in animals [5, 39]. Cytokines, such as TGF-β, could thus affect the perception of pain and fatigue through both direct and indirect effects. There is some recent evidence to support neuro-inflammation in CFS/ME [40]. TGF-β appears to have complex effects in the brain, but may promote a pro-inflammatory state and disturbance of blood brain barrier function [41].

It is known that sufficient exercise can modulate cytokines at the level of gene expression to protein ligand release and receptor activation, with associated local and systemic consequences in the healthy population [16, 42]. Although there is little evidence for this in patients with CFS [16, 43], it is possible that cytokines such as

TGF-β1 remain cell surface bound so bio-active and peripheral levels may not be fully represented either at rest or after exercise. By investigating RNA in this study it allowed us to determine the presence of such cytokine gene expression. However, we did not find RNA concentrations elevated in cases compared to controls with any of the cytokines for which it was measured, and it did not change differently between groups with either commuting or exercise in RNA extracted from whole peripheral blood. Finding no statistically significant difference in TGF-β RNA supports the spurious nature of the elevated TGF-β protein levels found in this study.

This study had some limitations. With 24 cases in the patient sample it is a relatively small sample particularly for the number of cytokines we wished to investigate; it is likely that we were underpowered. Finding cases without psychiatric comorbidity, who were not on medicines that excluded them, was a challenge. There is good evidence that CFS is a heterogeneous group of conditions [44], but our sample of cases was too small to divide into sub-groups, which a larger study has suggested may affect circulating cytokine concentrations [35]. Although we tried to match on a number of criteria, including smoking status and those taking the oral contraceptive pill (both known to effect cytokine levels), it was difficult to recruit sedentary and yet healthy participants who were not taking any excluding medicines. This is why we were only able to recruit 21 controls and they had a trend towards a higher BMI, were taking less vitamins and spent less time in bed, all of which could have confounded inter-group differences. In addition, because the protocol required a number of visits to the hospital, the CFS patients volunteering for this study would have been more physically able and those declining would have likely been the most disabled, leading to an increased risk of a type-2 error.

The strengths of this study include the reliability of the findings in having six measures of the same cytokines over nine days and testing for confounding factors such as taking the oral contraceptive. We also excluded participants with comorbid psychiatric disorders (a novel approach) that could have confounded cytokine levels, so our findings are more specific to CFS. We were able to analyse a good number of cytokines, including five in which more than one study had shown previous elevations, and others that had not been measured previously. We also suggest there is heuristic value in reporting this study, as an apparent bimodal distribution of a potential biomarker in CFS was found, which on a deeper analysis of confounders was found to be spurious.

Implications for future research

A systematic review of the association between circulating cytokines and CFS showed that only TGF-β was elevated in the majority of case control studies [10]; a finding we were unable to replicate. Another systematic review concluded that cytokine concentrations were not abnormal after exercise in CFS [9]; a finding we replicated. We suggest that circulating levels of cytokines are unlikely to be important in the pathophysiology of CFS.

Conclusions

We replicated the finding of consistently higher circulating TGF-β at rest in cases of CFS compared to healthy controls, but deeper analysis showed this to be a spurious finding, due to laboratory variation. Post-exertional symptoms and perception of effort with exercise were not associated with an increase in

circulating cytokine protein or RNA concentrations in patients with CFS. There have been many studies showing associations between various biomarkers and CFS, which have not been replicated; due caution should be taken before the next biomarker is reported.

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Conflicts of Interest

PDW is a member of the Independent Medical Experts Group, which advises the UK Minstitry of Defence regarding its Armed Forces Compensation Scheme, and is a consultant advisor to a re-insurance company. No other author declares a conflict of interest.

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Figure Legends

Fig. 1. Serum protein concentrations of TGF-beta in CFS cases compared to controls (median scores in ng/ml); single dots represent control outliers

Fig. 2. TGF-β protein concentrations in cases at each time point (ng/ml)

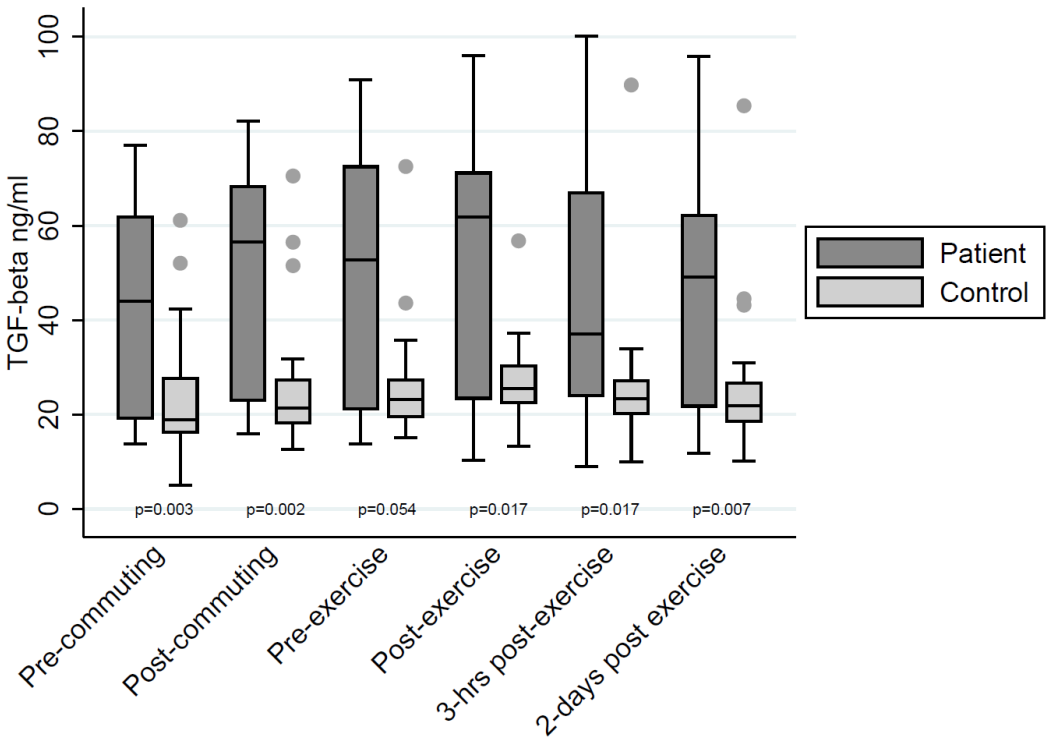

Fig. 1. Serum protein concentrations of TGF-beta in CFS cases compared to controls (median scores in ng/ml), single dots represent control outliers

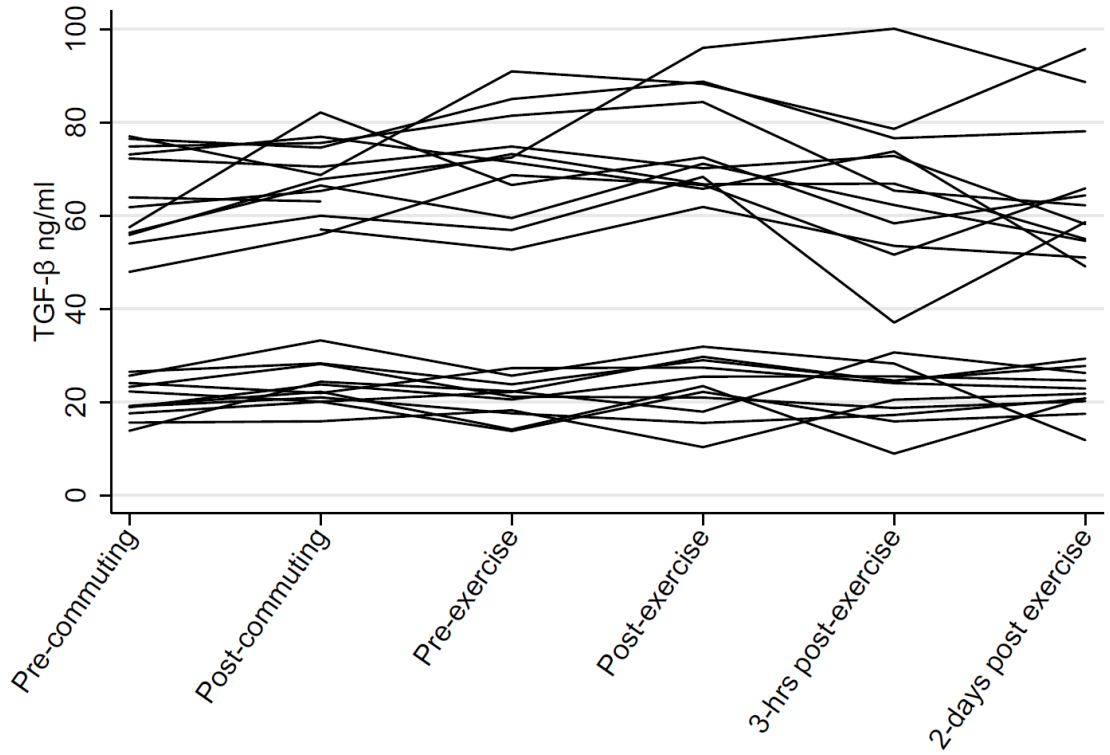

Fig. 2. TGF-β protein concentrations in cases at each time point (ng/ml)

Table 1. List of lowest level of quantification (LOQ) for cytokine proteins

Cytokine name	Lowest level of quantification (pg/ml)
IL-1β	4.2
IL-2	16.4
IL-4	20.8
IL-5	1.6
IL-6	1.2
IL-8	0.5
IL-10	1.9
IL-12p70	1.5
TGF-β	10.0
TNF	3.2
IFN-γ	1.6

Table 2. List of Genes and Gene Expression IDs

Gene name	Life Technologies Assay ID
18S	Hs99999901_s1
β2M	Hs99999907_m1
ATP5β	Hs00969567_g1
EIFA42	Hs00756996_g1
GAPDH	Hs99999905_m1
UBC	Hs00824723_m1
IL1β	Hs01555413_m1
IL6	Hs99999032_m1
IL8	Hs01553824_g1
TNF	Hs00174128_m1
TGFβ1	Hs00998133_m1

Table 3. Demographics and clinical data (n (%) unless otherwise stated)

	CFS **n = 24**	**Control** **n = 21**	**p value**
Age (years)[a]	40.3 (12.2)	39.3 (14.1)	0.81
Gender (Female)	15 (63%)	16 (76%)	0.32
BMI	23.2 (4.5)	25.5 (4.4)	0.08
Distance from hospital (miles)[b]	6.9 (3.8, 11.0)	7.8 (6.0, 11.7)	0.26
Taking antidepressants (SSRIs or TCAs)	7 (29%)	2 (10%)	0.14
Taking antihistamines	3 (13%)	1 (5%)	0.61
Taking thyroxine	1 (4%)	2 (10%)	0.59
Taking the oral contraceptive pill (females only)	4 (27%)	1 (6%)	0.17
Cigarette smoker	4 (17%)	4 (19%)	1.000
Taking vitamins, minerals and/or CAMs	13 (54%)	4 (20%)	0.015
Daily mins of activity [b]	21.8 (7.3, 46.4)	25.0 (7.9, 40.0)	0.91
Above recommendations for PA	10 (42%)	10 (44%)	0.69
Time spent sitting on usual day (minutes) [b]	600 (458, 803)	690 (495, 765)	0.91
Time spent in bed at night (minutes) [b]	540 (480, 600)	480 (420, 480)	0.001

a = Mean (SD); b = Median (quartiles)

BMI = body mass index; SSRI = selective serotonin reuptake inhibitors; TCAs = tricyclic antidepressant; PA = physical activity; CAMs = complementary and alternative medicines

Table 4. Questionnaire scores at baseline (median (quartiles)).

	CFS **N = 24**	**Control** **N = 21**	**P value**
Duration of illness (months)	60.5 (37, 122)	--	--
Chalder fatigue score	27 (22, 30)	11 (10, 11)	<0.001
SF-36 PF	60 (40, 75)	100 (95, 100)	<0.001
Jenkins sleep	9 (6, 12)	4 (2, 5)	<0.001
HADS Depression	5 (4, 7)	0 (0, 1.5)	<0.001
HADS D (excluding “slowed down”)	3 (1, 5)	0 (0, 1)	<0.001
HADS Anxiety	7.5 (5, 9)	3 (1.5, 7)	0.003
McGill Pain (discomforting or distressing) [a]	18 (75)	0 (0)	<0.001

a = n (%). SF-36 PF = SF-36 physical functioning subscale; HADS = Hospital anxiety and depression scale

Table 5. Exercise test variables (medians and quartiles).

	CFS (n = 23)	**Control (n = 20)**	**P value**
RPE Score at HR 70% peak	15 (14, 18)	13 (12, 13)	p = 0.002
RPE > median of 14 at HR 70% peak	16 (70%)	4 (20%)	p = 0.001
Time on bike (secs)	960 (675, 1530)	1530 (1080, 1620)	p = 0.027
$\dot{V}O_2$ peak (ml.kg.min^{-1})	20.4 (16.8 to 22.2)	19.5 (14.1 to 21.2)	p = 0.31
Peak HR (b.min^{-1})	143 (136 to 154)	151 (145 to 159)	p = 0.09
Max Power (watts)	90 (60 to 90)	90 (60 to 90)	p = 0.62
Competed test (yes)	9 (39%)	14 (70%)	p = 0.043

RPE = rating of perceived exertion, HR = heart rate. VO_2 = Volume of oxygen.

Table 6: Baseline cytokine (protein and RNA) concentrations at rest, (at home in bed, on day 7) using lowest level of quantification (LOQ) for undetected values (medians and quartiles)

	CFS (n = 24)		Control (n = 21)	
Cytokine pg/ml	**Undetectable (n)**	**All CFS, imputing LOQ for undetectable values [a, b]**	**Undetectable (n)**	**All control, imputing LOQ for undetectable values [b]**
IL-1β	20	4.2 (4.2, 4.2)	18	4.2 (4.2, 4.2)
IL-2	13	16.4 (16.4, 66.5)	17	16.4 (16.4, 16.4)
IL-4	17	20.8 (20.8, 23.2)	15	20.8 (20.8, 20.8)
IL-5	21	1.6 (1.6, 1.6)	16	1.6 (1.6, 1.6)
IL-6	24	1.2[c]	18	1.2 (1.2, 1.2)
IL-8	4	119.7 (38.1, 322.7)	5	82.8 (0.8, 261.1)
IL-10	15	1.9 (1.9, 8.8)	15	1.9 (1.9, 1.9)
IL-12p70	16	1.5 (1.5, 3.8)	18	1.5 (1.5, 1.5)
TGF-β (ng/ml)	0	43.9 (19.2, 61.8)	0	18.9 (16.1, 30.0)
TNF	15	3.2 (3.2, 11.9)	17	3.2 (3.2, 3.2)
IFN-γ	12	1.6 (1.6, 25.6)	15	1.6 (1.6, 6.7)
Cytokine RNA (relative expression)		**CFS (n = 24)**	**Control (n = 21)**	
IL-1β RNA		0.42 (0.29, 0.60)		0.37 (0.32, 0.43)
IL-6 RNA		0.00002 (0, 0.00003)		0.00001 (0, 0.00003)
IL-8 RNA		0.05 (0.03, 0.11)		0.07 (0.04, 0.13)
TGF-β RNA		0.40 (0.35, 0.53)		0.37 (0.32, 0.50)
TNF RNA		0.41 (0.27, 0.51)		0.36 (0.26, 0.45)

a = The only missing data was from one case of TGF-β. b = These values included the imputed lowest level of quantification (LOQ) score for a cytokine where a case had undetectable levels: this was repeated for all cytokine protein values. c = Constant at 1.2 as all undetectable, therefore no quartiles available.

Table 7: TGF-β assay values (pg/ml) by laboratory technician (medians; quartiles)

	N	TFG-β post-commuting	p value
Batch/Operator 1			
Patient	13	67833 (63066, 74606)	0.686
Control	2	63505 (56497, 70512)	
Batch 2			
Patient	11	22216 (20548, 26273)	0.766
Control	9	21361 (18683, 26376)	
Batch 3			
Patient	0	--	--
Control	10	19894 (14934, 27377)	
Operator 2			
Patient	11	22216 (20548, 26273)	0.471
Control	19	21298 (17361, 26554)	

Operator 1 vs Operator 2: Patient vs patient: $p < 0.001$; control vs control: $p = 0.010$